HARNESSING USEFUL RHIZOSPHERE MICROORGANISMS FOR PATHOGEN AND PEST BIOCONTROL

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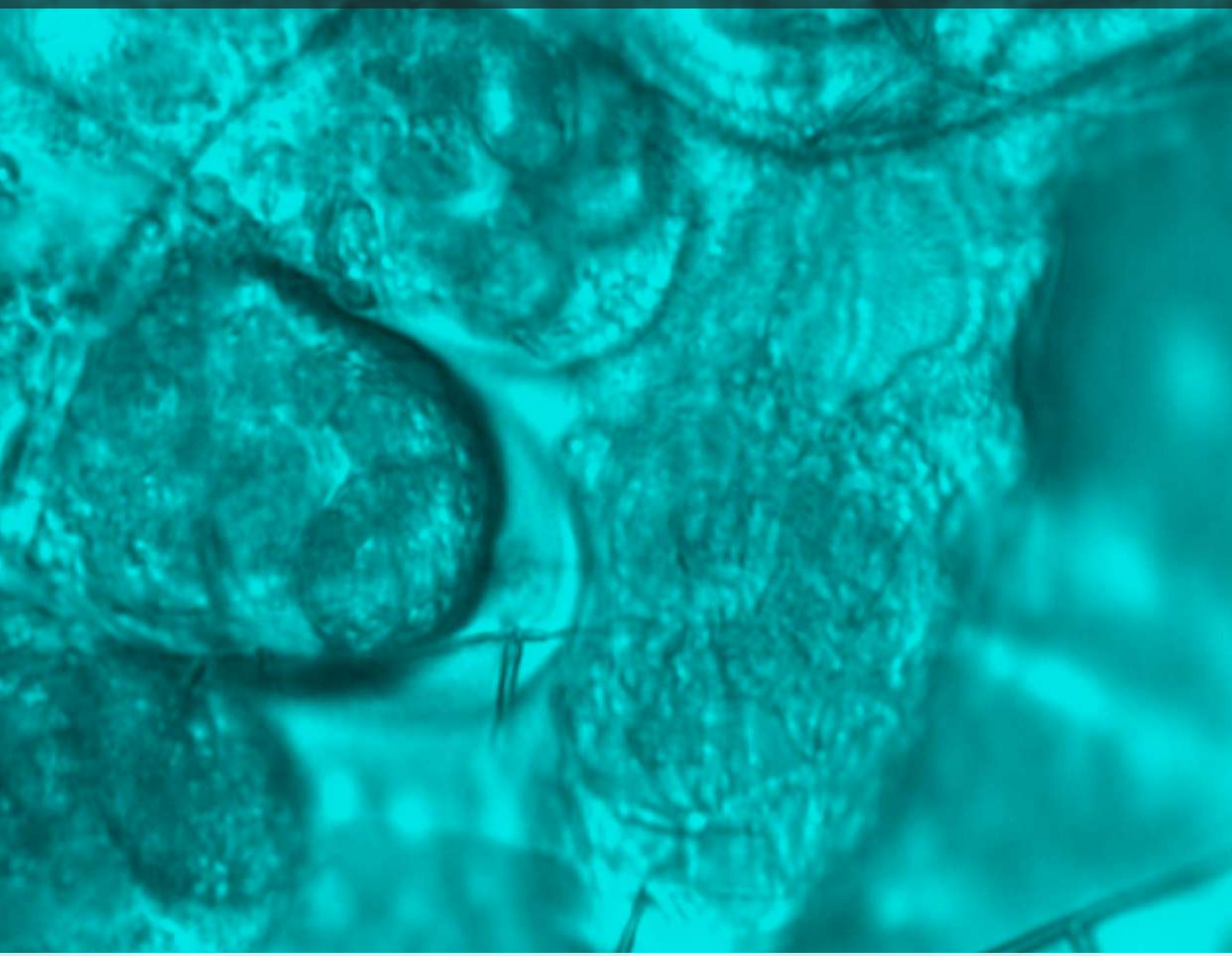

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HARNESSING USEFUL RHIZOSPHERE MICROORGANISMS FOR PATHOGEN AND PEST BIOCONTROL

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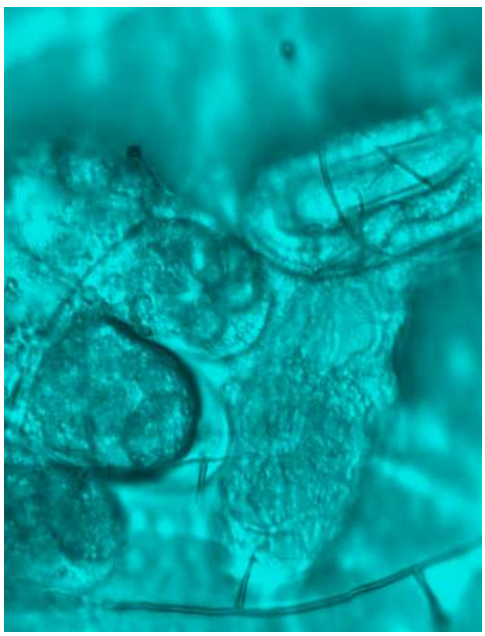

Root-knot nematode eggs parasitized by hyphae of an entomopathogenic Lecanicillium sp.
Image by Aurelio Ciancio

Growing demographic trends require sustainable technologies to improve quality and yield of future food productions. However, there is uncertainty about plant protection strategies in many agro-ecosystems. Pests, diseases and weeds are overwhelmingly controlled by chemicals which pose health risks and cause other undesirable effects.Therefore, an increasing concern on control measures emerged in recent years. Many chemicals became questioned with regard to their sustainability and are (or will be) banned. Alternative management tools are studied, relying on biological, and low impact solutions. This ResearchTopic concerns microbial biocontrol agents, root-associated microbiomes, and rhizosphere networks. Understanding how they interact or respond to (a)biotic environmental cues is instrumental for an effective and sustainable impact. The rhizosphere is in this regard a fundamental object of study, because of its role in plant productivity.

This e-book provides a polyhedral perspective on many issues in which beneficial microorganisms are involved. Data indeed demonstrate that they represent an as yet poorly-explored resource, whose exploitation may actively sustain plant protection and crop production. Given the huge number of microbial species present on the planet, the microorganisms studied represent just the tip of an iceberg. Data produced are, however, informative enough about their genetic and functional biodiversity, as well as about the ecosystem services they provide to underpin crop production. Challenges for future research work concern not only the biology of these species, but also the practices required to protect their biodiversity and to extend their application in the wide range of agricultural soils and systems present in the world. Agriculture cannot remain successfully and sustainable unless plant germplasm and useful microbial species are integrated, a goal for which new knowledge and information-based approaches are urgently needed.

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Section 6. -OMICS

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EDITORIAL
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Editorial: Harnessing Useful Rhizosphere Microorganisms for Pathogen and Pest Biocontrol

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The Editorial on the Research Topic

Harnessing Useful Rhizosphere Microorganisms for Pathogen and Pest Biocontrol

Growing demographic trends require sustainable technologies to improve quality and yield of future food productions. However, there is uncertainty about plant protection strategies in many agro-ecosystems. Pests, diseases, and weeds are overwhelmingly controlled by chemicals which pose health risks and cause other undesirable effects. Therefore, an increasing concern on control measures emerged in recent years. Many chemicals became questioned with regard to their sustainability and are (or will be) banned. Alternative management tools are studied, relying on biological, and low impact solutions.

This Research Topic concerns microbial biocontrol agents, root-associated microbiomes, and rhizosphere networks. Understanding how they interact or respond to (a) biotic environmental cues is instrumental for an effective and sustainable impact. The rhizosphere is in this regard a fundamental object of study, because of its role in plant productivity.

MICROBIAL VOLATILES AND OTHER COMPOUNDS

Roots are surrounded by a flow of molecules released by microorganisms. Some volatiles may affect growth through different mechanisms, such as biochemical signals eliciting local defense reactions or systemic resistance (Kai et al., 2007; Chung et al., 2016). Yi et al. studied the effects of 2,3-butanediol produced by a *Bacillus subtilis* isolate overexpressing the bud operon synthesis encoding genes. They showed that the isolate persisted on pepper roots more than the wild type, thereby interfering with colonization by fungi. Exudates from isolate pre-treated roots inhibited colonization by *Trichoderma* sp., by another *B. subtilis* strain, and by the soil-borne pathogen *Ralstonia solanacearum*. Application of 2,3-butanediol to roots followed by *R. solanacearum* exposure enhanced the expression of pathogenesis-related (PR) genes. The volatile triggered the secretion of root exudates modulating fitness of soil fungi and bacteria, thus acting as a plant defense inducer.

Bacteria metabolism affects nutrient assimilation. Aziz et al. reported a new mechanism in the plant growth-promoting (PGP) *Bacillus amyloliquefaciens* GB03, which activates genes involved in sulfur assimilation and uptake by *Arabidopsis*. Other transcripts encoding for proteins involved in the biosynthesis of sulfur-rich aliphatic and indolic glucosinolates were also expressed. The enhanced sulfur assimilation increased plant glucosinolate biosynthesis, and conferred protection against the beet armyworm *Spodoptera exigua*.

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Bacterial metabolites may also protect plants by inhibiting herbivores (Mithöfer and Boland, 2012). Ganassi et al. reported that also fungal metabolites may have this effect, as shown by a *Trichoderma citrinoviride* isolate, interfering with feeding of the cereal aphid *Rhopalosiphum padi*. Different long-chain primary alcohols (LCOHs) showed a phagodeterrent effect restraining aphids from settling on treated leaves. The LCOHs, perceived through taste receptor neurons and effective at low concentrations, hold potential for insect control in synergy with other compounds.

Lysobacter spp. may affect soil-borne pathogens through extracellular enzymes and other metabolites (Folman et al., 2003). Members of this genus appeared abundant in soil suppressive to the root pathogen *Rhizoctonia solani*. Some strains showed *in vitro* activity against *R. solani* and other phytopathogens such as *Pythium ultimum*, *Aspergillus niger*, *Fusarium oxysporum,* and *Xanthomonas campestris*. In soil, however, suppression of *R. solani* damping-off on sugar beet and cauliflower was low, and no PGP effect was found on sugar beet, cauliflower, onion and *Arabidopsis thaliana*, likely due to poor rhizosphere colonization. Antagonistic *Lysobacter* spp. are an important source of new enzymes and antimicrobial compounds, although their role in disease suppressiveness needs to be confirmed (Gómez Expósito et al.).

SOIL AMENDMENTS

The characterization of organic matter amendments enhancing biocontrol and influencing resident soil communities is a growing research field (Bailey and Lazarovits, 2003; Bonilla et al., 2012). Debode et al. studied the effect of chitin on lettuce growth and survival of pathogenic *Escherichia coli* O157:H7 and *Salmonella enterica* colonizing leaves. Chitin addition increased yields and reduced phyllosphere survival of both bacteria, increasing fungal and bacterial biomass in the rhizosphere. An increase was observed for bacterial genera *Cellvibrio*, *Pedobacter*, *Dyadobacter,* and *Streptomyces* and the fungi *Lecanicillium* and *Mortierella*. These taxa include species involved in biocontrol, PGP, nitrogen cycle, and chitin degradation, showing a potential for chitin-based amendments.

A positive effect of amendments on soil microbial communities was reported by Vida et al. Composted almond shells elicited suppression of *Rosellinia necatrix*, causal agent of white root rot on avocado, increasing Proteobacteria and Ascomycota, and reducing Acidobacteria and Mortierellales. Beneficial *Pseudomonas*, *Burkholderia* spp., or Actinobacteria were found only in the amended soil.

BIOCONTROL AND SUPPRESSION

Soil suppressiveness is an unfrequent condition, regulating noxious organisms, including root-knot nematodes (*Meloidogyne* spp.; Bent et al., 2008). In two organic horticulture greenhouses in Spain, nematodes decreased progressively in a crop rotation, a decline associated to eggs parasitism by the hyphomycete *Pochonia chlamydosporia*. Controlled assays confirmed suppressiveness, as indicated by lower egg densities and reduced *Meloidogyne* reproduction, observed in non-sterilized soil. A higher microbial diversity was also reported from suppressive soils (Giné et al.).

Entomopathogenic nematodes rely on phoretically associated, insect-killing bacteria (Lewis et al., 2015). In Florida, *Steinernema* sp. and *S. diaprepesi* occupy habitats with different soil properties and water potential. They have opposite migration behaviors in relation to soil water potential, the latter being attracted toward drier soil. After three migration cycles through soil to infect insect larvae, *S. diaprepesi* dominated the nematode community when soil was maintained at 6% moisture, whereas *Steinernema* sp. was dominant at 18% moisture. The nematode responses to water potential and osmotic gradients explained their geospatial patterns. *Steinernema* sp. was recovered only from shallow water tables and moist soils, whereas *S. diaprepesi* inhabited ecoregions with well-drained soils and deeper water tables. Differential expression of proteins involved in thermo or mechano-sensation and movement was associated to soil moisture. Proteins involved in metabolism, lectin detoxification, gene regulation, and cell division also differed between the two conditions. Modifying soil moisture in orchards may favor effective entomopathogens, useful in different environments (El-Borai et al.).

Late blight caused by the oomycete *Phytophthora infestans* is the most severe disease of potato (Nowicki et al., 2012). To find alternative solutions to copper-based products, three *Pseudomonas* isolates originating from potato phyllo—and rhizosphere were studied for their protective effect against late blight. When sprayed on potato leaves they survived 15 days in the greenhouse and 8 days in the field. *Pseudomonas chlororaphis* R47 was the most active *in vitro*, and the best protectant in the greenhouse. Its beneficial effects against *P. infestans*, high rates of phyllosphere survival and rhizosphere colonization suggested a potential for application as tuber treatment or leaf spray (Guyer et al.).

Pseudomonas aeruginosa is a model system due to endophytism and antagonism toward plant pathogens and pests. Nevertheless, it is also a human pathogen, a trait compromising its exploitation in agriculture (Deredjian et al., 2014). Thomas and Sekhar studied *P. aeruginosa* applications to control other phytopathogenic bacteria. Strain GNS.13.2a isolated from banana rhizosphere significantly reduced the density of native bacteria soon after inoculation. However, its density declined within a week, while a resilient response was observed for native soil bacteria. Assays under axenic conditions or with soil microbiota showed antagonism by the native microbial community, with varying interactive or antagonistic effects.

The use of bacteria, fungi and viruses for weed biocontrol received attention due to prevalence of herbicide-resistant weeds and pesticide bans. Benefits include low environmental impact, higher specificity, reduced costs, and identification of new herbicidal mechanisms. Harding and Raizada reviewed fungal bioherbicides from North America, based on genera *Colletotrichum*, *Phoma,* and *Sclerotinia*. Bioherbicides also include bacteria of genera *Xanthomonas* and *Pseudomonas* and some viruses. Weed suppression in field conditions is

a challenge, as bioherbicides with cytotoxic mechanisms are more sensitive to environmental variables than conventional herbicides.

Bacterial formulations are increasingly produced for sustainable agriculture using biocontrol and PGP species, including endospore-forming *Bacillus* spp. as alternatives to pesticides. Wu et al. reviewed the use of *B. amyloliquefaciens* type strain FZB42, as an application example.

MYCORRHIZAE, ENDOPHYTES, SYMBIOSES

Arbuscular Mycorrhizal Fungi (AMF) are primary soil components whose impairment affects the rhizosphere functioning. In their review, Berruti et al. highlighted AMF as valid alternatives to conventional fertilization in sustainable agriculture. Although soil inoculation appears a successful strategy, results may vary, depending on host plant, and fungi. Factors affecting AMF success and persistence include species compatibility with the soil environment, spatial competition and inoculation timing. Genomic and transcriptomic data advanced the knowledge on AMF interactions with the host-plant and other soil organisms, unraveling important factors. AMF can protect host plants against biotic stresses like plant-parasitic nematodes. Mechanisms include enhanced root tolerance, direct competition for nutrients and space, induced systemic resistance, and altered rhizosphere interactions. In a second AMF review, Schouteden et al. emphasized the importance of AMF-based systemic resistance for effective biocontrol of nematode pests.

Besides mycorrhizae, the root niche has been "discovered" also by other endophytic microorganisms contributing to plant health and disease containment (Mercado-Blanco and Lugtenberg, 2014; Hardoim et al., 2015). Bacterial root endophytes control the tomato resistance to *R. solanacearum*. Resistant cultivar Arka Abha showed a bacterial endophyte diversity higher than a susceptible cultivar, including species producing siderophores, HCN and antibiotics, of which three isolates (*Pseudomonas oleovorans*, *Pantoea ananatis,* and *Enterobacter cloacae*) were effective antagonists. Other resistant cultivars also showed higher prevalence for antagonistic than susceptible bacteria, confirming a role of root-associated endophytes in plant protection (Upreti and Thomas).

Colonization of lettuce roots and rhizosphere by five genetically modified *Streptomyces* spp. was studied by Bonaldi et al. The strains, transformed with a green fluorescent protein marker and apramycin resistance, inhibited *in vitro* the soil-borne pathogen *Sclerotinia sclerotiorum*. In a non-sterile substrate, a transformed strain colonized soil, roots and rhizosphere. When directly inoculated, the bacterium was re-isolated from rhizosphere and roots at densities higher than after seed coating, showing that it is either rhizospheric, and endophytic.

Microbial symbioses also contribute to the competitiveness of invasive plant species. In this regard, the interactions of their microbiome with native species may provide indications for effective biocontrol. A research agenda aiming at developing novel microbial-based biocontrol strategies was proposed by Kowalski et al. based on the invasive plant *Phragmites australis*.

VIRULENCE AND INDUCED RESISTANCE

Abscisic acid (ABA) is active in the rice interactions with the blast fungal pathogen *Magnaporthe oryzae* and the antagonistic bacterium *P. chlororaphis* EA105. Spence et al. reported that abscisic acid (ABA) affects plant defense by acting antagonistically on salicylic acid (SA), jasmonic acid (JA), and ethylene signaling. *Magnaporthe oryzae*-produced ABA enhanced plant susceptibility by accelerating pathogenesis through higher rates of spore germination and appressoria formation. Strain EA105 reduced the pathogen virulence by preventing its up-regulation of the ABA biosynthetic gene NCED3 in rice roots and of a β-glucosidase, activating ABA forms. EA105 counteracted the virulence-promoting effects of ABA on *M. oryzae* by inhibiting appressoria formation, preventing spores from increasing ABA biosynthesis and related signaling. ABA implication in plant protection by EA105 involved both direct mechanisms and plant signaling. The role of endogenous fungal ABA was confirmed through the inability of a knoc-kout mutant impaired in ABA synthesis to form lesions on rice, dramatically reducing fungal virulence. Studying the effect of virulence inducers (pH, temperature and acetosyringone concentration) on three homologous genes of *Agrobacterium tumefaciens* (VBP1, VBP2, and VBP3, involved in the T-DNA transfer), Yang et al. showed that vbp2 was affected by pH and by the deletion of vbp1. Results indicated that, in addition to T-complex recruitment, the three homologous genes were involved also in other biological processes.

Martínez-Hidalgo et al. reported how *Micromonospora* strains control fungal pathogens by stimulating plant immunity. This Gram-positive inhabits nitrogen fixing nodules of healthy leguminous plants, with PGP effects. Inoculation of tomato roots with antifungal *Micromonospora* isolates reduced leaf infection by *Botrytis cinerea*, with a durable induced systemic resistance. Gene expression analyses showed that *Micromonospora* stimulates plant defense, enhancing jasmonate-regulated defense pathways, an effects confirmed using defense-impaired tomato mutants. Nodule-isolated *Micromonospora* strains appear as excellent biocontrol agents, combining antifungal activity with the ability to elicit plant immunity.

Pérez et al. showed that the *Trichoderma parareesei* gene Tparo7 encodes a chorismate mutase (CM), an intermediate of aromatic amino acids, essential in protein synthesis and precursor of many secondary metabolites. Decreased levels of Tparo7 in silenced transformants showed reduced CM activity, lower growth rates and mycoparasite behavior against phytopathogenic fungi (*R. solani*, *F. oxysporum,* and *B. cinerea*) in dual cultures. Higher amounts of aromatic metabolites (tyrosol, 2-phenylethanol and SA) were produced from the silenced transformant, which inhibited growth of *F. oxysporum* and *B. cinerea*. The silenced transformants also showed reduced colonization of tomato root *in vitro*. In greenhouse assays the

plants colonized by the silenced transformants were reduced, with a higher susceptibility to *B. cinerea*. The treated plants became yellowish and were defective in JA- and ethylene-regulated signaling pathways, as shown by expression analysis of of lipoxygenase 1, ethylene-insensitive protein 2, and PR protein 1 genes.

Greenhouse trials with fermentation broth of *B. amyloliquefaciens* LJ02 showed effective reduction of cucurbits powdery mildew in China. Li et al. reported that treated seedlings produced superoxide dismutase, peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase at levels higher than control. Free SA, with the PR-1 gene product, increased after leaf treatments, suggesting SA-mediated defense. Secretions from treated cucumber leaves also inhibited fungal spores germination in the rhizosphere. The bacterium and its fermented products showed SA induction, available for powdery mildew biocontrol through systemic resistance.

-OMICS

Recent "-omics" research advances coupled to progressive cost reduction allow a better understanding on many trophic interactions in rhizosphere systems (Massart et al., 2015). The genomes of 12 *Bacillus subtilis* strains with PGP activity were sequenced and analyzed by Hossain et al. The strains exhibited high genomic diversity, except highly conserved *B. amyloliquefaciens* strains (*B. subtilis* group), with 32–90% gene family similarity among *Bacillus* genomes and 2839 core genome genes, similar to *B. amyloliquefaciens* subsp. *plantarum*. Comparative analyses identified genes linked to biocontrol and roots/leaf colonization, including 73 genes from subsp. *plantarum* with functions related to signaling, transport, secondary metabolites and carbon utilization. They encoded several secondary metabolites, with conserved polyketide biosynthetic clusters encoding difficidin and macrolactin. Deletion of secondary metabolite genes in *B. amyloliquefaciens* subsp. *plantarum* showed that expression of difficidin is critical to reduce damage by *Xanthomonas axonopodis* pv. *vesicatoria* on tomato.

Van der Voort et al. sequenced and analyzed the genome of *Pseudomonas* sp. SH-C52. This bacterium, from a *R. solani* suppressive soil, has antifungal activity attributed to the chlorinated 9-amino-acid lipopeptide thanamycin. Its 6.3 Mb genome showed 5579 predicted ORFs, matching *Pseudomonas corrugata* as closest taxon. In silico, secondary metabolite analysis showed six non-ribosomal peptide synthetase gene clusters, including clusters for thanamycin and the 2-amino acid antibacterial lipopeptide brabantamide. Thanamycin is an antifungal whereas brabantamide has anti-oomycete activity, affecting phospholipases of *Phytophthora infestans*. A third lipopeptide, thanapeptin, was discovered, with structural variants active against *P. infestans*. Remaining clusters encode for another unknown lipopeptide. Results indicated a potential for SH-C52 lipopeptides with different antimicrobial activities.

Lichen microbioms were studied by Cernava et al. to analyze antagonism against bacteria and fungi. The *Lobaria pulmonaria* antagonistic community was dominated by *Stenotrophomonas*, *Pseudomonas,* and *Burkholderia*. 24.5% of isolates were antagonists, accounting for 7% of the metagenome, indicative of an overrepresentation in the culturable fraction. *Stenotrophomonas* bioactive components like spermidine showed PGP effects. Lichens represent important reservoirs for antagonistic bacteria, useful for biotechnological applications.

OUTLOOK AND FUTURE CHALLENGES

Articles in this Research Topic provide a polyhedral perspective on many issues in which beneficial microorganisms are involved. Data indeed demonstrate that they represent an as yet poorly-explored resource, whose exploitation may actively sustain plant protection and crop production. Given the huge number of microbial species present on the planet, the microorganisms studied represent just the tip of an iceberg. Data produced are, however, informative enough about their genetic and functional biodiversity, as well as about the ecosystem services they provide to underpin crop production. Challenges for future research work concern not only the biology of these species, but also the practices required to protect their biodiversity and to extend their application in the wide range of agricultural soils and systems present in the world. Agriculture cannot remain successfully and sustainable unless plant germplasm and useful microbial species are integrated, a goal for which new knowledge and information-based approaches are urgently needed.

AUTHOR CONTRIBUTIONS

AC wrote the paper, JM and CP have made direct contributions to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Impact of a Bacterial Volatile 2,3-Butanediol on *Bacillus subtilis* Rhizosphere Robustness

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Volatile compounds, such as short chain alcohols, acetoin, and 2,3-butanediol, produced by certain strains of root-associated bacteria (rhizobacteria) elicit induced systemic resistance in plants. The effects of bacterial volatile compounds (BVCs) on plant and fungal growth have been extensively studied; however, the impact of bacterial BVCs on bacterial growth remains poorly understood. In this study the effects of a well-characterized bacterial volatile, 2,3-butanediol, produced by the rhizobacterium *Bacillus subtilis*, were examined in the rhizosphere. The nature of 2,3-butanediol on bacterial cells was assessed, and the effect of the molecule on root colonization was also determined. Pepper roots were inoculated with three *B. subtilis* strains: the wild type, a 2,3-butanediol overexpressor, and a 2,3-butanediol null mutant. The *B. subtilis* null strain was the first to be eliminated in the rhizosphere, followed by the wild-type strain. The overexpressor mutant was maintained at roots for the duration of the experiment. Rhizosphere colonization by a saprophytic fungus declined from 14 days post-inoculation in roots treated with the *B. subtilis* overexpressor strain. Next, exudates from roots exposed to 2,3-butanediol were assessed for their impact on fungal and bacterial growth *in vitro*. Exudates from plant roots pre-treated with the 2,3-butanediol overexpressor were used to challenge various microorganisms. Growth was inhibited in a saprophytic fungus (*Trichoderma* sp.), the 2,3-butanediol null *B. subtilis* strain, and a soil-borne pathogen, *Ralstonia solanacearum*. Direct application of 2,3-butanediol to pepper roots, followed by exposure to *R. solanacearum*, induced expression of *Pathogenesis-Related* (*PR*) genes such as *CaPR2*, *CaSAR8.2*, and *CaPAL*. These results indicate that 2,3-butanediol triggers the secretion of root exudates that modulate soil fungi and rhizosphere bacteria. These data broaden our knowledge regarding bacterial volatiles in the rhizosphere and their roles in bacterial fitness and as important inducers of plant defenses.

Keywords: PGPR, ISR, volatile, 2,3-butanediol, bacteria robustness

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INTRODUCTION

The rhizosphere is defined as the narrow area surrounding the plant root system. Root exudates within the rhizosphere act as a food source for other organisms (Bowen and Rovira, 1999). As a result, the rhizosphere is an important habitat for many different microbes, and acts as a competitive arena for roots and soil-borne-pathogenic and rhizosphere bacteria (rhizobacteria; Vespermann et al., 2007). Among the rhizobacteria, plant growth-promoting rhizobacteria (PGPR) have been the subject of much research in recent decades. PGPR colonization of roots promotes plant growth and enhances crop yields (biostimulants), and can help protect against plant pathogens (bioprotectants; Kloepper and Metting, 1992; Kloepper et al., 2004; Ryu et al., 2004; Calvo et al., 2014; Chung et al., 2016). PGPRs act as biostimulants and bioprotectants by (1) acting antagonistically to target pathogens, (2) producing plant hormone mimics, and (3) inducing systemic resistance (Kloepper and Ryu, 2006).

Volatile compounds such as isoprene, terpenes, alkanes, alkenes, alcohols, esters, carbonyls, and acids can influence communication between organisms, including between bacteria and plants (Kesselmeier and Staudt, 1999; Ryu et al., 2005a; Kai et al., 2007). Previous research reported that PGPR bacilli emitted volatiles that triggered plant growth promotion and induced systemic resistance (ISR; Ryu et al., 2003, 2004; Chung et al., 2016). After this discovery, numerous studies identified further bacterial volatiles and determined their effects on plant responses. Some volatiles are now available for field applications (Cortes-Barco et al., 2010a,b; Farag et al., 2013; Chung et al., 2016). One well-characterized volatile is 2,3-butanediol, which was examined in multiple Gram-negative and Gram-positive bacterial species such as *Bacillus* spp., *Aerobacter* spp., *Serratia* spp., *Enterobacter* spp., and *Klebsiella* spp. (Barrett et al., 1983; Voloch et al., 1985; Ryu et al., 2004; Han et al., 2006). Acetoin is the last synthesis intermediate for 2,3-butanediol, and metabolic conversion of acetoin to 2,3-butanediol is reversible in most bacteria but irreversible in fungi such as yeast (Syu, 2001). Acetoin and 2,3-butanediol also mediate plant-beneficial effects such as growth promotion and ISR in model plants and crops under *in vitro* and *in situ* conditions (Ryu et al., 2003, 2004; Han et al., 2006; Hahm et al., 2012). Recent metabolic engineering approaches facilitated increased production of 2,3-butanediol in non-producer or low-producer bacterial species such as *Klebsiella oxytoca*, *Escherichia coli*, and *Paenibacillus polymyxa* by introduction of new genes and modification of biosynthetic pathways (Ji et al., 2011, 2014; Yang et al., 2013; Bai et al., 2015). However, the benefit to bacteria of producing 2,3-butanediol in the anaerobic conditions of the rhizosphere remains unknown.

The role of 2,3-butanediol in bacterial fitness has not been intensively studied. Early research in a mouse model revealed that 2,3-butanediol synthesis in *Vibrio cholerae* conferred a survival advantage *in vivo* during infection of intestines (Yoon and Mekalanos, 2006; Xiao and Xu, 2007). It is thought that 2,3-butanediol acts as a neutralizer in the acidic conditions of the intestinal cells. A null mutant that was unable to produce 2,3-butanediol was unable to colonize or maintain the bacterial populations during infection (Xiao and Xu, 2007; Pradhan et al., 2010; Bari et al., 2011). We hypothesized that 2,3-butanediol might play a similar bacterial fitness role in the rhizosphere. In this study, the effects of 2,3-butanediol on rhizosphere colonization were examined using three strains: *Bacillus subtilis* 168, BSIP1174 [a 2,3-butanediol null mutant referred to as "2,3-B(−)"], and BSIP1171 [an overexpression strain referred to as "2,3-B(+++)"]. In addition, the indirect effects of 2,3-butanediol on secretion of root exudates were examined in pepper roots. Finally, the antimicrobial capacity of root exudates elicited by 2,3-butanediol treatment was assessed. Exudates exhibited selective antagonism against pathogenic bacteria such as *Ralstonia solanacearum*. To our knowledge, this is the first report to characterize a bacterial volatile under *in situ* conditions in plants and to validate *in vitro*.

MATERIALS AND METHODS

Plant Materials and Bacterial Preparation

Plants were grown were carried out as previously described (Kang et al., 2007). Briefly, seeds of *Capsicum annuum* were surface-sterilized with 6% sodium hypochlorite, washed four times with sterile distilled water (SDW), and then maintained at 25°C for 3 days until germination on Murashige and Skoog medium (Duchefa, Haarlem, the Netherlands). Germinated seeds were then transplanted to soilless media (Punong Horticulture Nursery Media LOW, Punong, Co. Ltd., Gyeongju, South Korea). Plants were grown at 25 ± 2°C under fluorescent light (12 h/12 h day/night cycle, 7000 lx light intensity) in a controlled-environment growth room. After establishment of seedlings, plants were transferred to the KRIBB greenhouse facility in Daejeon, South Korea.

Three *B. subtilis* strains were used to assess the role of 2,3-butanediol on bacterial rhizosphere competence: 168, BSIP1174 [2,3-butanediol null mutant referred to as 2,3-B(−)], and BSIP1171 [2,3-butanediol overexpression mutant referred to as 2,3-B(++); Cruz et al., 2000]. Bacterial suspension (5 ml at 10^8 colony forming units/ml) was used to inoculate pepper roots, as described previously (Lee et al., 2012, 2013). A spontaneous rifampicin resistance mutant of wild-type *B. subtilis* 168 was isolated previously (Ryu et al., 2005b). Bacterial strains were isolated from plant roots using specific antibiotics in the tryptic soy broth agar growth medium (TSA, BactoTM, BD, Sparks, MD, USA): 50 μg/ml rifampicin for strain 168, 10 μg/ml spectinomycin for 2,3-B(−), and 10 μg/ml spectinomycin plus 5 μg/ml chloramphenicol for 2,3-B(++). The experiment was repeated three times with five replications (one plant per replication).

The naturally occurring soil fungus was isolated from dilution plating method of pepper root system when we attempted to assess *B. subtilis* population described above.

Disease Assay of *Ralstonia solanacearum*

Spontaneous rifampicin resistant *R. solanacearum*, was grown on solid Casamino acid-Peptone-Glucose [CPG, 1 g casamino acid (casein hydrolysate), 10 g peptone, 5 g glucose, and 18 g

agar per 1 L water] medium containing 100 μg/ml rifampicin at 30°C for 2 days, scraped off the plates, re-suspended in sterilized distilled water and adjusted to 10^8 cfu/ml concentration for further experiments (Lee et al., 2012). The plants pretreated with 1 mM BTH was used a positive control. The 10 ml suspension of *R. solanacearum* was drenched on 3 weeks-old pepper seedlings at 1 week after 1 mM and 10 μM 2,3-butanediol and BTH drench-application as describe previously (Lee et al., 2012). To assess pathogen multiplication, the root sample at 0 and 3 days after pathogen challenge collected, macerated with sterile mortar and pestle, and plating on CPG agar medium containing 100 μg/ml rifampicin. The number of CFU was counted at 2–3 days after incaution of the plates.

Assessment of Bacterial Populations Isolated from Pepper Roots

Bacterial colonization on roots was determined at 0, 7, 14, 21, and 28 days after treatment, as described previously (Ryu et al., 2005b). Briefly, root samples were collected and, after removal of soil particles, roots were agitated in 20 ml of SDW in a flask. Samples of 10-fold serial dilutions were plated onto TSA containing appropriate antibiotics, as above. CFUs were counted after 1–3 days.

Assessing the Antifungal Capacity of Root Exudates

An *in vitro* assay was developed to test the antifungal capacity of root exudates (**Figure 2A**). Pepper seeds (cv. Bukwang) were prepared as described above. After 7 days of germination at 25°C, seeds were transferred to Petri dishes (diameter = 20 cm) and allowed to grow vertically. Plates were sealed with Saran wrap to retain moisture, and were half covered with aluminum foil (Daihan Eunpakgy Ind. Co., Ltd., Suwon, South Korea) to reduce exposure of roots to light from growth cabinets, which were set at 24 h light, 25°C (Vision Bio Tech., Seoul, South Korea). Four days after transplanting, 5 ml of 2,3-butanediol (1 mM or 1 μM) was drench-applied to the root system. Drenches with 1 mM benzothiadizole (BTH), which was commercialized SAR trigger by Syngenta as Actigard in the USA and BION in Europe and water were used as positive and negative controls, respectively. After cultivation for 2 days on potato dextrose broth agar (PDA, Becton, Dickinson and Company, Sparks, MD, USA) at 30°C, fungal spores were collected and their concentration was estimated using a hemocytometer. A sterilized cotton swab was used to inoculate the pepper root system with 10^5 CFU/ml fungus, avoiding direct contact with the root surface. To determine any inhibitory effects of the root exudate on fungal growth, growth of fungal mycelium was imaged daily for a week after spore inoculation using a digital camera (Nikon Coolpix 4500, Japan). Fungus-free zones around the pepper roots were measured, and the mean was calculated (n = 20). The experiments were repeated three times.

ITS-Based Fungus Identification

Fungus was isolated from pepper rhizosphere during assessing population density of *B. subtilis*. The fungus was cultured on the Poate Dextrose Broth agar (TSA, BactoTM, BD, Sparks, MD, USA) Total genomic DNA was extracted from the purified isolates using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, South Korea). The nuclear ribosomal internal transcribed spacer (ITS) region of genomic DNA was amplified with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers using Quick PCR Premix containing Taq DNA polymerase, dNTPs, reaction buffer, and tracking dye (Genenmed, Daejeon, South Korea). PCR analyses were conducted in a PTC100 Thermal Cycler (MJ Research, Watertown, MA, USA) using an initial denaturation step of 95°C for 5 min; followed by 29 cycles of denaturation for 1 min at 94°C, primer annealing for 30 s at 52°C, and extension for 30 s at 72°C; with a final extension for 10 min at 72°C. Amplified PCR products were detected by electrophoresis on a 0.75% agarose gel, and purified with AccuPrep® PCR Purification Kit (Bioneer, Daejeon, South Korea). The ITS region of the yeast isolates was sequenced using the same PCR primers and the ABI3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequence was submitted to NCBI[1] for identification of the fungus.

Collection of Root Exudates after Induction by 2,3-Butanediol Treatment

A new protocol was developed to collect root exudates from pepper seedlings. Seeds were germinated as described above, then positioned between two sterile filter papers (diameter = 120 mm) in a Petri dish (diameter = 150 mm and height = 20 mm). MS broth (10 ml) was applied to the Petri dish, which was then positioned vertically in an incubator at 25°C. After 4 days of incubation, excess MS broth was removed. A further 15 ml of MS broth containing 1 mM 2,3-butanediol, 1 μM 2,3-butanediol, or 1 mM BTH was drench-applied to filter papers. Treated Petri dishes were sealed with Saran wrap and partially covered with foil, as described above. Petri dishes were incubated at 25°C for a further 7 days before collection of root exudates. To collect root exudate in the hydroponic system, we modified our system previously described (**Figures 2D,F** inset; Song et al., 2015). Pepper seeds were surface-sterilized and germinated, as described above. Four-days-old seedlings were transferred to plates (60 mm × 15 mm, SPL) containing 26 ml of 0.5X MS liquid media. Plates were placed in the plastic container (phytohealth, 103 mm × 78.6 mm, SPL). 2 mM 2,3-butanediol, BTH and water control treatments were applied to plants as described above. The root exudates were collected at 7 and 14 days after treatments. For each replicate, containing 16 plants, 80 ml of root exudate was collected from plates. No media contamination was observed in the entire experiment.

Assessment of Root Exudates on Bacterial Growth

A 96-well based assay was used to assess the effects of root exudates on bacterial growth. *B. subtilis* strains 168, 2,3-B(−), and 2,3-B(++) were cultured in TSB containing antibiotics

[1]http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=TargLociBlast

as detailed above, then washed three times in 0.8% NaCl solution. Bacterial growth was monitored in 150 μl volumes containing TSB and root exudate (1:1 ratio). Optical density was determined every 15 min using a Bioscreen C system (Fluoroskan; Labsystems, Helsinki, Finland) at 30°C with continuous shaking.

Bacillus Growth on the Different pH

The phenotype analysis for different pH was carried out by using a new tool, Phenotype MicroArrays (PMs). The 2,3-butanediol over-producer [2,3-B (++)] and non-producer [2,3-B (−)] were assayed on PM (Biolog) lane A1 to A12 of microplates PM10, testing different pH range from 3.5 to 10. PM technology uses the irreversible reduction of tetrazolium violet to formazan as a reporter of active metabolism. All procedures were performed as indicated by the manufacturer and previous study (Zhang and Biswas, 2009). Strains were grown at 30°C on BUG agar (Biolog), and then, each strain was picked with a sterile cotton swab from the agar surface and suspended in 15 ml of inoculation fluid (IF-0; Biolog) until a cell density of 85% transmittance was reached on a Biolog turbidimeter. In order to inoculate microplates PM10, 1% tetrazolium violet (vol/vol) was added to the suspension and the mixture was inoculated (100 μl per well). The photo was taken at 24 h after bacterial inoculation.

Quantitative RT-PCR

Expression analysis of 2,3-butanediol-elicited defense genes was performed using quantitative real-time polymerase chain reaction (qRT-PCR), as described previously (Yang et al., 2011). Expression of *C. annum* basic β-1,3-glucanase (*CaPR 2*), *1*-aminocyclopropane-1-carboxylic acid (*CaACC*), *Systemic Acquired Resistance 8.2* (*CaSAR8.2*), *phenylalanine ammonia* (*CaPAL*), *lipid transfer protein* (*CaLTP 1*), *glutathione S-transferases* (*CaGST*), and *basic class II chitinase* (*CaChi2*) was reported previously during the defense response (Marrs, 1996; Garcia-Pineda and Lozoya-Gloria, 1999; Jung and Hwang, 2000; Lee et al., 2001; Hong and Hwang, 2002; Park et al., 2002; Mateos et al., 2009; Mazourek et al., 2009). The following primers were used: 5′-TAGTGAGACTAAGAAAGTTGGACG-3′ (*CaSAR8.2* Forward; GenBank accession no. AF327570.1), 5′-AAGAGTGCATGCAGTATCACAAAG-3′ (*CaSAR8.2* Reverse), 5′-ATTGGACGATGGAAGCCATCACCAG-3′ (*CaChi2* Forward; GenBank accession no. AF091235.1), 5′-ATATTCCGAATGTCTAAAGTGGTAC-3′ (*CaChi2* Reverse), 5′-TTTTAGCTATGCTGGTAATCCGCG-3′ (*CaPR2* Forward; GenBank accession no. AF227953.1), 5′-AAACCATGAGGACCAACAAAAGCG-3′ (*CaPR2* Reverse), 5′-CTCTAGGAAGGTGCTGTGGTGTC-3′ (*CaLTP1* Forward; GenBank accession no. AF118131.1), 5′-ACGGAAGGGCTGATTTCGGATG-3′ (*CaLTP1* Reverse), 5′-TCCACAAAGGGTCATGGTTT-3′ (*CaGST* Forward; GenBank accession no. HQ010689.1), 5′-GCCCTCTTCAATGACAGGAA-3′ (*CaGST* Reverse), 5′-ATTCGCGCTGCAACTAAGAT-3′ (*CaPAL* Forward; GenBank accession no. EU61657-5.1), 5′-CACCGTGTAAGGCCTTGTTT-3′ (*CaPAL* Reverse), 5′-AGTGGCCTTCAACTCCTCAA-3′ (*CaACC* Forward; GenBank accession no. AJ011109.1), and 5′-TTCCGTTTGTGATCACCTCA-3′ (*CaACC* Reverse). Relative mRNA levels were calibrated and normalized to the level of *CaActin* mRNA (GenBank accession no. AY572427). As a control, to ensure that equal amounts of RNA were used in each experiment, *CaActin* was analyzed using the primers 5′-CACTGAAGCACCCTTGAACCC-3′ and 5′-GAGACAACACCGCCTGAATAGC-3′. Candidate priming genes were amplified from 100 ng of cDNA by PCR using an annealing temperature of 55°C. A Chromo4 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) was used to carry out qRT-PCR. Reaction mixtures (20 μl) contained 10 μl of 2× Bril-liant SYBR Green QPCR master mix (Bio-Rad Laboratories, Hercules, CA, USA), cDNA, and 10 pmol of each primer. The thermocycle parameters were as follows: 10 min at 95°C, followed by 45 cycles of 30 s at 95°C, 60 s at 55°C, and 30 s at 72°C. Conditions were determined by comparing threshold values in a series of dilutions of the RT product, followed by a non-RT template control and a non-template control for each primer pair. Relative RNA levels were calibrated and normalized to the level of *CaACT1* mRNA (GenBank accession no. AY572427).

Statistical Analysis

Data were subjected to ANOVA using JMP software version 4.0 (SAS Institute, Cary, NC, USA). Significance of biological or chemical treatment effects was determined by the magnitude of the *F*-value at $P = 0.05$. When a significant *F*-value was obtained for treatments, separation of means was accomplished using Fisher's protected LSD at $P = 0.05$. Results of repeated trials of each experiment outlined above were similar, and one representative trial of each experiment is reported.

RESULTS

Effect of 2,3-Butanediol Production on Rhizosphere Competence of *Bacillus subtilis*

The role of bacterial volatile 2,3-butanediol *in situ* was examined in wild-type *B. subtilis* 168 and wild-type-derived null and overexpression strains. Wild-type *B. subtilis* 168 was previously shown to produce 2,3-butanediol (Ryu et al., 2004). A *pta-als* double mutant, 2,3-B(−), was unable to produce 2,3-butanediol, and the *pta* mutant 2,3-B(++) was an overproducer of 2,3-butanediol. Rifampicin resistance was generated in the three strains to allow selection from the pepper roots (data not shown). We hypothesized that the population densities of the three strains in the pepper rhizosphere at different time points (7 days intervals from inoculation) would differ. Total bacterial populations on pepper roots at inoculation were 10^7–10^8 cfu/g root and did not significantly differ between treatments (**Figure 1C**). Initial populations of each strain were $\sim 10^7$ cfu/g root [7.0, 7.2, and 7.0 log cfu/g root for 2,3-B(++), 2,3-B(−), and 168, respectively]. After 7 days, populations were 7.3, 6.1, and 5.3 log cfu/g root for 2,3-B(++), 2,3-B(−), and 168, respectively. After 14 days, the population densities of 2,3-B(++) and 168 were 2.8- and 3-fold higher than that of 2,3-B(−), respectively (**Figure 1A**). Populations of strains 168 and 2,3-B(++) remained at pepper

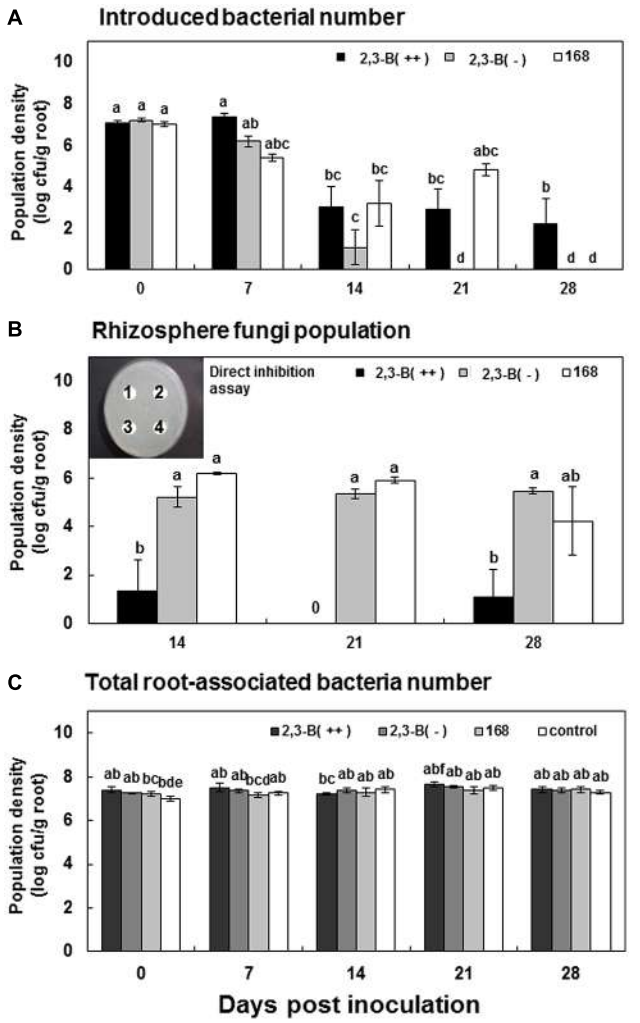

FIGURE 1 | The effect of 2,3-butanediol produced by *Bacillus subtilis* on bacterial rhizosphere competence. Pepper roots were inoculated with 5 ml of bacterial suspension at 10^8 cfu/ml (day 0). Pepper root samples were collected after removal of soil from roots. **(A)** Population densities of *B. subtilis* strains 168, 2,3-B(++), and 2,3-B(−) isolated from pepper roots 0, 7, 14, 21, and 28 days after inoculation. *B. subtilis* 168, the wild type; 2,3-B(++), 2,3-butanediol overexpression mutant derived from *B. subtilis* 168; 2,3-B(−), 2,3-butanediol null mutant derived from *B. subtilis* 168. **(B)** Fungal colonies on tryptic soy agar containing 100 μg/ml rifampicin recovered from pepper roots inoculated with strains 168, 2,3-B(++), and 2,3-B(−). **(C)** Direct inhibition test of 2,3-butanediol. 50 μl undiluted solution 1 (1), 10 fold diluted (2), and 100 fold diluted solution (3) 2,3-butanediol was spot-inoculated on the paper disks following spreading 10^5 cfu/ml *Trichoderma* sp., spores. The 50 μl water treatment was used as control (4).

roots 21 days after inoculation, at 2.8 and 4.8 log cfu/g root, respectively (**Figure 1A**). Strain 2,3-B(−) was not found at pepper roots 21 days after inoculation. After 28 days, only strain 2,3-B(++) was present at pepper roots, at 10^2 cfu/g root (**Figure 1A**). These results indicated that 2,3-butanediol facilitated maintenance of bacterial populations in the pepper rhizosphere. Unexpectedly, fungal colonies developed on the TSA plates used for isolation of *B. subtilis* from roots. The fungus was identified 98% as *Trichoderma* sp. from morphological characteristics and sequencing of the 18S ribosomal RNA ITS (data not shown). No fungal colonies were observed on isolation plates for 2,3-B(++) at 14, 21, and 28 days after inoculation (**Figure 1B**). Larger fungal populations were isolated from roots treated with strains 168 and 2,3-B(−) (**Figure 1B**). Fungal populations were 5.2, 5.3, and 5.4 log cfu/g root in the 2,3-B(−) treatment at days 14, 21, and 28, respectively (**Figure 1B**). For treatment with strain 168, fungal populations gradually decreased with time, at 6.1, 5.8, and 4.2 log cfu/g root at days 14, 21, and 28 respectively (**Figure 1B**). The number of *Trichoderma* sp. showed similar pattern when the repeated experiment was conducted. These results suggested that fungal growth could be directly inhibited by 2,3-butanediol. To test this, the fungus was challenged by pharmacological applications of 2,3-butanediol at different concentrations (10 μM–1 mM); however, no inhibition was observed, indicating that 2,3-butanediol did not directly affect fungal growth (**Figure 1B**). These results suggested that exposure of pepper roots to 2,3-butanediol might trigger the production of root exudates antagonistic to fungal growth.

Indirect Effect of 2,3-Butanediol on Inhibition of Soil Fungus

To determine whether exudates of pepper roots treated with 2,3-butanediol contained antifungal agents, a novel protocol was devised in which seedlings were cultivated on Petri dishes, drenched with 2,3-butanediol, and inoculated with fungus (**Figure 2A**). Fungal growth was inhibited with all treatments (1 mM 2,3-butanediol, 1 mM BTH, and water; **Figures 2B–D**), with clear root inhibition zones of 2.45, 1.41, and 0.875 mm, respectively (**Figure 2E**). These results indicated that root exudates elicited by treatment with 2,3-butanediol and BTH inhibited the growth of soil fungus. We therefore wished to test whether root exudates elicited by 2,3-butanediol could also inhibit the growth of other microorganisms such as saprophytic and pathogenic soil bacteria. To obtain the clear evidence of antifungal capacity in the root exudate from pepper plant treated with 2,3-butanediol, the hydroponic system was set-up and successfully obtained enough root exudates. The three time inoculation of root exudate from 2,3-butanediol pre-treated root only showed clear zone (No. 4 in **Figures 2E,F**) while one time root or chemical alone treatments did not show any inhibitory effect (**Figures 2E,F**).

Growth Kinetics of *B. subtilis* after Exposure to 2,3-Butanediol and Root Exudates

To understand the role of root exudates (**Figure 3A**), and their effects on growth of *B. subtilis* strains 168, 2,3-B(++) (strain BSIP1171), and 2,3-B(−) (strain BSIP1174) and other soil-borne bacterial species, the growth kinetics of each treatment were assessed. *B. subtilis* strains 168, 2,3-B(++), and 2,3-B(−) had similar growth patterns on TSB medium, with maximum optical density of $OD_{600} = 1$ indicating that the mutation of the garget genes did not affect bacterial robustness under ideal growth condition (**Figure 3B**).

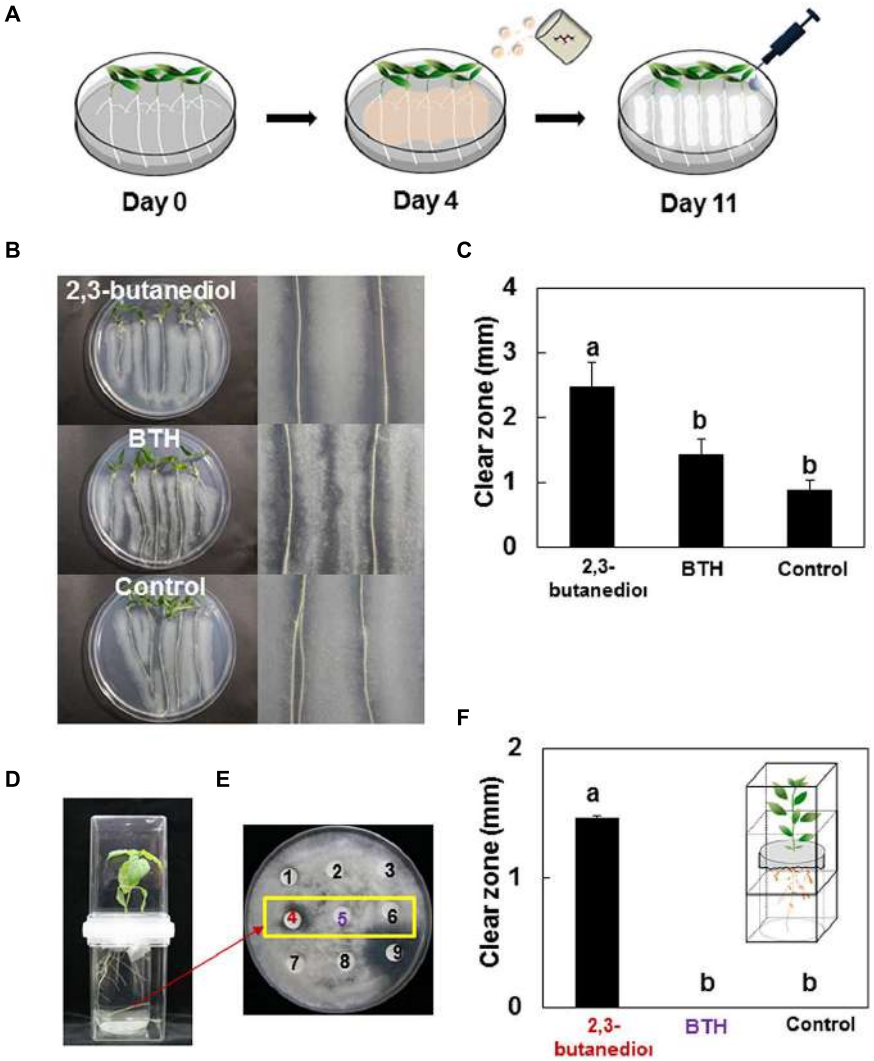

FIGURE 2 | Effect of 2,3-butanediol-elicited root exudates on fungal growth in the rhizosphere. (A) Schematic of protocol for assessing fungal growth inhibition by root exudate elicited by 2,3-butanediol pre-treatment. Images in **(B)**, were taken 3 days after inoculation with *Trichoderma* sp. upon 1 mM 2,3-butanediol, 1 mM BTH, and water control. Left panels, whole pepper seedlings; right panels, magnified images of pepper roots. **(C)** Quantification of clear fungal inhibition zones surrounding roots. **(D)** Schematic of protocol for hydroponic system to collect root exudates by 2,3-butanediol pretreatment. **(E)** The fungal growth inhibition assay by root exudate. 1 = one time inoculation of root exudate treated with 2 mM 2.3-butanediol, 2 = one time inoculation of root exudate treated with 1 mM BTH, 3 = one time inoculation of root exudate treated with water, 4 = two time inoculation of root exudate treated with 2 mM 2.3-butanediol, 5 = two time inoculation of root exudate treated with 1 mM BTH, 6 = two time inoculation of root exudate treated with water, 7 = 2 mM 2.3-butanediol alone, 8 = 1 mM BTH alone, 9 = 50 μg/ml kanamycin as positive control **(F)**. Quantification of clear fungal inhibition zones by root exudates from root treated by 2,3-butanediol, BTH, and water treatments. Different letters above bars indicate significant differences between treatments as determined using LSD at $P = 0.05$.

When exposed to root exudate from pepper root elicited by 2,3-butanediol treatment, growth of strains 168 (the wild type) and 2,3-B(++) (overexpression mutant) was less inhibited than that of 2,3-B(−) (null mutant; **Figure 3C**). After 2 days (48 h), growth of overexpresser strain 2,3-B(++) was higher when treated directly with 1 mM 2,3-butanediol (control) than when treated with exudates of 2,3-butanediol-treated pepper root (**Figure 3C**). Until log phase, the growth curve of strain 168 was similar between bacteria exposed to control and exudates (**Figure 3B**); nevertheless, after log phase, growth of control-treated 168 exceeded that of exudate-treated 168 (**Figure 3B**). Conversely, growth of exudate-treated 2,3-B(++) slightly exceeded that of control-treated 2,3-B(++) (**Figure 3C**). Furthermore, 2,3-B(−) was more sensitive to 2,3-butanediol itself while 2,3-B(++) was more resistant compared to wild type indicating that 2,3-butanediol non-producer can be less fitness than 2,3-butanediol producer (**Figure 3C**). The data suggested that 2,3-butanediol played an important role in protecting *B. subtilis* cells against harmful plant root exudates.

We next examined the effect of 2,3-butanediol on other soil bacteria, namely, the non-pathogenic biological control

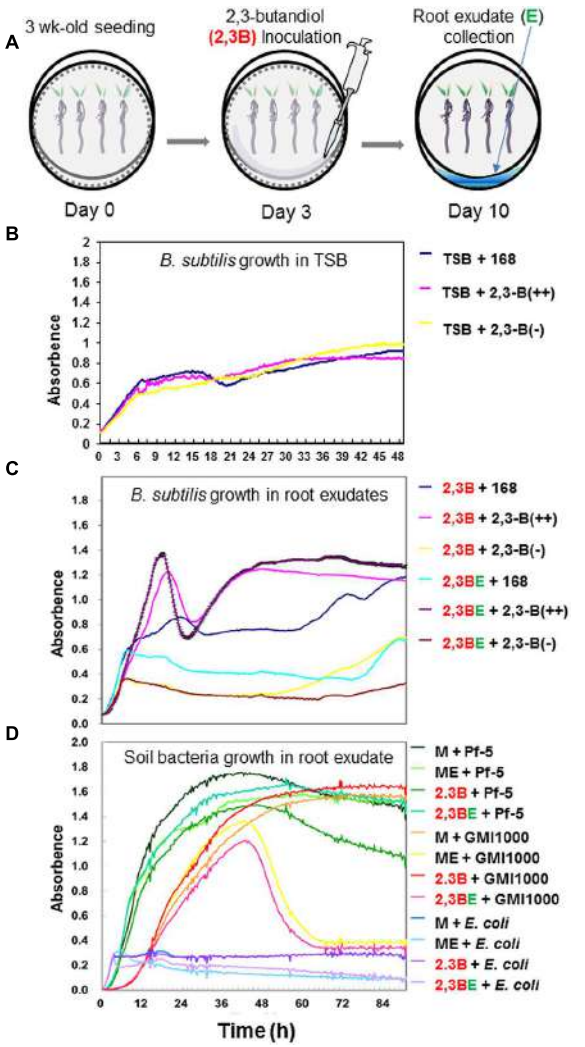

FIGURE 3 | Growth kinetics of *B. subtilis* wild-type and mutant strains after exposure to root exudates elicited by 2,3-butanediol. Initial cell culture concentrations were OD_{600} = 0.02 and data are shown as log-normal plots. Tryptic soy broth supplemented with root exudate at a 1:1 ratio was applied to pepper roots. Aliquots (150 μl) from each culture were transferred to 100 wells of a Bioscreen plate. Plates were incubated in a Bioscreen C (Fluoroskan; Labsystems, Helsinki, Finland) with shaking at 30°C for ∼4 days. The OD_{600} of each well was measured every 15 min. 2.3B = 2,3-butanediol; 23BE = root exudate collected from 2,3-butanediol-treated root system; 168 = *B. subtilis* 168; BSIP 1174 = *B. subtilis* BSIP 1174 (non-producer); BSIP 1171 = *B. subtilis* BSIP 1174 (overproducer); Pf-5 = *Pseudomonas protegens* Pf-5; GMI1000 = *Ralstonia solanacearum* GMI1000; M = MS broth; ME = MS broth amended with root exudate without treatment. **(A)** Schematic of protocol to extract root exudates after 2,3-butanediol application. **(B)** Growth kinetics of the three strains in control TSB medium. The figure indicate background expression of three strains 168, 2,3-B(++), and 2,3-B(−). **(C)** Growth of bacterial strains 168, 2,3-B(++), and 2,3-B(−) after treatment with 2,3-butanedol alone (referred to as 2,3B) or 2,3-butanediol-elicited root exudate (referred to as 2,3BE). **(D)** Growth kinetics of *P. protegens* Pf-5, *Ralstonia solanacearum* GMI1000, and *E. coli*. *P. protegens* Pf-5 is a non-pathogenic saprophyte that inhabits soil, water, and plant surface environments. Growth of *P. protegens* Pf-5 was not inhibited by 2,3-butanediol-elicited exudate. Growth of GMI1000, a soil-borne bacterial wilt pathogen, was inhibited by root exudates. *E. coli* was included as a bacterial control. Data shown are mean ± SEM of triplicate experiments.

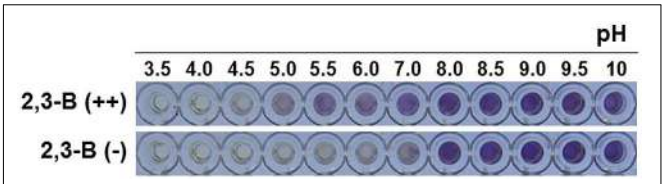

FIGURE 4 | Growth of *B. subtilis* 2,3-butanedol over-producer and non-producer under different pH condition. The phenotype analysis for different pH was carried out by PM10 plate of Phenotype MicroArrays (PMs). The 2,3-butanediol over-producer [2,3-B (++)] and non-producer [2,3-B (−)] were assayed on PM (Biolog) lane A1 to A12 of microplates PM10, testing different pH range from 3.5 to 10. The photo was taken at 24 h after bacterial inoculation.

agent *Pseudomonas protegens* Pf-5, the bacterial wilt pathogen *R. solanacearum* GMI1000, and *E. coli*. Growth of the non-pathogenic saprophyte Pf-5 did not much affected by amendment of 2,3-butanediol-elicited root exudate (M + Pf-5, ME +Pf5, 2,3B +Pf5, and 2,3BE + Pf-5; **Figure 3D**). However, bacterial growth upon 2,3BE + Pf-5 gradually decreased after 60 h. In contrast growth of the pathogen GMI1000 was inhibited by exudates from pepper roots treated with 2,3-butanediol at 42 h after root exudate treatment (2,3B + GMI1000 vs. 2,3-BE + GMI1000). Although, the treatment ME (MS media solution plus root exudate without 2,3-butanediol treatment) also showed inhibitory effect on growth of strain GMI1000, the inhibition by root exudate collected from 2,3-butanediol treatment was greater (ME + GMI1000 vs. 2,3-BE + GMI1000). Growth of *E. coli* was totally inhibited by all treatment including control 2,3-butanediol treatments, TSB, MS, and root exudate mixtures (**Figure 3D**). These results indicate that 2,3-butanediol-elicited root exudate contains compounds that allow selective inhibition of bacterial growth depending on bacterial species. The bacterial growth upon different pH condition using by Phenotype Microarray system showed that the growth of 2,3-B(++) and 2,3-B(−) was indicated at pH 5 and pH 7 respectively (**Figure 4**). This results clearly showed that 2,3-butanediol production acts an important role on bacterial fitness under the acidic pH condition.

Expression of Defense Genes in 2,3-Butanediol-Treated Pepper Roots

Induction of plant defense genes by 2,3-butanediol was assessed in pepper roots using qRT-PCR. At 3, 6, and 12 h after treatment, transcription of *basic* β-*1,3-glucanase* (*CaPR 2*) was higher in pepper roots treated with 1 mM 2,3-butanediol than in those treated with water (**Figure 5**). The effects of 2,3-butanediol on pathogen populations and gene expression in pathogen-challenged pepper roots were also assessed. Roots were exposed to the wilt pathogen *R. solanacearum* GMI1000 for 3 days, and bacterial populations were then determined. Fewer GMI1000 bacteria were recovered from roots treated with 1 mM and 10 μM 2,3-butanediol than from roots treated with water (**Table 1**). Root exudates of 2,3-butanediol-treated pepper were therefore able to inhibit growth of the bacterial pathogen GMI1000. Three days after pathogen challenge, expression levels of *Basic pathogenesis systemic acquired resistance gene 8.2* (*CaSAR8.2*) and

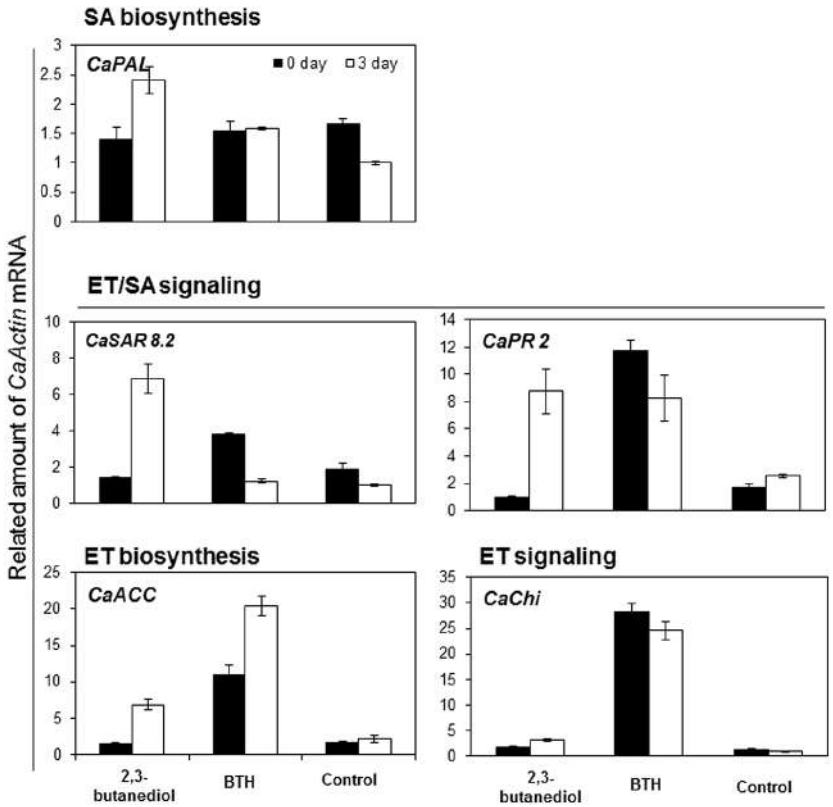

FIGURE 5 | Induction of defense genes in pepper roots after exposure to 2,3-butanediol. Expression levels of nine defense genes were quantified by qRT-PCR. Expression of pepper defense-related genes, a salicylic acid (SA) biosynthesis gene *Capsicum annum phenylalanine ammonia* (*CaPAL*), two ethylene (ET) and SA signaling related genes *systemic acquired resistance gene 8.2* (*CaSAR8.2*) and *basic* β*-1,3-glucanase* (*CaPR 2*), an ET biosynthesis gene *1-aminocyclopropane-1-carboxylic acid* (*CaACC*), and an ET signaling-related *basic class II chitinase* (*CaChi 2*) were evaluated 0 and 3 days after *R. solanacearum* drench application at 7 days following. Bars indicate standard deviation ($n = 4$).

phenylalanine ammonia (*CaPAL*) were higher in roots treated with 1 mM 2,3-butanediol than in positive control roots treated with 1 mM BTH (**Figure 5**). Conversely, expression levels of *1-aminocyclopropane-1-carboxylic acid* (*CaACC*), *lipid transfer protein* (*CaLTP 1*), and *basic class II chitinase* (*CaChi 2*) were lower in roots treated with 1 mM and 10 μM 2,3-butanediol than in positive control roots, but higher than in negative control roots treated with water (**Figure 5**). Transcription of *basic* β*-1,3-glucanase* (*CaPR 2*) was similar in roots treated with 1 mM and 10 μM 2,3-butanediol and roots treated with 1 mM BTH (**Figure 5**).

DISCUSSION

The first examinations of bacterial volatile-mediated plant growth and ISR, which used *B. subtilis* and *Arabidopsis thaliana* (Ryu et al., 2003, 2004), were followed by numerous studies examining the effects of bacterial volatiles on plants. Of the many volatile compounds identified from bacteria, 2,3-butanediol generated particular interest due to its broad spectrum effects on bacterial cells and induction of host responses (Ryu et al., 2004; Xiao and Xu, 2007; Rudrappa et al., 2010; Moons et al., 2011; Hahm et al., 2012). However, the impact of 2,3-butanediol on bacterial cells is yet to be elucidated. This prompted us to ask why bacteria, and soil bacteria in particular, might secrete 2,3-butanediol. Our results suggest that 2,3-butanediol promotes bacterial cell robustness against the effects of harmful compounds, such as root exudates (**Figures 1A** and **3C**). Both 2,3-butanediol and its precursor acetoin were shown to trigger ISR in plants (Han et al., 2006; Cortes-Barco et al., 2010a,b; Rudrappa et al., 2010; Hahm et al., 2012). Our study provides new information regarding the roles of 2,3-butanediol in root-associated bacteria *in situ*.

The volatile compound 2,3-butanediol is produced by many bacterial species as a result of a synthetic cascade, termed butanediol fermentation (Xiao and Xu, 2007). The exact role that butanediol fermentation plays in bacterial fitness is largely unknown. Classic literature suggested that 2,3-butanediol was formed to divert the cellular metabolism away from production of acidic compounds (Johansen et al., 1975). It was later discovered that 2,3-butanediol provided an alkaline environment during cell multiplication and protected bacterial cells against unfavorable acidic conditions, such as are found in eukaryotic hosts (Yoon and Mekalanos, 2006; Pradhan et al., 2010; Bari et al., 2011). Our results showed that a 2,3-butanediol null *B. subtilis* mutant was eliminated from the rhizosphere by 21 days

TABLE 1 | Effect of 2,3-butanediol on elicitation of plant immunity against *Ralstonia solanacearum*.

Treatments	Pathogen population (x 10^7 cfu/g root)	
	Day 0	Day 3
1 mM 2,3-butanediol	8.10[a]	8.35[a]
10 μM 2,3-butanediol	8.32[a]	8.26[a]
1 mM BTH	8.45[a]	7.92[a]
Control	7.96[a]	8.62[b]

The bacterial cell count was measured at 14 days after pathogen inoculation. Different letters indicate significant differences between treatments ($P = 0.05$ according to least significant difference). The experiment was repeated three times with similar results (sample size, $n = 5$ replications per treatment).

after inoculation; however, corresponding wild-type and 2,3-butanediol overexpressing strains persisted for 21 and 28 days, respectively (**Figure 1A**). This can be hypothesized that 2,3-butanediol production by *B. subtilis* increased robustness of the acidic rhizosphere environment similar with rhizosphere microorganism (Huang and Chen, 2003; Hinsinger et al., 2003). Root exudates, which include acidic root secretion products, contribute to lowering the rhizosphere pH by releasing H^+ or OH^- to compensate for unbalanced cation–anion uptake at the root surface (Hinsinger et al., 2003). In our system, the pH of root exudate was changed to 4.5 at 2 weeks after treatments compared to pH 5.8 at the beginning of experiment (data not shown). However, there are no difference upon pH between pretreatment of 2,3-butaendiol and water control. In addition to the proposed protective role of 2,3-butanediol in bacteria, 2,3-butanediol was recently shown to be critical for virulence of soft-rot plant pathogenic *Pectobacterium* spp. and *Dicheya* spp. Cell wall-degrading enzymes produced by the bacteria, such as protease, pectinase, and cellulose, require neutral pH for optimal function (Kwan et al., 2013). Bacterial robustness under acidic conditions and on normal artificial medium was compromised in a *Serratia plymuthica* budB mutant (Wevers et al., 2009).

In addition to the role of 2,3-butanediol as a bacterial protectant, 2,3-butanediol directly affects plant physiology and immunity (Han et al., 2006; Cortes-Barco et al., 2010a,b; Rudrappa et al., 2010; Hahm et al., 2012). In this study, the 2,3-butanediol null mutant could not stimulate plant defenses; however, wild-type *B. subtilis* successfully elicited a plant defense response against pathogens, indicating that bacterial 2,3-butanediol production played an important role in plant protection (Ryu et al., 2004; Rudrappa et al., 2010). No direct inhibition was observed when pathogenic bacteria and fungi were exposed to 2,3-butanediol, indicating that plant immunity rather than the direct effect of 2,3-butanediol provided inhibition (**Figure 1C**). Root application of 2,3-butanediol triggered root exudation and secretion of unknown compounds that differentially affected different species of bacteria in the rhizosphere. Exudate from roots treated with 2,3-butanediol suppressed growth of the soil-borne pathogen *R. solanacearum*, but enhanced growth of the saprophytic biocontrol bacterium *P. protegens* Pf-5 (**Figure 3D**). Our extra bioinformatics analyses revealed support the role of microbial production of 2,3-butanediol upon its robustness in rhizosphere: the *P. protegens* Pf-5 genome contains three major genes, acetolactate synthase (*alsS*), alpha-acetolactate decarboxylase (*alsD*), and 2,3-butanediol dehydrogenase (*bdhA*), needed for 2,3-butanediol production but *R. solanacearum* contains only the *alsS* gene with the result that less or no 2,3-butanediol can be produced (data not shown). Similarly, 2,3-butanediol null mutant of *B. subtilis* became more sensitive to pepper root exudates while the overexpressor of 2,3-butanediol was more resistance compared to wild type strain (**Figure 3C**). Moreover, 2,3-butanediol production help bacterial cells tolerate against acidic pH such as pH5 (**Figure 4**). Interestingly, the root exudate pH was stabilized as pH 4.5 in our hydroponic system indicating that *B. subtilis* may optimize the robustness using 2,3-butanediol production to acidification around root. Another possible explanation is that the two species may have different sensitivities to unknown compounds within the 2,3-butanediol-elicited root exudate. In our previous research, aboveground infestation of sucking insects like whitefly and aphids modulated the secretion of plant root exudates, leading to the recruitment of specific microbiota such as Gram-positive *Bacillus* spp. (Yang et al., 2011; Lee et al., 2012). Further research revealed that whitefly-infested tobacco plants secreted salicylic acid, which repressed *Agrobacterium tumefaciens* virulence genes and resulted in the suppression of crown gall formation (Song et al., 2015). We propose that plant defenses were induced by soil application of 2,3-butanediol, and that this induced the secretion of unknown compounds that targeted bacteria in a species-dependent manner. Detailed profiling of root exudates is required to characterize the compounds involved. The root exudate profiling was failed due to limitation to obtain large scale root exudates following 2,3-butanediol application.

Finally, one additional explanation is that different species may have different utilization capacities for root exudates. *P. protegens* Pf-5 is a saprophyte and encodes numerous enzymes that degrade organic materials, while *R. solanacearum* is a plant pathogen that primarily obtains nutrition from specific plant materials within xylem sap during the infection process (Salanoubat et al., 2002; Loper et al., 2007, 2012; Remenant et al., 2010).

The direct effect of 2,3-butanediol on pepper roots was demonstrated by changes in gene expression. Transcription of *CaPAL*, *CaSAR8.2*, *CaACC*, and *CaPR2* was affected when roots were drenched with 2,3-butanediol (**Figure 5**). This suggested that bacterial secretion of 2,3-butanediol activated plant defenses in the roots, mainly via salicylic acid and ethylene signaling pathways. This is supported by recent research in which direct soil application of 2,3-butanediol stimulated defense responses against foliar pathogenic anthracnose fungus and *Pseudomonas syringae* (Han et al., 2006; Cortes-Barco et al., 2010a,b). Previous studies showed 2,3-butanediol to be a signaling molecule involved in activation of immune responses in animal hosts. In mammals, 2,3-butanediol produced by pathogenic bacteria was closely associated with lung infections, including those caused by *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Serratia marcescens*. Under these conditions, 2,3-butanediol produced an anti-inflammatory effect via inhibition of NF-κB signaling (Hsieh et al., 2007). Taken together, these

data suggest that 2,3-butanediol is highly important for host colonization.

In summary, this study demonstrates that the bacterial volatile 2,3-butanediol has two key roles in the rhizosphere. First, 2,3-butanediol-elicited root exudates selectively affect different bacterial species, and, secondly, 2,3-butanediol protects bacterial cells against putative harmful plant root exudates and low pH. To our knowledge, this study is the first to demonstrate the significance of 2,3-butanediol on bacterial robustness *in planta*.

AUTHOR CONTRIBUTIONS

H-SY, Y-RA, S-YG, and C-MR conceived and designed research. H-SY, Y-RA, GCS, GL, and C-MR conducted all experiments. The manuscript was written by H-SY, Y-RA, and C-MR and approved by all other authors. SL conducted the pH sensitivity and provide **Figure 4**. The **Figure 4** was input during revising the manuscript.

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Augmenting Sulfur Metabolism and Herbivore Defense in *Arabidopsis* by Bacterial Volatile Signaling

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Sulfur is an element necessary for the life cycle of higher plants. Its assimilation and reduction into essential biomolecules are pivotal factors determining a plant's growth and vigor as well as resistance to environmental stress. While certain soil microbes can enhance ion solubility via chelating agents or oxidation, microbial regulation of plant-sulfur assimilation has not been reported. With an increasing understanding that soil microbes can activate growth and stress tolerance in plants via chemical signaling, the question arises as to whether such beneficial bacteria also regulate sulfur assimilation. Here we report a previously unidentified mechanism by which the growth-promoting rhizobacterium *Bacillus amyloliquefaciens* (GB03) transcriptionally activates genes responsible for sulfur assimilation, increasing sulfur uptake and accumulation in *Arabidopsis*. Transcripts encoding for sulfur-rich aliphatic and indolic glucosinolates are also GB03 induced. As a result, GB03-exposed plants with elevated glucosinolates exhibit greater protection against the generalist herbivore, *Spodoptera exigua* (beet armyworm, BAW). In contrast, a previously characterized glucosinolate mutant compromised in the production of both aliphatic and indolic glucosinolates is also compromised in terms of GB03-induced protection against insect herbivory. As with *in vitro* studies, soil-grown plants show enhanced glucosinolate accumulation and protection against BAW feeding with GB03 exposure. These results demonstrate the potential of microbes to enhance plant sulfur assimilation and emphasize the sophisticated integration of microbial signaling in plant defense.

Keywords: plant growth-promoting rhizobacteria (PGPR), *Bacillus amyloliquefaciens* GB03, bacterial volatile organic compounds (VOCs), glucosinolates (GSL), sulfur assimilation, plant-defense priming

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Abbreviations: 1MOI3M, 1-methoxyindol-3-ylmethyl glucosinolate; 4MOI3M, 4-methoxyindol-3-ylmethyl glucosinolate; 4-MSOB, 4-methylsulfinylbutyl glucosinolate; 4-MTB, 4-methylthiobutyl glucosinolate; 5-MSOP, 5-methylsulfinylpentyl glucosinolate; 6-MSOH, 6-methylsulfinylhexyl glucosinolate; 7-MSOH, 7-methylsulfinylheptyl glucosinolate; 7-MTH, 7-methylthioheptyl glucosinolate; 8-MSOO, 8-methylsulfinyloctyl glucosinolate; 8-MTO, 8-methylthiooctyl glucosinolate; APK, APS kinase; APR, APS reductase; APS, adenosine 5′-phosphosulfate; ATPS, ATP sulfurylase; BAW, beet armyworm; DMDS, dimethyl disulfide; BCAT, branched-chain amino acid amino transferase; CFU, colony forming unit; CYP, cytochromes $P6_{450}$; DDW, double-distilled water; ESI-MS, electrospray ionization-mass spectrometry; FMO_{GS-OX}, flavin monooxygenase; GST, glutathione-*s*-transferase; I3M, indol-3-ylmethyl glucosinolate; IGMT, indole glucosinolate methyl transferase; IPMDH, isopropyl malate dehydrogenase; IPMI, isopropyl malate isomerase; MAM, methylthioalkyl malate synthase; MSG, methylsulfinylalkyl glucosinolates; MTG, methylthioalkyl glucosinolates; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PDA, photodiode array; PGPR, plant growth-promoting rhizobacteria; RT-PCR, reverse-transcription PCR; SOT, sulfotransferase; SUR, super root; TSA, tryptic soy agar; UHPLC, ultra-HPLC; VOCs, volatile organic compounds.

INTRODUCTION

Sulfur, a crucial element for plants, is ubiquitous in proteins, present in the antioxidant tripeptide glutathione, the Cys-rich peptides phytochelatins that function in heavy metals detoxification and thioredoxins that are the major disulfide reductases responsible for maintaining the reduced state of proteins inside cells (Arnér and Holmgren, 2000; Cobbett, 2000). Sulfur can also be present in chloroplastic membrane lipids as well as certain coenzymes/vitamins (Falk et al., 2007). Sulfur is taken up by plants as inorganic sulfate via sulfate transporters and incorporated into APS by ATPS (Mugford et al., 2009). APS is then sequentially reduced by APR and sulfite reductase to sulfite and sulfide, and subsequently incorporated into *O*-acetylserine to form the sulfur containing amino acid cysteine. APS can also be phosphorylated to PAPS by the action of APK. PAPS is the sulfate donor for the formation of sulfated metabolites including glucosinolates, select flavonoids, phytosulfokines, and certain hormones.

From an ecological context, sulfur metabolites function in plant defense against pathogens and herbivores (Falk et al., 2007). Defensin and thionin peptides are sulfur-containing antimicrobial defenses with widespread plant distribution (Broekaert et al., 1995), whereas anti-feedant glucosinolates are limited to the Brassicale order (Falk et al., 2007). *Brassica* crops including cabbage, broccoli, cauliflower (*Brassica oleracea*) and rapeseed (*B. napus*) as well as *Arabidopsis* are rich in glucosinolates. In addition to these amino acid derivatives functioning in plant defense, glucosinolates are a nutritional source of sulfur and possess cancer-preventive properties (Sønderby et al., 2010). Glucosinolates are classified based on their amino acid precursor with aliphatic glucosinolates derived from Met, Ala, Leu, Ile, or Val; indolic glucosinolates derived from Trp; and aromatic glucosinolates derived from Phe or Tyr (Kliebenstein et al., 2001).

With plant damage, glucosinolates are rapidly converted into an array of toxic derivatives that can obfuscate phytochemical analysis. Enzymatically generated glucosinolate derivatives including isothiocyanates, epithionitriles, nitriles, and thiocyanates are produced in proportion to the amount of leaf damage as well as the reaction time (Halkier and Gershenzon, 2006; Wittstock and Burow, 2010; Winde and Wittstock, 2011). Therefore quantifying the pool of original glucosinolates requires deactivating the myrosinase enzyme before glucosinolates are enzymatically converted (Koroleva et al., 2000; Andréasson and Jørgensen, 2003; Zhao et al., 2008; Winde and Wittstock, 2011).

In addition to constitutive glucosinolate accumulation serving in chemical defense against herbivore damage, soil-borne microbes such as mycorrhizal fungi and PGPR can induce plant defense responses (van Loon, 2007; van Wees et al., 2008; Yang et al., 2009; Pineda et al., 2010, 2012). PGPR are naturally occurring soil microorganisms that colonize roots and stimulate plant growth. Such bacteria are applied to a wide range of agricultural crops for the purpose of growth enhancement, including increased seed germination, plant weight, harvest yields, and disease resistance (Kloepper et al., 1980, 1991, 1999). *Bacillus subtilis* (GB03), recently re-named as *B. amyloliquefaciens* is a commercially available PGPR strain that can be introduced into the soil at the time of planting via seed coating since spores are stable over time (Choi et al., 2014). Unlike many plant-growth promoting rhizobacterial strains that activate plant growth by directly producing and releasing indole-3-acetic acid and/or gibberellins, GB03 emits a bouquet of volatile metabolites, devoid of classic phytohormones that are capable of triggering plant growth promotion (Ryu et al., 2003; Paré et al., 2005). These VOCs have been shown to activate differential expression of approximately 600 transcripts related to cell wall modifications, primary and secondary metabolism, stress responses, hormone regulation, and iron homeostasis (Ryu et al., 2003; Farag et al., 2006; Zhang et al., 2007). This *Arabidopsis* profiling of GB03-induced transcripts has resulted in a new paradigm for PGPR-mediated iron uptake. While some soil microbes are proposed to enhance iron mobility and uptake solely via production of bacterial siderophores (Neilands and Leong, 1986; Bar-Ness et al., 1992; Briat, 1992; Glick et al., 1999; Sharma et al., 2003), GB03 enhances *Arabidopsis* iron accumulation via activation of the plant's own iron acquisition machinery including the iron uptake-related genes *FRO2* and *IRT1* that encode for ferric reductase and iron transport enzymes, respectively (Zhang et al., 2009). GB03 also transcriptionally regulates the Fe-deficiency-induced transcription factor 1 (*FIT1*) that is necessary and sufficient for ferric reductase and iron transporter induction (Zhang et al., 2009). More recently, an upstream iron acquisition-related transcription factor *MYB72* has been shown to be transcriptionally induced in *Arabidopsis* by bacterial VOCs with activation of the iron uptake-related genes *FIT1, FRO2,* and *IRT1* (Zamioudis et al., 2015).

The current study reports a novel mechanism in which the growth-promoting rhizobacterium *B. amyloliquefaciens* strain GB03 induces *Arabidopsis* sulfur assimilation and accumulation by inducing the plant's own sulfur assimilation machinery. Moreover, the impact of GB03 in regulating primary and secondary sulfur metabolites to enhance plant defense against herbivory is examined.

MATERIALS AND METHODS

Plant Material and Treatments

Arabidopsis thaliana seeds were surface sterilized and stratified for 2 days at 4°C in the absence of light. Seeds were planted in plastic Petri dishes (100 × 15 mm) containing a central partition (I-plates; Fisher Scientific), covered Magenta boxes (75 mm × 75 mm × 100 mm) or standard Petri dishes (150 mm × 15 mm), based on the specific experimental requirements. The bacterial culture is inoculated on the unplanted side of the partitioned plate, a glass vial (4 dr.) or a plastic plate (35 mm × 10 mm). All chambers contained half-strength MS solid media prepared according to Murashige and Skoog (1962) with 1.5% (w/v) sucrose and 0.8% (w/v) agar (except where noted otherwise). Plants were grown under a 14-/10-h light/dark cycle with metal halide and high pressure sodium lamps for a total light intensity of 200 μmol photons m^{-2} s^{-1}; temperature was 21 ± 4°C and relative humidity 40 ± 10%. For

plant growth, the media surface was oriented horizontally for I-plates and Magenta boxes and vertically for the larger plates with media agar increased to 1.5% (w/v).

Bacillus amyloliquefaciens (GB03) was streaked onto TSA plates and incubated at 28°C in the absence of light for 24 h. Cells were harvested in double distilled water (DDW) to yield 10^9 CFU mL^{-1}, as determined by optical density ($OD_{600} = 0.7$). Two days after seed germination, the bacterial suspension culture or DDW (25 μL for plates and 50 μL for Magenta boxes) was added to the non-plant portion of the chamber. Vials containing bacterial culture were replaced with fresh culture every 14 days.

For soil experiments, bacterial liquid cultures were mixed with sterile growing mix (Sunshine LC1 Mix; Sun Gro Horticulture, Canada) to a final PGPR concentration of 10^8 – 10^9 CFU/g soil. For water control, the bacterial suspension was replaced with sterile DDW. Seeds were sown in growing mix and fertilized weekly using 13:13:13 (N:P:K) fertilizer.

Semi-quantitative RT-PCR

Plants were harvested 48- or 72-h after GB03 or water treatment. Total RNA was extracted using RNeasy plant mini kit (Qiagen, Valencia, CA, USA) with genomic DNA contamination excluded by DNase digestion. First strand cDNA was synthesized from 3-5 μg total RNA using MuMLV-RT (Fisher Scientific, Houston, TX, USA); primer sequences are shown (**Table 1**). The PCR reaction included an initial 3 min denaturation at 94°C, followed by 30 s at 94°C, 30 s at 54°C and 1 min at 72°C with 24–27 cycles (based on the optimized linear range for each pair of specific primers), a final 10 min extension at 72°C (T100 Thermal Cycler, Bio-Rad, Hercules, CA, USA). No-reverse-transcription controls were included with the PCR runs to confirm the absence of DNA contamination. Agarose gel electrophoresis were imaged with a Kodak Gel Logic 100 Imaging System (Fisher Scientific, Houston, TX, USA) and quantified using Image J 1.33u[1] (National Institute of Health, USA). *TUB8* and *UBQ10* were employed for normalization as they were uniformly expressed in all tissues examined.

TABLE 1 | Sequence of primers employed in the semi-quantitative RT-PCR analysis.

Gene Name	Primer Sequence (5′ to 3′)
ATPS1	Forward: GTTTCCTTCCCTTCCAAATC
	Reverse: GAGCCAGTTTCCAGCATTAG
ATPS3	Forward: GAATGAAACAGCACGAGAAG
	Reverse: CCAGGGCACATAAATCCATC
ATPS2	Forward: ATGCTGTTTTTGCGTTTCAG
	Reverse: ACGGCTTGTTGTTTTGCTTC
ATPS4	Forward: GCGTATGAGACAGCACGAG
	Reverse: AACCAACACCTTCCAACCAG
APR1	Forward: AGGTTTGGATGGTGGAGTTG
	Reverse: CATAAAGCACGACGATCCAAG
APR2	Forward: CGAATCTTGGGTTACTCGTG
	Reverse: CCTCCTTGATGTTCCCTTTG
APR3	Forward: GAGATGGTGGTGGGAAGATG
	Reverse: TGGAACGAGACTGGATGGTC
APK1	Forward: TCCACCACCGTGAGATATGA
	Reverse: ATCCGCAAAAAGCTTAGCAA
APK2	Forward: TGGCACGAGAGTTCGATATG
	Reverse: CAGCACTACCTCGCAATTCA
CYP79F1	Forward: TCCATGGCATCAATCACTCTAC
	Reverse: CATCAACATTCCAACCTCTCAA
SUR1	Forward: TCGTGCTGCTTACAGTGGTC
	Reverse: ACACAGGGGATGTCCTTGAG
FMO_{GS-OX3}	Forward: ACCAATGTCCCGAGAGAAAGTA
	Reverse: GGAACGGAAATCTTCTCGTATG
UBQ10	Forward: CGATTACTCTTGAGGTGGAG
	Reverse: AGACCAAGTGAAGTGTGGAC
TUB8	Forward: CGTGGATCACAGCAATACAGAGCC
	Reverse: CCTCCTGCACTTCCACTTCGTCTTC

Total Sulfur Determination

Shoots and roots were separated, oven-dried, pulverized, and converted to dry-ash by heating at 550°C for 3 h in the presence of Ag_2O and $NaHCO_3$ based on Kalra (1998). Dried tissue was then neutralized, diluted, and analysis via a barium chloride-gelatin turbidimetric assay (Tabatabai and Bremner, 1970). Standards were prepared as tissue material and diluted to a final concentration of 0–32 μg mL^{-1}. Total sulfur was quantified spectrophotometrically at 420 nm based on a sulfur standard curve.

$^{35}SO_4^{-2}$ Uptake Assay

For sulfate uptake measurements, plants were germinated on nylon mesh and grown vertically on media-containing plates with GB03 or water exposure for 11 days. Radio-labeling was initiated by submerging the roots into liquid media containing 37 MBq L^{-1} $^{35}SO_4^{-2}$ (Perkin–Elmer). After incubation for 30 min, roots were briefly rinsed with non-labeled medium to remove apoplastic radioactivity (modified protocol from Kataoka et al., 2004; Maruyama-Nakashita et al., 2004; Yoshimoto et al., 2007). After blotting, shoots and roots were weighed separately, transferred to scintillation vials and covered with 1 mL of 0.1 M HCl. Overnight-extracted samples were mixed with universal scintillation cocktail (4 mL; Fisher Scientific) and incorporated radioactivity measured by liquid scintillation counting.

Cysteine Measurements

Whole plant tissue (0.1 g) was ground in liquid nitrogen and thiols were acid extracted using ice-chilled 0.1 N HCl (200 μL). The homogenate was centrifuged at 12,000 × *g* for 10 min at 4°C. Supernatant aliquots were neutralized with 200 mM HEPES (pH 12.4), reduced with dithiothreitol and sulfhydryl groups derivatized with monobromobimane (VWR). Separation, detection and quantification of fluorescent adducts was based on Schupp and Rennenberg (1988).

Glucosinolates Analysis

Plants were shoot and root separated, frozen in liquid nitrogen and lyophilized. Tissue (20–50 mg) was extracted for 15 min in boiling aqueous 7.5 mM $Pb(OAc)_2/Ba(OAc)_2$ (4 mL) with

[1]http://rsb.info.nih.gov/ij/

0.57 μmol internal standard (sinigrin, Sigma–Aldrich) based on Reintanz et al. (2001). At room temperature, samples were gently shaken for 30 min, centrifuged at 4000 × *g* for 10 min and the supernatant was loaded on DEAE Sephadex A-25 column (120 mg, Sigma–Aldrich). Resin was rinsed with aqueous methanol (67%) and water and subsequently incubated with 50 μL sulfatase solution overnight (Graser et al., 2000). The resulting desulfoglucosinolates were eluted with 60% aqueous methanol (800 μL) and water (800 μL). The pooled extract was evaporated to dryness *in vacuo* and the residue was dissolved in HPLC-grade water (100 μL).

Desulfoglucosinolates were separated by HPLC on a Dionex Ultimate 3000 UHPLC system equipped with auto-sampler, column oven, and diode array detector. A C_{18} reversed phase column (Acclaim 120 mm × 3.0 mm, 150 mm × 3.0 mm i.d., 3-μm particle size) was run with a 400 μL/min flow rate at 25°C; the injection volume was 10 μL. Elution was performed with a gradient (solvent A water; B acetonitrile) of 1.5 to 5% solvent B (6 min), 5 to 7% solvent B (2 min), 7 to 21% solvent B (10 min), 21 to 29% solvent B (5 min), and 29 to 57% solvent B (14 min), followed by a cleaning cycle (57 to 93% solvent B for 3 min, 6 min of hold, 93 to 1.5% solvent B for 3 min with a 5 min hold). Compounds were monitored at 229 nm.

Desulfoglucosinolates were identified by HPLC-PDA-MS based on method of Kusznierewicz et al. (2013). Samples were analyzed on a LCQ Fleet HPLC system equipped with PAL autosampler, Surveyor PDA detector, and Surveyor MS pump using an Alltima C_{18} reversed phase column (250 mm × 2.1 mm i.d., 5-μm particle size) with a 200 μL/min flow rate. The injection volume was 10 μL. Elution was performed with a gradient (solvent A water/0.1% formic acid; B acetonitrile/0.1% formic acid) of 1.5% solvent B (3 min) 1.5 to 13% solvent B (15 min), 13 to 33% solvent B (12 min), 33 to 57% solvent B (7 min), followed by a cleaning cycle (57 to 93% solvent B for 3 min, 6 min of hold, 93 to 1.5% solvent B for 3 min with a 5 min hold). Compounds were monitored by PDA at 229 nm, then subsequently by ESI-MS (LCQ Fleet Ion Trap MS) operated in positive ion mode, an acquisition time of 40 min with scanning from *m*/*z* 150 to 800 amu.

Previously reported desulfoglucosinolates were identified by MS via characteristic $[M+H]^+$ and $[M+Na]^+$ peaks except for 3-methylsulfinylpropyl glucosinolate (3MSOP) which could not be identified because of poor resolution. Positional isomers 4MOI3M and 1MOI3M with equivalent masses were differentiated based on retention time comparisons with literature values (Reintanz et al., 2001). Glucosinolates were quantified based on response factors established for individual desulfoglucosinolates relative to the internal standard at 229 nm (Brown et al., 2003).

Herbivore Feeding

Spodoptera exigua (BAW) eggs were purchased from Benzon research (Carlisle, PA, USA). After hatching, neonate larvae were transferred to feed on artificial media for 6 days with a transfer to fresh media every 2–3 days. Since an acclimation period is required whenever larvae are transferred from one diet to another, 1 day before the experiment, third-instar larvae were transferred to feed on non-experimental wild-type *Arabidopsis* plants (Mewis et al., 2005). After this pre-feeding, larvae of the same developmental stage were weighted and transferred to 29-day-old GB03- or water-treated plants (one larva/plant); the initial average weight of larvae was recorded for both GB03 and water treatments. Shoot biomass was recorded after 56 h of feeding. Additional GB03-treated and untreated plants were reserved to serve as undamaged controls. Plants were harvested, rinsed, and weighted. Milligrams eaten per plant were calculated based on the weight difference between BAW eaten and uneaten plants. The quadruple glucosinolate knock-out mutant (*myb28 myb29 cyp79b2 cyp79b3*) was treated the same as Col-0.

For soil experiments, herbivore weights were collected. Neonate larvae were transferred to 28-day-old GB03- or water-treated plants with a transfer to fresh plants every 2–3 days. Larvae weight was measured at 7 and 9 days after feeding.

Statistical Analysis

For herbivore feeding experiments, statistical analyses were performed using R software[2]. First, a Levene's test was performed to check the homogeneity of variance (Levene, 1960); homogeneous variance was achieved after transforming the data into the corresponding square root. Then, two-way ANOVAs were performed separately for wild-type and knock-out mutant lines. Tukey's method was used to do pair-wise comparisons of means and an "lsmeans" package was used for means' grouping. For all other experiments, pair-wise comparison of means was performed using Excel 2007 with significant difference between treatments was based on Student's *t*-test at *P*-values ≤ 0.05. The number of biological replicates is shown in each figure legend with minimum of three replicates.

RESULTS

Elevated Sulfate Assimilation with GB03 Exposure

The sulfate assimilation pathway with previously identified genes is depicted in **Figure 1A**. Mining whole-plant microarray data of GB03-exposed *Arabidopsis* seedlings identified sulfate-assimilation gene induction for *ATPS* and *APR*. Of the four ATPS and the three APR isozymes present, *ATPS1* and *ATPS3* as well as *APR1* and *APR2* were found to be induced at 72 h post GB03 exposure (Supplementary Figure 1). RT-PCR analysis confirmed GB03 induction for *ATPS1*, *ATPS2*, and *ATPS3* and all *APR* genes (**Figure 1B**). Another branch of sulfur assimilation involves APS conversion to PAPS by APK. There are four functional APK isoforms in *Arabidopsis*, among them APK1 and APK2 are the most active isoforms (Mugford et al., 2009). From the microarray data, both *APK1* and *APK2* are GB03 up regulated relative to controls (Supplementary

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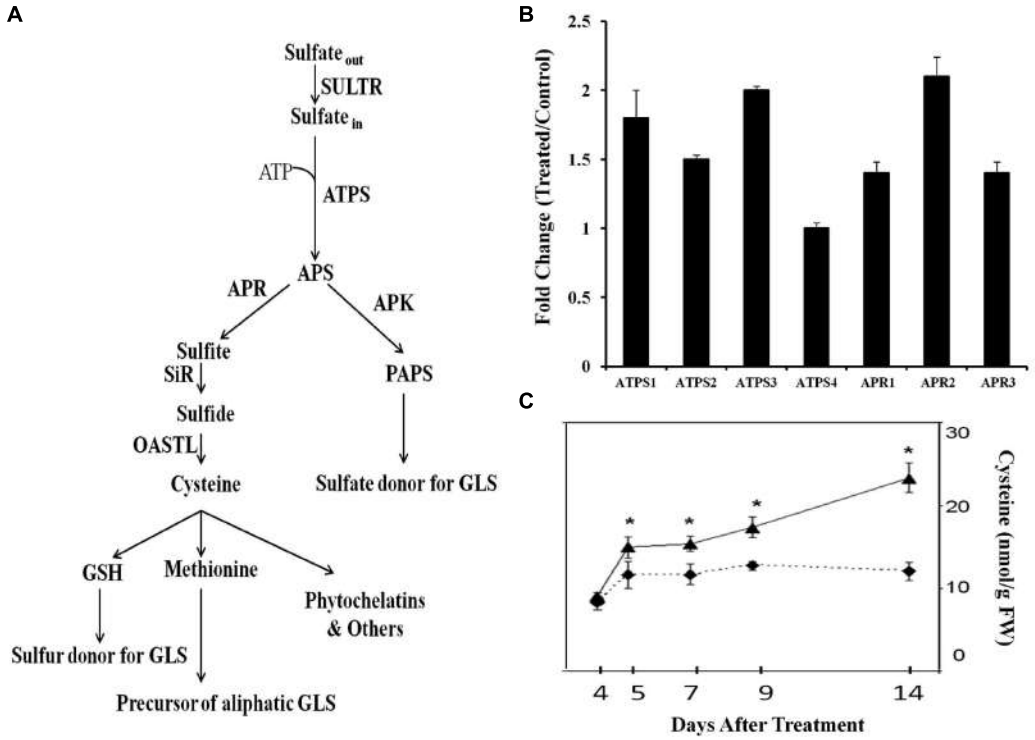

FIGURE 1 | Transcriptional regulation of *Arabidopsis* sulfur assimilation genes by GB03. Depicted sulfate assimilation pathway adapted from Mugford et al. (2009) **(A)**. Semi-quantitative RT-PCR analysis of whole-plant sulfur assimilation gene expression at 72 h post GB03 treatment **(B)**; data are the averages of three biological replicates with error bars representing standard error. The amino acid cysteine increases with GB03 treatment (solid line) relative to the water controls (dashed lines; **C**); an asterisk (*) indicate statistically significant difference between treatments (*t*-test, *P*-value ≤ 0.05, $n = 6$, mean $\pm$ SE). Sulfur assimilation pathway includes SULTR, sulfate transporter; ATPS, ATP sulfurlyase; APS, adenosine 5′-phosphosulfate; APR, APS reductase; SiR, sulfite reductase; OASTL; *O*-acetylserine (thiol) lyase; GSH, glutathione; APK, APS kinase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

Figure 1). *APK* transcript induction confirmation via RT-PCR analysis showed GB03 induction only in shoots (*APK1*, 1.3 $\pm$ 0.08; *APK2*, 1.5 $\pm$ 0.05). In addition, the amino acid cysteine, a precursor of many organic sulfur metabolites increased 28 $\pm$ 11, 32 $\pm$ 8, 37 $\pm$ 10, and 93 $\pm$ 15 % with GB03 exposure at 5, 7, 9, and 14 days, respectively (**Figure 1C**).

GB03 Enhances Sulfur Accumulation and Uptake

As sulfate assimilation and reduction genes were GB03 induced, sulfur accumulation was examined. While total sulfur accumulation per tissue weight decreased *ca.* twofold in shoots 11 days post GB03 exposure, shoot sulfur accumulation per plant increased *ca.* 75% (**Figure 2A**). In roots, increases of *ca.* 50-fold and *ca.* 100-fold on a dry-weight and per-plant basis, respectively, were observed (**Figure 2B**). To better characterize the process of inducible sulfur metabolism, plant sulfur movement was monitored with radioactive sulfate ($^{35}SO_4^{-2}$) to examine sulfur uptake and translocation. Although there was a *ca.* 30% reduction in total sulfur uptake per tissue weight, GB03 exposure enhanced total sulfur uptake per plant by *ca.* twofold, relative to untreated controls, within 30 min of radio-labeling (**Figure 3A**). Shoot sulfur translocation per tissue weight was *ca.* twofold less with GB03 treatment; however, similar translocation rate per plant was observed for both GB03 and controls (**Figure 3B**). And in roots, sulfur uptake and retention was higher with GB03 exposure on both a tissue weight and per-plant basis (**Figure 3C**).

Since select bacterial volatiles such as 2,3-butandiol have been previously shown to induce growth promotion and induced systemic resistance in *Arabidopsis* (Ryu et al., 2003, 2004), an array of 2,3-butandiol concentrations were assayed to examine for enhanced sulfur accumulation albeit no sulfur-associated changes were detected (data not shown). Similarly, collected bacterial volatiles re-introduced to plants also did not enhance sulfur accumulation.

GB03 Induces Glucosinolate Biosynthetic Transcripts

The aliphatic and indolic glucosinolate biosynthetic pathways with previously identified genes is depicted in **Figures 4A,B**, respectively. Mining microarray data for transcripts encoding glucosinolate biosynthesis revealed that the majority of aliphatic

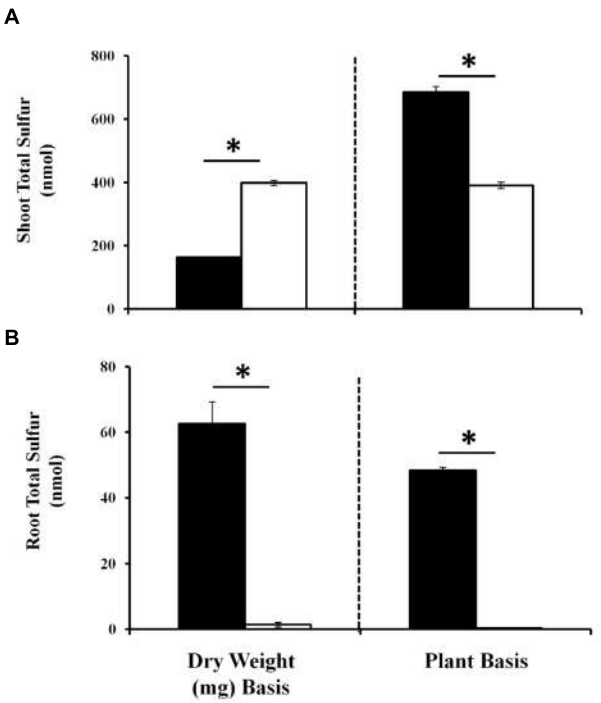

FIGURE 2 | Sulfur accumulation in *Arabidopsis* with GB03 exposure. Shoot **(A)** and root **(B)** sulfur accumulation in 13-day-old plants that are GB03- (black bars) or water-treated (white bars) on a dry-weight and per-plant basis. Root sulfur values for water-treated plants are 1.36 ± 0.68 nmol/mg DW and 0.4 ± 0.05 nmol/plant, although values are not perceivable in the figure. An asterisk (*) indicates statistically significant difference between treatments (*t*-test, *P*-value ≤ 0.05, $n = 4$, mean ± SE).

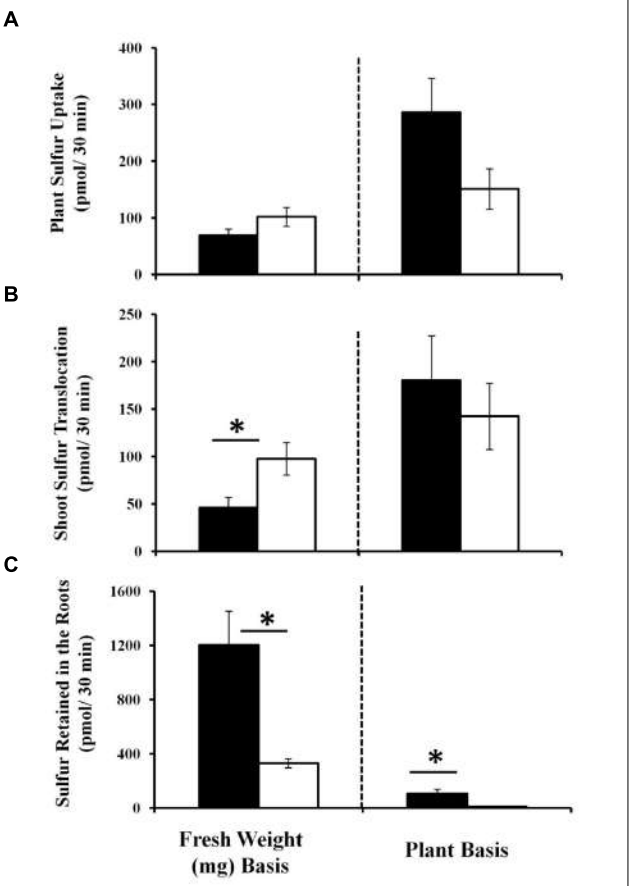

FIGURE 3 | Sulfur uptake and translocation monitored by radioactive sulfate ($^{35}SO_4^{-2}$ 30 min pulse) in *Arabidopsis* with GB03 exposure. Whole plant sulfur uptake **(A)**, shoot sulfur translocation **(B)** and sulfur root retention **(C)** is shown in 13-day-old plants that are GB03- (black bars) or water-treated (white bars) on a fresh-weight and per-plant basis. The value of sulfur retained in the roots for water-treated plants is 8.6 ± 0.98 pmol/30 min/plant, although values are not perceivable in the figure. An asterisk (*) indicates statistically significant difference between treatments (*t*-test, *P*-value ≤ 0.05, $n \geq 4$, mean ± SE).

pathway genes are GB03 induced (Supplementary Figure 2A). For indolic glucosinolate biosynthesis, microarray data showed transcript induction limited to *GSTF9*, *SUR1*, *UGT74B1*, and *SOT16* (Supplementary Figure 2B). GSTF9 is a GST which is responsible for the conjugation of the activated aldoximes to the sulfur donor glutathione, where the resulting *S*-alkylthiohydroximates are converted to thiohydroximates by a carbon-sulfur lyase, SUR1. Thiohydroximates are in turn *S*-glucosylated by the glucosyltransferases UGT74B1 to form desulfoglucosinolates. Finally, desulfoglucosinolates are sulfated to the corresponding glucosinolates by the sulfotransferase SOT16. Monitoring select shoot and root transcripts separately by RT-PCR confirmed gene induction with *CYP79F1* induction in shoots *ca.* threefold, while root induction was *ca.* 30% (**Figures 4C,D**). CYP79F1 catalyzes the first committed step in biosynthesis of the aliphatic glucosinolate core structure that involves conversion of amino acids to corresponding aldoximes (a rate-limiting step in glucosinolates biosynthesis; Mikkelsen and Halkier, 2003). FMO_{GS-OX3}, a gene that encodes one of the five flavin monooxygenases responsible for *S*-oxygenation of aliphatic glucosinolates resulting in conversion of MTG to MSG (Sønderby et al., 2010) was induced in shoots within 48 h while root induction was delayed to 72 h (**Figures 4C,D**). *SUR1* gene expression was induced *ca.* threefold in shoots (**Figures 4C,D**). To link transcriptional regulation with downstream glucosinolate accumulation, qualitative and quantitative glucosinolate analysis was performed.

GB03 Induces Glucosinolate Accumulation

Desulfoglucosinolates were separated by HPLC based on relative polarity, with MSG eluting first in increasing order of their side-chain length, followed by indolic glucosinolates; long chain MTGs eluted last off the column (**Figure 5A**). GB03 exposure resulted in *ca.* 33 and 70% greater glucosinolate accumulation in shoots and roots, respectively (**Figure 5B**). Specifically, GB03

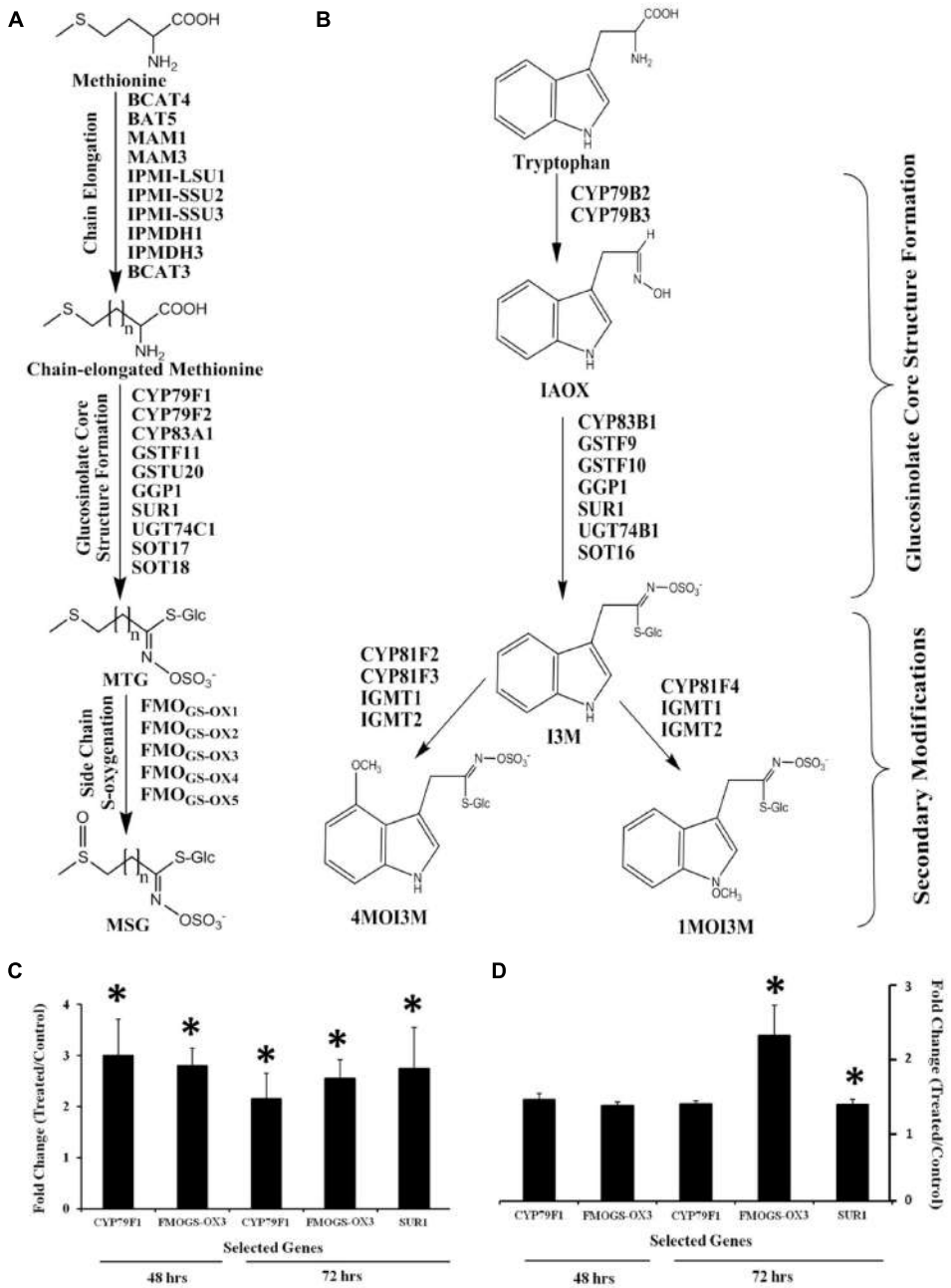

FIGURE 4 | Glucosinolate biosynthesis transcriptional regulation in *Arabidopsis* by GB03. Depicted aliphatic **(A)** and indolic **(B)** glucosinolate biosynthetic pathways adapted from Sønderby et al. (2010). Semi-quantitative RT-PCR analysis of *CYP79F1* and FMO_{GS-OX3} gene expression in both shoots **(C)** and roots **(D)** at 48 and 72 h, and *SUR1* at 72 h post GB03 exposure; an asterisk (*) indicates statistically significant difference between treatments (*t*-test, *P*-value ≤ 0.05, $n = 3$, mean $\pm$ SE).

increased indolic glucosinolates in shoots (55%) and roots (twofold) while MSGs were induced in shoots by 45%. MTG accumulation differences with regard to tissue or GB03 treatment was not observed. In shoots, I3M was the most GB03 induced indolic glucosinolate (68%; **Table 2**); while among MSG, there was a 35, 37, 69, 73, and 65% GB03 induction of 4-MSOB, 5-MSOP, 6-MSOH, 7-MSOH, and 8-MSOO, respectively. For roots, the most abundant glucosinolate, 1MOI3M, increased threefold with no other statistically significant accumulation changes. Glucosinolate accumulation was not induced with plant exposure to 2,3-butandiol or collected bacterial volatiles (data not shown).

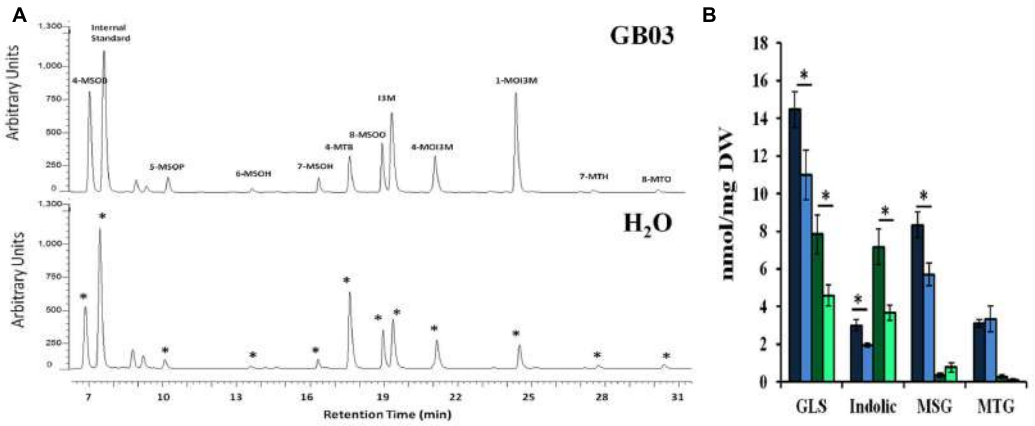

FIGURE 5 | Glucosinolate accumulation in *Arabidopsis* by GB03. Representative UHPLC-PDA chromatogram showing shoot glucosinolate profile in both GB03- and water-treated 30-day-old plants **(A)**; an asterisk (*) indicates peak alignment between the lower and upper chromatograms. GB03 induces total glucosinolate (GLS) accumulation in shoots (dark blue-GB03 versus light blue bar-H_2O control) and roots (dark green-GB03 versus light green bar-H_2O control) in 30-day-old plants **(B)**. The aliphatic MSG and MTG as well as indolic glucosinolates are shown; an asterisk (*) indicates statistically significant difference between treatments (*t*-test, *P*-value ≤ 0.05, $n = 6$, mean $\pm$ SE). Internal standard, sinigrin; 4-MSOB, 4-methylsulfinylbutyl glucosinolate; 5-MSOP, 5-methylsulfinylpentyl glucosinolate; 6-MSOH, 6-methylsulfinylhexyl glucosinolate; 7-MSOH, 7-methylsulfinylheptyl glucosinolate; 4-MTB, 4-methylthiobutyl glucosinolate; 8-MSOO, 8-methylsulfinyloctyl glucosinolate; I3M, indol-3-ylmethyl glucosinolate; 4MOI3M, 4-methoxyindol-3-ylmethyl glucosinolate; 1MOI3M, 1-methoxyindol-3-ylmethyl glucosinolate; 7-MTH, 7-methylthioheptyl glucosinolate; and 8-MTO, 8-methylthiooctyl glucosinolate.

TABLE 2 | GB03-induced glucosinolate (nmol/mg DW) accumulation with 30-day-old plants.

Systematic Name	Common Name	Shoot			Root		
		GB03	H_2O	Fold Change	GB03	H_2O	Fold Change
4-MSOB	Glucoraphanin	**4.43 ± 0.40**	**3.28 ± 0.26**	1.35	Nd	Nd	–
5-MSOP	Glucoalyssin	**0.657 ± 0.061**	**0.48 ± 0.038**	1.37	Nd	Nd	–
6-MSOH	Glucohesperin	**0.147 ± 0.014**	**0.087 ± 0.012**	1.69	Nd	Nd	–
7-MSOH	Glucoibarin	**0.623 ± 0.057**	**0.359 ± 0.051**	1.73	0.12 ± 0.027	0.32 ± 0.11	0.38
4-MTB	Glucoerucin	2.74 ± 0.19	2.98 ± 0.64	0.92	Nd	Nd	–
8-MSOO	Glucohirsutin	**2.48 ± 0.22**	**1.50 ± 0.277**	1.65	0.25 ± 0.088	0.45 ± 0.13	0.56
I3M	Glucobrassicin	**1.61 ± 0.12**	**0.958 ± 0.083**	1.68	0.16 ± 0.033	0.30 ± .066	0.53
4MOI3M	4-Methoxygluco-brassicin	**0.825 ± 0.046**	**0.657 ± 0.025**	1.25	1.16 ± 0.21	1.28 ± 0.36	0.90
1MOI3M	Neoglucobrassicin	0.56 ± 0.178	0.32 ± 0.048	1.71	**5.85 ± 0.74**	**2.10 ± 0.29**	2.78
7-MTH	–	0.17 ± 0.0062	0.16 ± .022	1.10	0.086 ± .028	0.036 ± .017	2.37
8-MTO	–	0.20 ± 0.0064	0.20 ± 0.029	0.97	0.198 ± 0.069	0.083 ± .024	2.37

Bold values indicate statistically significant difference between treatments (t-test, P-value ≤ 0.05, n = 6, mean ± SE).

GB03 Induces Plant Biomass Protection with Herbivory

GB03-treated plants were approximately twice the weight of water controls (**Figure 6A**). For feeding experiments, third instar larvae were pre-fed for 1 day on non-exposed *Arabidopsis* seedlings and initial BAW weight was monitored for larvae that were to feed on GB03 (46.39 ± 2.48) or water (46.48 ± 1.68) treated plants to exclude the effect of larvae weight and developmental stage variation on larval feeding. In addition, vials containing GB03 were removed from plant chambers before introducing BAW to avoid any direct interactions between bacterial volatiles and larvae as well as to avoid unequal PGPR-mediated growth for plants without BAW. With larval feeding, GB03-treated plants lost 24% shoot weight while controls lost 62% weight within 56 h of BAW feeding; plant tissue eaten per plant was 469 ± 54 and 658 ± 20 mg for GB03 and water treatments, respectively (*t*-test, $P = 0.004$, $n = 11$).

GB03 Induces Glucosinolate-Dependant Plant Biomass Protection with Herbivory

For plants without herbivory, similar GB03-induced growth promotion was observed for both the Col-0 and a glucosinolate knock-out line (**Figures 6A–D**) compromised in both aliphatic and indolic glucosinolate production, *myb28 myb29 cyp79b2 cyp79b3* (Müller et al., 2010). With larval feeding on the knock-out line, shoot weight loss of 55% was observed for both GB03 and water treated plants; tissue consumed per plant was 962 ± 13

and 364 ± 11 mg for GB03 and water treatments, respectively (*t*-test, $P = 1.89E - 20$, $n \geq 11$).

GB03 Induces Plant Biomass Protection with Herbivory *In Vivo*

GB03-treated soil-grown Col-0 plants accumulate *ca*. 25% higher levels of glucosinolates compared to water controls in shoots for 35-day-old plants (**Figure 7A**). In addition, larval weight was lower when fed on GB03-treated plants for 7 and 9 days compared with controls (**Figures 7B–D**). Moreover, in the presence BAW, plant tissue eaten per plant was less for GB03-treated plants versus water controls (**Figures 7E,F**). A model for GB03-conferred protection against herbivory is proposed (Supplementary Figure 3).

DISCUSSION

Several responses are induced in *Arabidopsis* by the PGPR strain GB03 including enhanced photosynthetic efficiency (Zhang et al., 2008), increased iron assimilation (Zhang et al., 2009) and elevated reproductive success (Xie et al., 2009), however, the ability of PGPR to induce sulfur assimilation via established mechanisms operational in plants has not been previously reported. Here is described that sulfur assimilation and glucosinolate biosynthetic genes are transcriptionally up regulated with GB03 exposure in *Arabidopsis*, from literature extracted microarray data (Zhang et al., 2007) and RT-PCR analysis. At the metabolite level, enhanced sulfate uptake along with elevated total sulfur, cysteine and sulfated aliphatic/indolic glucosinolate accumulation is observed. GB03-exposed plants also exhibit greater protection against the generalist herbivore BAW, while enhanced protection is compromised in a glucosinolate quadruple knockout line. Consistent with *in vitro* studies, GB03 enhanced glucosinolate accumulation and protection against larval feeding with soil-grown plants.

The PGPR strain *Bacillus* sp. B55 has previously been shown to promote tobacco growth by enhancing sulfur nutrition via uptake of sulfur volatiles including the major bacterial volatile component, DMDS (Meldau et al., 2013). B55 DMDS sulfur uptake observed in tobacco is subsequently incorporated into plant proteins and accompanied by reduced gene expression involved in sulfur assimilation, Met biosynthesis and sulfur recycling. In contrast, GB03 VOCs are low in sulfur emissions (Farag et al., 2006) and up-regulate genes that mediate sulfur assimilation. Since the volatilome has only been chemically characterized for GB03 (Farag et al., 2006), a unified mechanism for chemical incorporation and/or signaling inducing sulfur metabolism by GB03 and B55a is not possible. Moreover, different sulfur demands between glucosinolate-rich cruciferous plants such as *Arabidopsis* and glucosinolate-deficient tobacco (Falk et al., 2007), also prevents direct comparisons between the two sulfur induction studies.

In *Arabidopsis*, sulfate is taken up by roots and although root plastids contain the enzymatic machinery for sulfate reduction, sulfate conversion to sulfide and subsequent incorporation into cysteine predominantly takes place in shoot chloroplasts (Davidian and Kopriva, 2010). GB03 induces several *Arabidopsis* sulfate reduction genes including the key drivers of sulfate assimilation ATPS1 and APR2 (Loudet et al., 2007; Koprivova et al., 2013). While low-level gene activation does not constitute transcriptional regulation, the comprehensive induction of sulfate assimilation genes observed with GB03 exposure is consistent with coordinated transcriptional control. GB03-induced sulfur assimilation correlates with enhanced sulfur uptake and accumulation in roots. In shoots, sulfur uptake and accumulation per tissue weight is lower with GB03 exposure (**Figures 2** and **3**) which may be in part due to a dilution of plant sulfur with enhanced growth induced by GB03. On a whole-plant basis, sulfur uptake and accumulation is uniformly higher with GB03 treatment: 286.37 ± 59 versus 151 ± 35 pmol/30 min sulfur uptake and 732.6 ± 20 versus 391.36 ± 10 nmol accumulation for GB03 versus water treatments, respectively. Sulfur content in for *in vitro* grown *Arabidopsis* with and without GB03 exposure are consistent with published ICP-MS sulfur quantification under the same experimental conditions (Kwon et al., 2010). In addition to enhancing sulfur accumulation, GB03 has been previously shown to enhance *Arabidopsis* iron and copper accumulation (Zhang et al., 2009; Kwon et al., 2010), suggesting that this may be a coordinated effort by bacteria to increase plant growth by effectively enhancing the accumulation of essential elements.

GB03-induced sulfur assimilation enhances accumulation of cysteine, the precursor of methionine, GSH and subsequently select glucosinolates. Methionine is the main substrate for aliphatic glucosinolates (Ravanel et al., 1998) while the active sulfate donor for glucosinolate biosynthesis is PAPS, a phosphorylated derivative of APS produced by APK (Mugford et al., 2009; Sønderby et al., 2010). The crucial role of APK1 and APK2 in glucosinolate biosynthesis has previously been established using an *apk1 apk2* double mutant which resulted in an 80% glucosinolate reduction and a concomitant increase in desulfoglucosinolates (Mugford et al., 2009). The transfer of the sulfate group from PAPS to the free hydroxyl group of desulfoglucosinolates is catalyzed by SOTs (Sønderby et al., 2010). The parallel transcriptional up-regulation of *APKs* and *SOTs* suggest a coordinated regulation of the sulfate donor formation and the sulfate transfer reaction by GB03.

The induction of glucosinolate accumulation in response to herbivore attack has been extensively studied (Mewis et al., 2006; Hopkins et al., 2009), however, much less is known with regard to microbial glucosinolate induction (van de Mortel et al., 2012). With the root-colonizing *Pseudomonas fluorescens* strain SS101 (*Pf*.SS101), the phytoalexin camalexin and glucosinolates were correlated with induced systemic resistance in *Arabidopsis* against several bacterial pathogens, including *Pseudomonas syringae* pv *tomato* (*Pst*). In addition, herbivore mortality rate was greater with BAW feeding on *Pf*.SS101-root-colonized *Arabidopsis* and mortality-rate differences with SS101 root inoculation were lost when an indolic glucosinolate deficient line was assayed (van de Mortel et al., 2012). In this current report, inducible sulfur assimilation and/or partitioning is/are linked with elevated endogenous glucosinolates, foliar plant biomass with herbivory

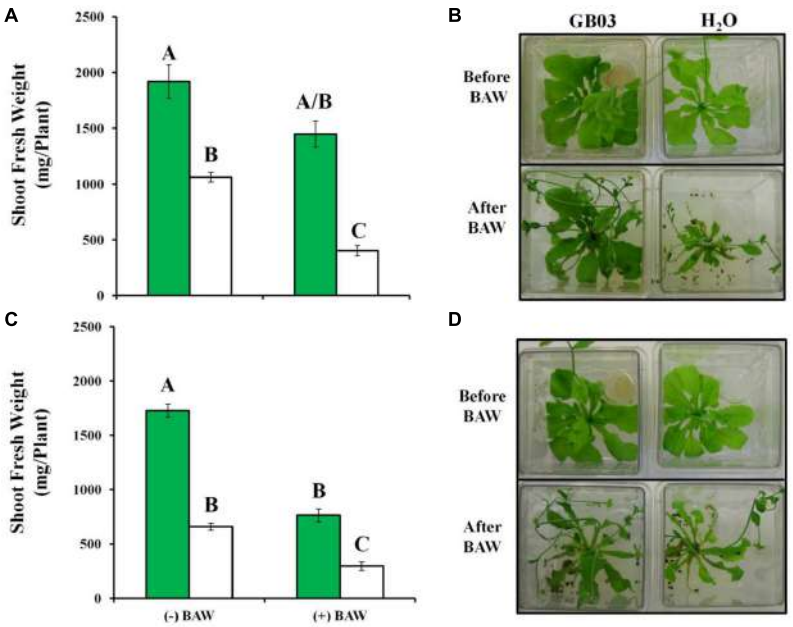

FIGURE 6 | *Arabidopsis* protection against the generalist herbivore *Spodoptera exigua* (BAW) by GB03. Twenty nine-day-old wild-type **(A,B)** and glucosinolate quadruple *myb28 myb29 cyp79b2 cyp79b3* mutant plants **(C,D)** without (–BAW) and with (+BAW) larval feeding (third instar) for 56 h. GB03-treated (green bars) and H_2O-control plants (white bars) are shown. Data were analyzed by two-way ANOVA; different letters indicate statistically significant differences between treatments (Tukey's test, P-value ≤ 0.01, $n \geq 11$, mean $\pm$ SE). Representative plant images **(B,D)** are shown.

and larval weight. By monitoring enhanced plant protection against BAW feeding by bacterial volatiles albeit devoid of direct plant–bacteria contact, induced plant defense responses independent of potential confounding bacterial anti-feedant effects can be identified. Without BAW larvae present, GB03 induced plant growth in both the wild-type and glucosinolate mutant line (**Figure 6**), indicating that glucosinolates play no role in GB03-triggered growth promotion. However, greater GB03-induced growth promotion in the mutant line compared to Col-0 (**Figure 6C**) may be in part due to additional energy available for growth promotion without glucosinolate biosynthesis operative. With BAW herbivory, GB03-treated Col-0 plants lost less shoot weight than water controls (**Figures 6A,B**), indicating GB03-induction of plant defense(s). With such GB03 plant protection against larval feeding compromised in the glucosinolate mutant line (**Figures 6C,D**), a causal relationship is established between GB03-enhanced glucosinolate accumulation and conferred plant protection. Interestingly, in the mutant line without glucosinolate defenses present, larval-consumed plant tissue per plant was greater with versus without GB03 exposure; tissue consumed per plant was 962 $\pm$ 13 and 364 $\pm$ 11 mg for GB03 versus water treatments, respectively. Future experiments will examine if GB03-induced plants contain greater amounts of young leaves that have yet to accumulate non-glucosinolate based chemical defenses or if such plants dilute non-inducible chemical defenses making the GB03-induced glucosinolate mutant line more palatable for feeding larvae.

Soil-grown GB03-treated plants exhibited enhanced glucosinolate accumulation and plant protection against BAW is consistent with I-plate experiments; however, elicitation differences limit direct comparisons between *in vitro* and *in vivo* systems. For example, chemical signaling is confined to bacterial VOCs *in vitro* while non-volatile metabolites can also serve as potential signaling molecules in the *in vivo* soil system. Moreover, although the soil is sterilized before planting and bacterial inoculation, the non-sterile environment in which soil-grown plants are exposed is conducive to bacterial proliferate of leaves and roots by other bacterial strains besides GB03. Down-stream signaling pathways can also be differentially regulated in media and soil systems. For example, ethylene signaling is operative with *in vivo* PGPR signaling but not with *in vitro* growth promotion (Ryu et al., 2005). Future studies will examine several mutant lines to elucidate which of the different plant signaling pathways are involved in eliciting enhanced sulfur metabolism and protection against herbivores by GB03 both *in vitro* and *in vivo*. Moreover, since it has been widely recognized that the plant hormone jasmonic acid (JA) plays a crucial role in plant defense against pathogens and herbivores as well as in glucosinolate accumulation (van Dam et al., 2004; van Dam and Oomen, 2008), JA mutant lines will be assayed.

Since the growth promotion signal 2,3-butandiol (Ryu et al., 2003, 2004) as well as collected GB03 VOCs re-introduced to plants do not exhibit enhanced sulfur assimilation or glucosinolate accumulation a more effective absorbent may be necessary to trap biologically active bacterial volatiles. Alternatively, as the genome sequence of GB03 has been recently identified (Choi et al., 2014), testing different GB03 mutant lines

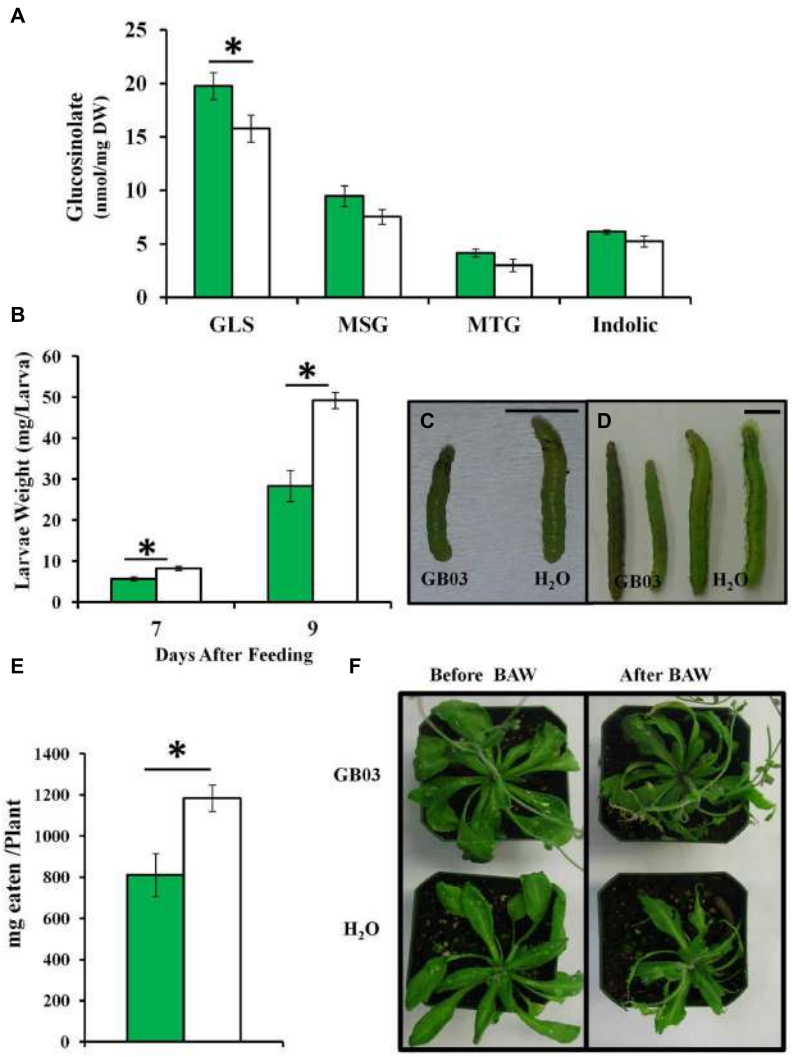

FIGURE 7 | Glucosinolate accumulation, BAW weight and leaf damage in soil-grown *Arabidopsis* with GB03 treatment. Shoot total GLS accumulation in 35-day-old plants that are GB03- (green bars) or water-treated (white bars; $n = 5$; **A**). The aliphatic MSG and MTG as well as indolic glucosinolates are shown. Forth instar larvae were weighed 7 ($n \geq 18$) and 9 days ($n \geq 11$) after feeding on 28-day-old plants **(B)**; representative images at 7 **(C)** and 9 days **(D)** are shown with the bar scale representing 0.5 cm. Milligrams eaten per plant after 3 days of larvae feeding on 37-day-old plants ($n = 10$; **E**). Representative plant images are shown **(F)**. An asterisk (*) indicates statistically significant difference between treatments (*t*-test, *P*-value ≤ 0.05, mean $\pm$ SE).

could help deciphering the effect of different VOCs products on inducing sulfur metabolism.

Glucosinolate accumulation differs between shoots and roots. Without GB03 exposure, total glucosinolates are higher in shoots with aliphatic glucosinolates being the most abundant compared with roots. The subclass of indolic glucosinolates accumulates predominately in roots as has been reported previously in *Arabidopsis* (Brown et al., 2003) as well as in other *Brassica* species (Rosa, 1997; Kirkegaard and Sarwar, 1998). An absence of detectable short chain aliphatic glucosinolates in roots (**Table 2**) is consistent with recent findings where rosette leaves are the major source and storage site for short chain aliphatic glucosinolates (Andersen et al., 2013). GB03 induces a 33 and 70% increase in total glucosinolate content in shoots and roots, respectively. Although tissue perception of bacterial VOCs is unknown, GB03-induced increase in glucosinolates is higher in roots, suggesting that GB03 may have initially been recognized as a pathogen with glucosinolates potentially induced as a defense mechanism. In fact, *Arabidopsis* indolic glucosinolates are pathogen-induced by *Erwinia carotovora* (Brader et al., 2001).

In agriculture, in addition to generating defense-rich plants, sulfur-rich cruciferous crops such as canola (*Brassica napus* L. cv) require uniform sulfur uptake independent of soil sulfur content (Scherer, 2001). Other plant specific soil bacteria, active in triggering canola growth promotion have been examined, although their role in regulating sulfur assimilation has yet

to be characterized (Kloepper et al., 1988; Bertrand et al., 2001). Commercial canola inoculants have been developed that oxidize elemental sulfur to the sulfate form that is more readily taken up by plants. Such bacterial inoculants are agriculturally relevant since elemental sulfur, an industrial by-product, is economically viable for regenerating sulfur deficient soils. Here, GB03 transcriptionally induces sulfate assimilation and coordinates this process with enhanced sulfate uptake as well as elevated sulfur, cysteine, and glucosinolate accumulation. In addition to the role of glucosinolates in plant defense, select sulfur metabolites possess cancer-preventive properties. For humans, isothiocyanates derived from the hydrolysis of MSG are potent cancer-preventive agents (Hansen et al., 2007; Li et al., 2008). The cancer-preventive properties of MSG have been targeted for elevated production by plant breeders of cruciferous crops (Li et al., 2008) and here they are shown to be selectively induced by GB03, relative to other aliphatic glucosinolates.

AUTHOR CONTRIBUTIONS

MA designed the project, performed experiments, collected data, analyzed results, and wrote up the study; RN performed experiments, collected data, and analyzed results; XX designed the project, performed experiments, collected data, and analyzed data; YS collected data; KS collected data; J-LZ designed the project and analyzed the results; and PP designed the project, analyzed results, and wrote up the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00458

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Long Chain Alcohols Produced by *Trichoderma citrinoviride* Have Phagodeterrent Activity against the Bird Cherry-Oat Aphid *Rhopalosiphum padi*

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In this study we report the effects of fungal metabolites isolated from cultures of the fungus *Trichoderma citrinoviride* ITEM 4484 on the feeding preference of the aphid *Rhopalosiphum padi*, a major pest of cereal crops. Different phagodeterrent metabolites were purified by a combination of direct and reverse phase column chromatography and thin-layer chromatography. Chemical investigations, by spectroscopic and chemical methods, led to the identification of different long chain primary alcohols (LCOHs) of the general formula R-OH, wherein R is a long, unbranched, unsubstituted, linear aliphatic group. LCOHs have been reported as components of lepidopteran pheromone blends, but their phagodeterrent effect to aphids is herein reported for the first time. The effects of LCOHs on *R. padi* were studied by behavioral and electrophysiological bioassays. Feeding preference tests that were carried out with winged and wingless morphs of *R. padi* showed that LCOHs had high phagodeterrent activity and restrained aphids from settling on treated leaves at a concentration as low as 0.15 mM (0.036 g/l). The results of different electrophysiological analyses indicated that taste receptor neurons located on the aphid tarsomeres were involved in the LCOHs perception. Behavioral assays carried out with some commercial agrochemicals, including azadirachtin A, pyrethrum and a mineral oil-based product, in combination with 1-hexadecanol, the LCOH most abundantly produced by *T. citrinoviride* ITEM 4484, showed that these different active principles could be applied together, resulting in a useful increase of the phagodeterrent effect. The data shown indicate that these compounds can be profitably utilized for novel applications in biotechnical control of aphid pests. Furthermore, the tested LCOHs have no chiral centers and therefore can be obtained with good yield and at low cost through chemical synthesis, as well as from natural sources.

Keywords: *Trichoderma*, long-chain alcohols, biocontrol, aphids, phagodeterrence

INTRODUCTION

Aphids are insect pests of great economic importance for agriculture and represent one major cause of damage and loss of both quantity and quality of produce in horticultural, cereal and tree crops (Blackman and Eastop, 2007; Dedryver et al., 2010). Aphids use their piercing sucking mouthparts to feed on host plants causing damage by subtraction of sap, injection of saliva which has phytotoxic effects, and spread of insect-transmitted virus diseases (Hull, 2002; Ng and Perry, 2004; Katis et al., 2007). Moreover, aphids produce large amounts of honeydew, a sugary liquid waste on which sooty molds often grow hindering the plant photosynthetic capability (Dedryver et al., 2010). Various insecticides can be used to control aphids, however the intensive use of chemical insecticides has led to environmental pollution and onset of resistant populations of various aphid species (Vanlerberghe-Masutti and Guillemaud, 2007). In addition, the phasing out of some active principles and the more and more strict registration procedures for new agrochemicals have in recent times led to a progressive reduction of the number of insecticides available for aphid control (Dedryver et al., 2010; Roubos et al., 2014). Therefore, there is a growing interest in the development of innovative control strategies that may lead to a progressive reduction of chemicals, with the aim to minimize the environmental impact of pest management and improve the safety of the agro-food chain.

Signaling chemicals produced by plants (Reddy and Guerrero, 2004; Honda et al., 2010) and microorganisms (Davis et al., 2013) may evoke different behavioral responses in insects, such as attraction, repellence or aggregation stimulation. The concept of using nontoxic compounds as insect antifeedants in crop protection has grown with the demonstration of the potent feeding deterrent effect of azadirachtin and neem seed extracts to a large number of pest species (Isman, 2006). The identification of new compounds with phagodeterrent activity, which possess the capability to interfere with aphid host plant selection and host acceptance, is currently becoming of great interest for the design of innovative biotechnical strategies in control of phytophagous insects.

Different species of fungi of the genus *Trichoderma* have long been known as effective biocontrol agents of a wide range of important soil-borne and air-borne plant pathogens (Kubicek and Harman, 1998; Lorito et al., 2010). Furthemore it has been shown that some *Trichoderma* species also have a potential for biocontrol of insects (Jassim et al., 1990; Ganassi et al., 2001, 2007; Shakeri and Foster, 2007; Verma et al., 2007). In previous studies (Ganassi et al., 2002, 2007, 2009), we showed that cultures of fungal isolates belonging to four species of *Trichoderma*, namely *T. atroviride*, *T. citrinoviride*, *T. harzianum* and *T. viride,* had significant phagodeterrent activity toward different species of aphids, including *Schizaphis graminum* (Ganassi et al., 2007), one of the most important pests of cereal crops, and the polyphagous species *Myzus persicae* (Ganassi et al., 2009). Further investigations on fungal compounds produced by the isolate *T. citrinoviride* ITEM 4484 led to the isolation and structure determination by spectroscopic methods (essentially NMR and MS) of two new metabolites, namely citrantifidiene and citrantifidiol, which exhibited a potent antifeedant effect toward both winged and wingless aphid morphs (Evidente et al., 2008). Later on, two more compounds with phagodeterrent activity were isolated from the same source and identified as bislongiquinolide and dihydrotrichodimerol, two compounds belonging to the chemical family of bisorbicillinoids (Evidente et al., 2009). In this paper we report the chemical and biological characterization of one more group of phagodeterrent metabolites obtained from *T. citrinoviride* ITEM 4484, which consists of primary alcohols of the general formula R-OH, wherein R is a long, unbranched, unsubstituted linear aliphatic group. Long chain alcohols (LCOHs), varying between C10 and C18, are commonly detected especially in lepidopteran pheromone blends emitted by females to attract males (Francke and Schulz, 2010; Ando and Yamakawa, 2011), but their phagodeterrent effect to aphids and their possible use for control of this pest is herein reported for the first time. We tested the LCOHs identified in cultures of *T. citrinoviride* ITEM 4484 and other commercially available LCOHs in feeding preference assays against the bird cherry-oat aphid *Rhopalosiphum padi*, one of the most important pests of all the major cereals (Blackman and Eastop, 2000) and the most important vector of the viruses responsible of Barley Yellow Dwarf Disease (BYD) in autumn-sown cereals in Europe (Dedryver and Harrington, 2004). In addition, some LCOHs were also tested in combination with commercial agrochemicals in order to compare the effectiveness of LCOHs with that of a technology currently in use and to assess the compatibility of the active principles. Finally, we investigated the mode of perception of LCOHs by *R. padi* using a standard electroantennogram (EAG) technique and single chemosensory cell (from single tarsal sensillum) recordings.

MATERIALS AND METHODS

Fungal Strain

The producing strain used in this study was isolated from soil under *Abies* sp. in Tyrol (Austria). After reisolation from a single germinated conidium, the strain was maintained in purity in the culture collection of the Institute of Sciences of Food Production with the accession number ITEM 4484. The strain was identified as belonging to the species *Trichoderma citrinoviride* on the basis of morphological characters and sequencing of a region of the nuclear rDNA comprising the two diagnostic regions ITS-1 and ITS-2 and of a fragment of the translation elongation factor gene (TEF-1α), as previously reported (Evidente et al., 2008).

Aphids

The aphids used in tests belonged to the species *Rhopalosiphum padi*. They were reared in the laboratory on durum wheat plants (*Triticum durum*) for several generations in a thermostatic chamber at 20°C under 16/8 h day/night photoperiod to induce parthenogenesis. Trials were carried out with winged and wingless adult morphs. Winged offsprings were obtained in the laboratory by crowding.

Production, Extraction, Purification and Identification of Metabolites with Phagodeterrent Activity

The fungal metabolites with phagodeterrent activity against aphids were purified from cultures of *T. citrinoviride* ITEM 4484 through a bioassay-guided chromatographic fractionation of the culture organic extract. ITEM 4484 was cultured on sterile rice kernels and the culture was processed and extracted with methanol (MeOH)-H_2O 55:45 (v/v) and dichloromethane (CH_2Cl_2) and purified through chromatographic procedures previously developed and reported by Evidente et al. (2008, 2009). The organic extract was dried on sodium sulfate (Na_2SO_4) and evaporated under reduced pressure, yielding 15 g of a brown oil that showed a significant phagodeterrent activity. The extract was fractionated by column chromatography (CC) using chloroform ($CHCl_3$)-*iso*-propanol (*i*-PrOH) 85:15 as eluent, yielding 10 groups (A–L) of homogeneous fractions. The residue of the fraction F1 (7.5 mg) was further purified by preparative thin layer chromatography (TLC) using petroleum ether- acetone (Me_2CO) 9:1 as eluent and yielded a mixture of LCOHs (5.4 mg) as below reported, which appeared as a homogeneous spot (*Rf* 0.29).

Spectroscopic Data of LCOHs

IR $\nu_{max}cm^{-1}$ 3349, 2922, 2852, 1466; UV λ_{max} nm < 220;^{1}H NMR, δ: 5.38 (m), 5.35 (m), 3.64 (t, $J = 6.3$ Hz), 2.01 (m), 1.57–1.26 (m), 0.88 (t, $J = 6.8$ Hz). ^{13}C NMR, δ: 130.5 (d), 130.3 (d), 129.9 (d), 129.8 (d), 63.1 (t), 32.8–22.7 (t), 14.1 (q); EI MS (rel. int) *m/z*: 265 $[M_{C16}+Na]^+$, 293 $[M_{C18}+Na]^+$, 291 $[M_{C18:1}+Na]^+$.

Conversion of LCOHs into the Corresponding Esters

An aliquot of the mixture of LCOHs (0.8 mg) was dissolved in Me_2CO (0.20 ml) at 0°C and oxidized with Jones reagent (Bowden et al., 1946) until a persistent orange color was obtained. After 10 min, all the starting compounds had reacted, as determined by TLC (eluent petroleum ether-Me_2CO, 9:1) and the reaction was stopped by addition of *i*-PrOH. The suspension was diluted with iced H_2O to obtain a brilliant green solution, which was extracted with ethyl acetate (EtOAc) (3 × 0.5 ml). The organic extracts were combined, dried with Na_2SO_4, and evaporated under reduced pressure. The residue was dissolved in MeOH (300 µl) and esterified with an ethereal solution of diazomethane (CH_2N_2) until a persistent yellow color was obtained. After 15 min the reaction was monitored by TLC (eluent petroleum ether-Me_2CO, 9:1), and having all the starting compounds reacted, the reaction was stopped by evaporation under a N_2 stream. The residue was analyzed by gas chromatography–mass spectrometry (GC-MS) as below described.

Preparation of *cis*-9-octadecen-1-ol

Lithium aluminum tetrahydride ($LiAlH_4$) (113.0 mg, 3.20 mM) was added to a solution of methyl oleate (850.0 mg, 2.87 mM) in anhydrous diethyl ether (Et_2O) (20 ml). The reaction was stirred for 2 h at 0°C, then stopped by addition of ethyl acetate (EtOAc) to remove the excess of $LiAlH_4$ and diluted with water. The aqueous solution was extracted 3 times with EtOAc and the organic layers were combined and dried with anhydrous Na_2SO_4. After removal of the solvent, the residue (803.5 mg) was purified by CC on silica gel, eluted with the eluent system *n*-hexane-Me_2CO (7:3, v:v) giving 651.2 mg of *cis*-9-octadecen-1-ol with a yield of 76.6%.

Preparation of *trans*-9-octadecen-1-ol

Methyl elaidate (888.4 mg; 3.00 mM) was converted in *trans*-9-octadecen-1-ol like previously reported for the *cis* isomer, with a yield of 75.6% (671.0 mg).

General Experimental Procedures

Infrared spectroscopy (IR) spectra were recorded as glassy film on a Perkin-Elmer (Norwalk, CT, USA) Spectrum One FT-IR Spectrometer and ultraviolet (UV) spectra were taken in acetonitrile (MeCN) solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 600 or 400 MHz and at 125 or 100 MHz, respectively, in deuterochlorofom ($CDCl_3$), on Bruker (Karlsruhe, Germany) spectrometers. The same solvent was used as internal standard. Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded on Waters Micromass Q-TOF Micro (Milford, MS, USA).

Analytical and preparative TLC was performed on silica gel, Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively, (Merck, Darmstadt, Germany). The spots were visualized by spraying first with 10% sulphuric acid (H_2SO_4) in MeOH and then with 5% phosphomolybdic acid in ethanol (EtOH), followed by heating at 110°C for 10 min. CC was performed on silica gel column, Kieselgel 60, 0.063–0.200 mm (Merck). Standards of 1-hexadecanol and 1-octadecanol and of methyl esters of oleic and elaidic acids were purchased from Larodan Fine Chemicals (Malmö, Sweden).

The GC-MS analyses were carried out on a QP5050 Shimadzu (Kyoto, Japan) instrument equipped with a Supelcowax TM10 column, 60 m × 0.32, 0.5 µm (Supelco, Bellefonte, PA, USA). Helium was used as carrier gas at a flow rate of 2.1 ml/min, with an initial pressure of 52 Kpa. The instrument was programmed at 180°C for 15 min, increasing 10°C/min to 230°C for 20 min; the injector temperature was 270°C. The MS analysis was carried out by electron ionization (EI) at 70 eV; the interface temperature was 270°C; the source ionic temperature was 200°C; mass ranged from 40 to 450 amu; the scan rate was 0.5 scan/s.

Behavioral Assays

The phagodeterrent activity of the LCOHs 1-tetradecanol, 1-pentadecanol, 1-hexadecanol and 1-heptadecanol, were assessed by feeding preference tests on both winged and wingless morphs of *R. padi*; 1-octadecanol, *cis*-9-octadecen-1-ol, *trans*-9-octadecen-1-ol, 1-nonadecanol and 1-eicosanol, due to their limited supply, were tested only on winged morphs. For testing purposes, batches of pure LCOHs 1-tetradecanol, 1-pentadecanol, 1-heptadecanol, and 1-eicosanol were purchased by Sigma-Aldrich (St. Louis, MO, USA).

To perform the assays, the LCOHs were first solubilized in 5% (v/v) aqueous MeOH and then diluted with 5% MeOH to obtain different test concentrations. Excised wheat leaves about 5 cm in length were dipped in LCOH or control (5% MeOH) solutions for 10 s. Then, the leaves were placed on wet filter paper in 12 cm-diameter Petri dishes. Each dish contained two leaves, one treated with the test solution and one dipped in the control solution, arranged in parallel at a distance of 4 cm. Aphids were placed between the two leaves with a fine brush, and their position was recorded every hour for 8 h, starting from the initial access that began less than 1 h after leaf excision. In the tests on winged morphs the LCOHs 1-tetradecanol, 1-pentadecanol, 1-hexadecanol and 1-heptadecanol were tested at concentrations of 0.037, 0.075, 0.15, 0.3, 0.6, and 1.2 mM. Due to the limited availability of 1-octadecanol, *cis*-9-octadecen-1-ol, *trans*-9-octadecen-1-ol, 1-nonadecanol and 1-eicosanol, these compounds were tested only at the highest concentration of 1.2 mM. In tests on wingless morphs 1-tetradecanol, 1-pentadecanol, 1-hexadecanol and 1-heptadecanol were tested at concentrations of 0.075, 0.15, 0.3, 0.6, and 1.2 mM. Limitedly to winged morphs, behavioral bioassays were also carried out with three different combinations of two LCOHs chosen among the compounds that had given the best results, when assayed separately: 1-tetradecanol, 1-pentadecanol, 1-hexadecanol and 1-heptadecanol. The blends of LCOHs were made as follows: 1-tetradecanol 0.6 mM plus 1-hexadecanol 0.6 mM; 1-pentadecanol 0.6 mM plus 1-hexadecanol 0.6 mM; 1-pentadecanol 0.6 mM plus 1-heptadecanol 0.6 mM.

In addition, in order to compare the effect of LCOHs with substances that are currently in use and to assess the compatibility of active principles, behavioral assays on winged morphs were carried out with some commercial agrochemicals used in integrated pest management, and with a combination of these agrochemicals with 1-hexadecanol, the LCOH more abundantly produced by *T. citrinoviride* ITEM 4484. The tested agrochemicals were three insecticides that act by contact against a broad spectrum of insects, including aphids: viz. Pyganic®1.4 that contains natural pyrethrum, UFO®Ultra Fine Oil, containing a refined mineral oil, and NeemAzal®-T/S, containing azadirachtin A. In addition, Gondor®, a soy lecithin-based adjuvant often used in combination with the above agrochemicals was also included in the bioassay. All the agrochemicals were provided by CBC (Europe) S.R.L.-Biogard division. The dosages of the agrochemicals used for testing were those recommended for field use, that are Pyganic®1.4 2.0 ml/l; UFO® 1% (v/v); Gondor® 2.5 ml/l; NeemAzal®-T/S 2.5 ml/l. Pyganic®1.4 was tested in combination with 0.15, 0.30. 0.6, and 1.2 mM of 1-hexadecanol; UFO® was tested in combination with 0.3 and 1.2 mM of 1-hexadecanol; UFO® was tested in combination with 0.30 and 0.60 mM of 1-hexadecanol; NeemAzal®-T/S was tested in combination with 0.15 mM of 1-hexadecanol. For the assays, the agrochemicals were solubilized in distilled water and the control was distilled water. In tests carried out with 1-hexadecanol, the agrochemicals were added to solutions of 1-hexadecanol in 5% MeOH; 5% MeOH solution was used as control.

Experimental Design and Statistical Analysis

In the behavioral assays, 10 individuals per each treatment were tested and the experiments were repeated 12 times, except for the assays carried out on winged morphs with 1-hexadecanol 1.2 mM, 1-hexadecanol 0.075 mM, *cis*-9-octadecen-1-ol, 1.2 mM, and *trans*-9-octadecen-1-ol 1.2 mM, where 15, 21, 48, and 30 replicated experiments were run, respectively.

The raw data obtained from the feeding preference tests were analyzed by the Generalized Linear Model (GLM) for repeated measures (over time) procedure and compared by using a test of within-subjects effects. The differences between the means of the number of aphids per leaf in each of the experimental treatments and those of the number of aphids on the relevant controls over time were analyzed and adjusted by the Bonferroni test (Mukhopadhyay, 2009) for the number of comparisons.

The data of the feeding preference tests carried out with the agrochemicals and with the agrochemicals combined with different concentrations of 1-hexadecanol were utilized also to calculate the Feeding Preference Index (FPI). FPI values were calculated from the total number of aphids counted on treated (T) and control (C) leaves at hourly observations over a 8-h time interval as FPI = (C – T)/(C + T). Possible values for the index range between 1 (complete preference for control leaves) and −1 (complete preference for treated leaves), with a value equal or close to zero indicating no effect (Powell et al., 1997). For each treatment, the FPI values of the hourly observations were averaged and the data were analyzed by one-way analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) multiple comparison *post-hoc* test. All the statistical analyses were done with the SPSS software for Windows, release 22.0 (SPSS, Chicago, IL, USA).

Electrophysiological Bioassays

Electrophysiological bioassays were carried out with 1-hexadecanol (1.2 mM), 1-octadecanol (1.2 mM), *cis*-9-octadecen-1-ol (1.2 mM) and *trans*-9-octadecen-1-ol (1.2 mM) as taste and antennal stimuli. Recordings by single tarsal sensilla were performed according to previous studies (Solinas et al., 2001; Ganassi et al., 2007; Evidente et al., 2009). Electrophysiological responses from the mesothoracic distal tarsomere were recorded by combining different techniques previously used to study single chemosensory sensilla (Solinas et al., 2001; Maher and Thiery, 2004; Ganassi et al., 2007). The indifferent electrode, a glass micropipette containing an Ag/AgCl silver wire (tip 4–5 μm diameter) filled with a solution of NaCl 100 mM, was inserted into the aphid prothorax. The recording electrode, a glass micropipette (tip 2 μm diameter) containing one of the test stimuli, was connected to a sensillum. Action potentials were preamplified, filtered, and recorded with commercially available electrophysiological equipment (Tasteprobe Type DTP-1, Syntech, Hilversum, The Netherlands). Taste stimuli were diluted in a 100 mM NaCl solution in 5% (v/v) MeOH; as control stimuli a 100 mM NaCl solution in distilled water or in 5% MeOH were used. Test solutions were put into the micropipette 10 s before the experiment. Single-cell recordings

were carried out at 22 ± 2°C and 70 ± 10% room humidity. Electrical activity was recorded for 1 s after stimulus onset, and 5 min was allowed to elapse between presentation of successive stimuli to the same sensillum. Test and control solutions were applied in a random series on the same sensillum. Action potentials (spikes) were stored on a computer or a magnetic tape (CditII, IEC II/Type II, High Bias 70 ms EQ, position chrome, Sony, Pontonx sur l'Adour, France) by a double-channel recorder (Sony, TC-D5M) and successively analyzed with the AutoSpike 3.1 program (Syntech) on the basis of their amplitude, shape, and frequency. Sensilla that failed to respond to the tested solutions were considered not functioning and were discarded (Crnjar and Prokopy, 1982; Ganassi et al., 2007). Responses of the sensory cells were evaluated as spike frequency (spikes per second) during the first second of stimulation, 100 ms after stimulus onset. Firing frequencies were compared by means of the Student's *t*-test.

In order to verify the possible occurrence of olfactory responses, test stimuli were also tested on antennae of living aphids. Stimuli were absorbed on a piece of filter paper (1 cm^2) inserted in a Pasteur pipette. Antennal responses were obtained by using a standard electroantennographic (EAG) technique (Ambrosi et al., 2001; De Cristofaro et al., 2004; Anfora et al., 2014), and the green leaf volatile (*Z*)-3-hexen-1-ol (25μl of a 1:10 v/v diluted solution in pure MeOH) was used as an EAG active control stimulus.

RESULTS

Identification of LCOHs Produced by *T. citrinoviride* Item 4484

The organic extract obtained from the solid culture of *T. citrinoviride* 4484 was purified by combined CC and TLC on silica gel as described in detail in the Materials and Methods section, yielding the already known compounds citrantifidiene (acetic acid 4-acetoxy-6-hydroxy-1-(2-diydroxy-ethyl)-hexa-1,3-dienyll ester and citrantifidiol (1,2,3-trimethyl-4-(4-methylpent-3-enyl)-cyclohexane-1,3-diol (Evidente et al., 2008), trichodimerol, dihydrotrichodimerol, bislongiquinolide, and dihydrobislongiquinolide (Evidente et al., 2009) and a homogeneous oily mixture of metabolites which were identified as LCOHs as below reported.

Their IR spectrum showed bands that are typical of hydroxy and olefinic groups (Nakanishi and Solomon, 1977) while the UV spectrum consistently exhibited an end-absorption (Pretsch et al., 2000). The ^{1}H and ^{13}C NMR spectra of this mixture showed signals characteristic of olefinic protons and carbons [δ: 5.38 (m), 5.35 (m), and 130.5 (d), 130.3 (d), 129.9 (d), 129.8 (d)], of hydroxy and aliphatic methylene groups [3.64 (t, J = 6.3 Hz and 63.1 (t), 32.8–22.7 (t), and 1.57–1.26 (m)], and of terminal methyl groups [0.88 (t, J = 6.8 Hz) and 14.1 (q)] (Breitmaier and Voelter, 1987; Pretsch et al., 2000). Their ESI MS spectrum showed sodium clusters at *m/z* 265 $[M_{C16}+Na]^+$, 293 $[M_{C18}+Na]^+$, 291 $[M_{C18:1}+Na]^+$ consistent with the presence of C_{16} and C_{18} saturated and C_{18} unsaturated fatty alcohols.

The LCOHs were identified by GC-MS analysis as follows. They were first converted into the corresponding acids by oxidization with Jones reagent and then converted in the corresponding methyl esters by reaction with an ethereal solution of diazomethane. The esters so obtained were subjected to GC-MS analysis, as reported in the experimental section, in comparison with chemical reference standards. They were identified as the methyl esters of hexadecanoic and octadecanoic acids and the *cis* and *trans* stereoisomers of the 9-octadecenoic acid.

Therefore, the alcohols produced by *T. citrinoviride* and purified as a mixture as above reported were identified as 1-hexadecanol, 1-octadecanol and *cis*- and *trans*-9-octadecen-1-ol. These latter compounds were also prepared with high yields in one step from the corresponding commercially available esters (methyl oleate and methyl elaidate, respectively) by reduction with $LiAlH_4$.

Behavioral Assays

Data of behavioral assays were analyzed with the GLM repeated measures procedure. This analysis assesses whether the interaction between either test conditions (treated or untreated) and the changes over the time of the number of aphids that visited the treated leaves or the control leaves is statistically significant. No time × treatment interaction effect indicates that the number of aphids per treated leaf and the number of aphids per control leaf did not change over time. In our bioassays a significant time × treatment interaction effect at a given concentration indicated that the number of aphids counted on control leaves increased over the time of the trial. The Bonferroni test was used to assess whether the average number of aphids on treated leaves was significantly smaller than that on corresponding control leaves, over time, indicating a phagodeterrent effect of the test solution.

The results of phagodeterrent effect of LCOHs on *R. padi* winged morphs are shown in **Table 1**.

1-tetradecanol

The GLM analysis of the data revealed time × treatment interaction effects at the concentrations 1.2, 0.6, 0.15, and 0.075 mM. On the contrary, at the concentrations 0.3 and 0.037 mM no time × treatment interaction effect was found. Over time, the average number of aphids on leaves treated with 1-tetradecanol at the concentrations of 1.2, 0.6, 0.3, 0.15, and 0.075 mM was significantly smaller than the number of aphids on the relevant control leaves, but no significant difference was found at the concentration of 0.037 mM (Bonferroni test).

1-pentadecanol

The GLM analysis of the data revealed time × treatment interaction effects at the concentrations 1.2, 0.6, 0.3, and 0.15 mM, while no time × treatment interaction effect was found at the concentrations of 0.075 and of 0.037 mM. The average number of aphids on leaves treated with 1-pentadecanol at all the tested concentrations, except 0.037 mM, was significantly smaller than that on corresponding control leaves over time (Bonferroni test).

TABLE 1 | Effect of long-chain alcohols at different concentrations on feeding preference of *Rhopalosiphum padi* winged morph.

Test compound	Concentration (mM)	GLM (time × treatment)[a]		Bonferroni test[b]	
				Mean treatment	Mean control
1-Tetradecanol	1.2	$F_{(7, 154)} = 4.230$	$P < 0.0001$	1.938 ± 0.322	6.271 ± 0.322**
	0.6	$F_{(7, 154)} = 2.318$	$P < 0.05$	2.365 ± 0.264	5.292 ± 0.264**
	0.3	$F_{(7, 154)} = 1.186$	$P > 0.05$	3.271 ± 0.217	5.552 ± 0.217**
	0.15	$F_{(7, 154)} = 10.72$	$P < 0.0001$	2.375 ± 0.370	5.980 ± 0.370**
	0.075	$F_{(7, 154)} = 5.173$	$P < 0.0001$	3.500 ± 0.384	4.958 ± 0.384*
	0.037	$F_{(7, 154)} = 1.210$	$P > 0.05$	3.969 ± 0.414	5.146 ± 0.414n.s.
1-Pentadecanol	1.2	$F_{(7, 154)} = 2.354$	$P < 0.05$	1.365 ± 0.301	7.615 ± 0.301**
	0.6	$F_{(7, 154)} = 11.052$	$P < 0.0001$	2.385 ± 0.237	6.188 ± 0.237**
	0.3	$F_{(7, 154)} = 4.686$	$P < 0.0001$	2.271 ± 0.283	6.292 ± 0.283**
	0.15	$F_{(7, 154)} = 4.265$	$P < 0.0001$	3.406 ± 0.405	5.083 ± 0.405**
	0.075	$F_{(7, 154)} = 1.576$	$P > 0.05$	3.073 ± 0.335	5.594 ± 0.335**
	0.037	$F_{(7, 154)} = 0.674$	$P > 0.05$	4.954 ± 0.383	4.854 ± 0.383n.s.
1-Hexadecanol	1.2	$F_{(7, 196)} = 5.488$	$P < 0.01$	1.308 ± 0.315	7.717 ± 0.315**
	0.6	$F_{(7, 154)} = 4.367$	$P < 0.01$	2.281 ± 0.371	6.531 ± 0.371**
	0.3	$F_{(7, 154)} = 1.336$	$P > 0.05$	2.635 ± 0.323	6.635 ± 0.323**
	0.15	$F_{(7, 154)} = 2.837$	$P > 0.05$	3.938 ± 0.429	5.479 ± 0.429*
	0.075	$F_{(7, 280)} = 0.791$	$P > 0.05$	4.024 ± 0.349	5.113 ± 0.349*
	0.037	$F_{(7, 154)} = 1.390$	$P > 0.05$	3.719 ± 0.352	4.125 ± 0.352n.s.
1-Heptadecanol	1.2	$F_{(7, 154)} = 6.940$	$P < 0.0001$	1.094 ± 0.294	6.427 ± 0.294**
	0.6	$F_{(7, 154)} = 0.863$	$P > 0.05$	2.177 ± 0.298	5.240 ± 0.298**
	0.3	$F_{(7, 154)} = 1.265$	$P > 0.05$	3.063 ± 0.532	5.448 ± 0.532**
	0.15	$F_{(7, 154)} = 2.892$	$P < 0.01$	3.240 ± 0.410	5.677 ± 0.410**
	0.075	$F_{(7, 154)} = 2.654$	$P < 0.05$	4.250 ± 0.373	5.406 ± 0.373*
	0.037	$F_{(7, 154)} = 1.912$	$P > 0.05$	3.854 ± 0.274	4.417 ± 0.274n.s.
1-Octadecanol	1.2	$F_{(7, 154)} = 1.012$	$P > 0.05$	2.469 ± 0.333	5.927 ± 0.333**
cis-9-Octadecen-1-ol	1.2	$F_{(7, 658)} = 9.093$	$P < 0.0001$	3.518 ± 0.235	5.826 ± 0.235**
trans-9-Octadecen-1-ol	1.2	$F_{(7, 406)} = 1.557$	$P > 0.05$	3.938 ± 0.291	5.067 ± 0.291**
1-Nonadecanol	1.2	$F_{(7, 154)} = 1.573$	$P > 0.05$	3.427 ± 0.391	4.792 ± 0.391*
1-Eicosanol	1.2	$F_{(7, 154)} = 5.048$	$P < 0.01$	3.042 ± 0.303	5.073 ± 0.303**

[a] *Values of $P < 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was statistically significant. Values of $P > 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was not statistically significant.*

[b] *The average number of aphids on treated leaf and the number of aphids on the control leaf over the duration of the assay were analyzed and adjusted with Bonferroni test for the number of comparisons. In each treatment, significant difference between the means are indicated with *($P < 0.05$) or with **($P < 0.01$), ns indicates a not significant difference.*

1-hexadecanol

The GLM analysis revealed time × treatment interaction effects at the concentrations of 1.2 and 0.6 mM. No time × treatment interaction effects were found with concentrations of 0.3, 0.15, 0.075, and 0.037 mM. The average number of aphids on leaves treated with 1-hexadecanol 1.2, 0.6, 0.3, 0.15, or 0.075 mM was significantly smaller than that on control leaves over time, while at concentration 0.037 mM it was not significantly different from control (Bonferroni test).

1-heptadecanol

Time × treatment interaction effect was found at the concentrations 1.2, 0.15, and 0.075 mM, while no time × treatment interaction effects were found with 1-heptadecanol 0.6, 0.3, and 0.037 mM. The average number of aphids on leaves treated with 1-heptadecanol at all the concentrations tested, except 0.037 mM, was significantly smaller than that on control leaves over time (Bonferroni test).

1-octadecanol

The GLM analysis of the data obtained in tests carried out with 1-octadecanol tested at concentration 1.2 mM, revealed no time × treatment interaction effects. The Bonferroni test showed that the average number of aphids on treated leaves was significantly smaller than that on corresponding control leaves over time.

cis-9-octadecen-1-ol

The GLM analysis of the data obtained in tests carried out with *cis*-9-octadecen-1-ol 1.2 mM revealed time × treatment interaction effects. The Bonferroni test showed that the average number of aphids on treated leaves was significantly smaller than that on corresponding control leaves over time.

trans-9-octadecen-1-ol

The GLM analysis revealed no time × treatment interaction effects of *trans*-9-octadecen-1-ol applied at the concentration 1.2 mM. The Bonferroni test showed that the average number of aphids on treated leaves was significantly smaller than that on corresponding control leaves over time.

1-nonadecanol

No time × treatment interaction effect was found for 1-nonadecanol tested at 1.2 mM. The Bonferroni test showed that the average number of aphids on treated leaves was significantly smaller than that on control over time.

1-eicosanol

The GLM analysis of the data obtained in tests carried out with 1-eicosanol 1.2 mM revealed time × treatment interaction effects. The Bonferroni test showed that the average number of aphids on treated leaves was significantly smaller than the number of aphids on corresponding control leaves over time.

The results of phagodeterrent effect of LCOHs on *R. padi* wingless morphs are shown in **Table 2**.

1-tetradecanol

The GLM analysis of the data obtained in tests carried out with 1-tetradecanol 1.2, 0.3, 0.15, and 0.075 mM revealed time × treatment interaction effects. No time × treatment interaction effect was found with the concentration 0.6 mM. The average number of aphids on leaves treated with 1-tetradecanol at the concentrations 1.2, 0.6, 0.3, and 0.15 mM was significantly smaller than the number of aphids on corresponding control leaves, over time, while no significant difference was found with 0.075 mM (Bonferroni test).

1-pentadecanol

Time × treatment interaction effect was found for 1-pentadecanol applied at 1.2, 0.6, and 0.15 mM, but not at 0.3 and 0.075 mM. The average number of aphids on leaves treated with 1-pentadecanol 1.2, 0.6, 0.3, and 0.15 mM, but not 0.075 mM, was significantly smaller than that on the relevant control leaves, over time (Bonferroni test).

TABLE 2 | Effect of long-chain alcohols at different concentrations on feeding preference of *Rhopalosiphum padi* wingless morph.

Test compound	Concentration (mM)	GLM (time × treatment)[a]		Bonferroni test[b]	
				Mean treatment	Mean control
1-Tetradecanol	1.2	$F_{(7, 154)} = 4.527$	$P < 0.01$	2.208 ± 0.259	7.104 ± 0.259**
	0.6	$F_{(7, 154)} = 1.953$	$P > 0.05$	2.813 ± 0.308	5.594 ± 0.308**
	0.3	$F_{(7, 154)} = 9.320$	$P < 0.01$	3.583 ± 0.239	5.271 ± 0.239**
	0.15	$F_{(7, 154)} = 3.430$	$P < 0.01$	3.542 ± 0.253	5.177 ± 0.253**
	0.075	$F_{(7, 154)} = 2.566$	$P < 0.01$	4.083 ± 0.557	4.667 ± 0.557n.s.
1-Pentadecanol	1.2	$F_{(7, 154)} = 11.926$	$P < 0.01$	2.313 ± 0.321	6.719 ± 0.321**
	0.6	$F_{(7, 154)} = 6.624$	$P < 0.01$	2.125 ± 0.378	5.958 ± 0.378**
	0.3	$F_{(7, 154)} = 0.940$	$P > 0.05$	2.563 ± 0.163	6.104 ± 0.163**
	0.15	$F_{(7, 154)} = 3.382$	$P < 0.05$	3.083 ± 0.301	5.188 ± 0.301**
	0.075	$F_{(7, 154)} = 0.0931$	$P > 0.05$	4.625 ± 0.412	4.333 ± 0.412n.s.
1-Hexadecanol	1.2	$F_{(7, 154)} = 8.041$	$P < 0.01$	2.698 ± 0.291	5.354 ± 0.291**
	0.6	$F_{(7, 154)} = 2.660$	$P < 0.05$	2.604 ± 0.347	5.385 ± 0.347**
	0.3	$F_{(7, 154)} = 0.499$	$P > 0.05$	3.167 ± 0.460	5.479 ± 0.460**
	0.15	$F_{(7, 154)} = 1.192$	$P > 0.05$	3.906 ± 0.421	5.240 ± 0.421*
	0.075	$F_{(7, 154)} = 2.017$	$P > 0.05$	4.135 ± 0.430	4.646 ± 0.430n.s.
1-Heptadecanol	1.2	$F_{(7, 154)} = 1.168$	$P < 0.01$	2.188 ± 0.247	6.561 ± 0.247**
	0.6	$F_{(7, 154)} = 11.885$	$P < 0.01$	2.354 ± 0.231	6.094 ± 0.231**
	0.3	$F_{(7, 154)} = 3.647$	$P < 0.01$	3.771 ± 0.393	4.979 ± 0.393*
	0.15	$F_{(7, 154)} = 0.744$	$P > 0.05$	3.958 ± 0.377	5.344 ± 0.377*
	0.075	$F_{(7, 154)} = 0.996$	$P > 0.05$	4.375 ± 0.280	4.750 ± 0.280n.s.

[a] *Values of $P < 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was statistically significant. Values of $P > 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was not statistically significant.*

[b] *The average number of aphids on treated leaf and the number of aphids on the control leaf over the duration of the assay were analyzed and adjusted with Bonferroni test for the number of comparisons. In each treatment, significant difference between the means are indicated with *($P < 0.05$) or with **($P < 0.01$), ns indicates a not significant difference.*

1-hexadecanol

The GLM analysis of the data of 1-hexadecanol applied at 1.2 and 0.6 mM revealed time × treatment interaction effects. No time × treatment interaction effects were found at the concentrations 0.3, 0.15, and 0.075 mM. The Bonferroni test showed that the average number of aphids on leaves treated with 1-hexadecanol 1.2, 0.6, 0.3, and 0.15 mM, but not with 1-hexadecanol 0.075 mM, was significantly smaller than the number of aphids on corresponding control leaves over time.

1-heptadecanol

Time × treatment interaction effects were found for 1-heptadecanol at 1.2, 0.6, and 0.3 mM, but not at 0.15, and 0.075 mM (GLM analysis). The average number of aphids on treated leaves treated with 1-heptadecanol at the concentrations 1.2, 0.6, 0.3, and 0.15 mM was significantly smaller than that on corresponding control leaves, while at a concentration of 0.075 mM the effect was not statistically significant.

Phagodeterrent effect of LCOHs blends are reported in **Table 3**. The blends tested on winged morphs of *R. padi* were indicated in the proper Materials and Methods section. In all the combinations, the GLM analysis revealed time × treatment interaction effects. The Bonferroni test revealed that the average number of aphids on treated leaves was significantly smaller than the number of aphids on corresponding control leaves, respectively, over time (**Table 3**).

Phagodeterrent effect of hexadecanol in combination with agrochemicals are reported in **Table 4**. The GLM analysis of the data obtained in experiments carried out with the test solutions as described in the Materials and Methods section, revealed time × treatment interaction effects (**Table 4**). For the treatments Gondor® 2.5 ml/l, NeemAzal®-T/S 2.5 ml/l and NeemAzal®-T/S 2.5 ml/l plus 1-hexadecanol 0.15 mM, the GLM analysis showed that the interaction between the number of aphids on treated leaf and the number of aphids on control leaf did not change over time (**Table 4**). The Bonferroni test revealed that in all the above treatments the average number of aphids on treated leaves was significantly smaller than the number of aphids on the control leaves, indicating that all the agrochemicals and all the combinations of agrochemicals and different concentrations of 1-hexadecanol that were tested had phagodeterrent effect (**Table 4**).

The FPI values obtained from feeding preference tests carried out with the agrochemicals and with the agrochemicals combined with different concentrations of 1-hexadecanol were subjected to ANOVA and SNK test for multiple comparisons; results are reported in **Figures 1A–D**. The results of comparison of FPI values of Pyganic®1.4 at the dosage for field application (2.0 ml/ml), different doses of 1-hexadecanol, and a combination thereof are shown in **Figure 1A**. The addition of 1-hexadecanol in a range of concentrations from 0.15 to 1.2 mM to Pyganic®1.4, significantly ($P < 0.05$) increased the phagodeterrent effect of the agrochemical. The addition of 1-hexadecanol increased the efficacy of Pyganic®1.4 regardless of the concentration. The combination of UFO® 1% with either 1.2 or 0.3 mM of 1-hexadecanol significantly increased the phagodeterrent activity of the agrochemical ($P < 0.05$; **Figure 1B**). Gondor® was tested at 2.5 ml/l in combination with 0.6 and 0.3.M mM of 1-hexadecanol, and these mixtures increased the phagodeterrent activity, as determined by the FPI (**Figure 1C**). The combination of NeemAzal®-T/S with 0.15 mM of 1-hexadecanol significantly ($P < 0.05$) increased the phagodeterrent effect of the agrochemical (**Figure 1D**).

Electrophysiological Bioassays

EAG responses showed that the tested LCOHs were not able to stimulate significantly the olfactory sensilla of either winged or wingless morphs of *R. padi* in respect of the solvent (MeOH). The antennae, on the contrary, were stimulated by the control stimulus, the green leaf volatile *cis*-3-hexen-1-ol that elicited significant responses (0.6 ± 0.2 mV).

Electrophysiological bioassays carried out on sensilla of the mesothoracic distal tarsomere revealed that the applications of either control solutions, i.e., NaCl (100 mM) or NaCl (100 mM) in 5% MeOH, evoked action potential frequencies similar to those obtained in resting activity. Single taste tarsal cells of winged and wingless morphs were significantly stimulated by 1-hexadecanol (1.2 mM) and 1-octadecanol (1.2 mM) and highly significantly stimulated by *cis*-9-octadecen-1-ol (1.2 mM) and *trans*-9-octadecen-1-ol (1.2 mM) with a significant increase ($P < 0.01$) of the frequency of action potentials over the controls

TABLE 3 | Effect of long chain alcohols blends on feeding preference of *Rhopalosiphum padi* winged morph.

Test blend	GLM (time × treatment)[a]		Bonferroni test[b]	
			Mean treatment	Mean control
1-Tetradecanol 0.6 mM+ 1-Hexadecanol 0.6 mM	$F_{(7, 154)} = 5.703$	$P < 0.05$	1.448 ± 0.851	7.698 ± 0.851**
1-Pentadecanol 0.6 mM + 1-Hexadecanol 0.6 mM	$F_{(7, 154)} = 9.247$	$P < 0.05$	1.979 ± 0.367	7.240 ± 0.367**
1-Pentadecanol 0.6 mM + 1-Heptadecanol 0.6 mM	$F_{(7, 154)} = 5.554$	$P < 0.05$	1.854 ± 0.346	7.115 ± 0.346**

[a] *Values of $P < 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was statistically significant.*

[b] *The average number of aphids on treated leaf and the number of aphids on control leaf over the duration of the assay were analyzed and adjusted with Bonferroni test for the number of comparisons. In each treatment, significant difference between the means are indicated with **($P < 0.01$).*

TABLE 4 | Effect of 1-hexadecanol (He) in combination with different agrochemicals on feeding preference of *Rhopalosiphum padi* winged morph.

Test solution[a]	GLM (time × treatment)[b]		Bonferroni test[c]	
			Mean treatment	Mean control
Pyganic® 1.4 (Py)	$F_{(7, 154)} = 4.341$	$P < 0.01$	1.042 ± 0.310	6.469 ± 0.310**
Py + He 1.2 mM	$F_{(7, 238)} = 3.202$	$P < 0.01$	0.486 ± 0.197	6.625 ± 0.197**
Py + He 0.6 mM	$F_{(7, 238)} = 10.787$	$P < 0.01$	0.347 ± 0.251	7.444 ± 0.251**
Py + He 0.3 mM	$F_{(7, 238)} = 16.337$	$P < 0.01$	0.319 ± 0.219	6.819 ± 0.219**
Py + He 0.15 mM	$F_{(7, 196)} = 3.241$	$P < 0.01$	0.396 ± 0.664	6.264 ± 0.542**
UFO® (UFO 1%)	$F_{(7, 154)} = 2.573$	$P < 0.05$	1.25 ± 0.365	6.27 ± 0.365**
UFO + He 1.2 mM	$F_{(7, 196)} = 13.118$	$P < 0.01$	0.367 ± 0.247	8.250 ± 0.247**
UFO + He 0.3 mM	$F_{(7, 196)} = 7.137$	$P < 0.01$	0.683 ± 0.259	7.108 ± 0.259**
Gondor® (Go)	$F_{(7, 196)} = 0.396$	$P > 0.05$	2.16 ± 0.243	6.28 ± 0.243**
Go + He 0.6 mM	$F_{(7, 196)} = 9.471$	$P < 0.01$	0.692 ± 0.184	7.933 ± 0.184**
Go + He 0.3 mM	$F_{(7, 196)} = 6.307$	$P < 0.01$	1.267 ± 0.232	6.592 ± 0.232**
NeemAzal®-T/S (Ne)	$F_{(7, 154)} = 0.525$	$P > 0.05$	3.76 ± 0.709	5.33 ± 0.709*
Ne + He 0.15 mM	$F_{(7, 154)} = 0.182$	$P > 0.05$	1.281 ± 0.184	2.875 ± 0.184**

[a] *The agrochemicals were tested at the dosages recommended by the manufacturers: Pyganic® 1.4, 2.0 ml/l; UFO®, 1% (v./v.); NeemAzal®-T/S, 2.5 ml/l. Gondor® was tested at 2.5 ml/l.*
[b] *Values of $P < 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was statistically significant. Values of $P > 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was not statistically significant.*
[c] *The average number of aphids on treated leaf and the number of aphids on the control leaf over the duration of the assay were analyzed and adjusted with Bonferroni test for the number of comparisons. In each treatment, significant difference between the means are indicated with *($P < 0.05$) or with **($P < 0.01$), ns indicates a not significant difference.*

(**Table 5**). Results of single chemosensory cell recordings indicate that the structures involved in the LCOHs perception are taste cells located on the aphid tarsomeres.

DISCUSSION

LCOHs are known signaling molecules in different insect groups, especially in Lepidoptera where they are part of pheromone blends (Francke and Schulz, 2010; Ando and Yamakawa, 2011). In particular, primary alcohols and their derivatives (mainly acetates and aldehydes) with a long straight chain (C10–C18) are commonly detected in pheromone-gland extracts of lepidopteran females and have been found to explicate an attractive action toward males (Ando and Yamakawa, 2011). Our results show that the LCOHs obtained from the isolate *T. citrinoviride* ITEM 4484 and LCOHs chemically related to them also have a strong phagodeterrent activity toward winged and wingless morphs of *R. padi* and interestingly, in several trials, the number of aphids on the control leaves increases over time.

Behavioral assays carried out with some insecticides in combination with 1-hexadecanol, the LCOH most abundantly produced by *T. citrinoviride* ITEM 4484, showed that 1-hexadecanol is compatible with different commercial active principles and the phagodeterrent effect of the mixture increases significantly. The agrochemicals Pyganic® 1.4 and NeemAzal®-T/S are natural compound-based insecticides utilized in integrated pest management. Their phagodeterrence is due to the presence of pyrethrum and azadirachtin A, respectively. Pyrethrum and its analogs pyrethrins are widely used as insecticides in agricultural, public health, and domestic applications (Wesseling et al., 2001; Ray and Fry, 2006) and have also a repellent effect toward different species of insects (Baumler and Potter, 2007; Patel et al., 2012; Prota et al., 2014). Azadirachtin blocks the synthesis and release of molting hormones from the prothoracic gland, leading to incomplete ecdysis in immature insects and to sterility in adult females and is a potent antifeedant compound to many insects species (Isman, 2006; Gahukar, 2014). Mineral oils, like the main active principle of UFO® (Ultra Fine Oil), have also been reported to exhibit repellent action against some insect pests (Mounts et al., 1988; Yang et al., 2010). In our experiments, 1-hexadecanol increased significantly the phagodeterrent effect of Pyganic® 1.4, NeemAzal®-T/S and Gondor®, applied at the dosage recommended for field application. The increasing effect of 1-hexadecanol in combination with these products was already significant at a dose as low as 0.15 mM (0.036 g/l).

The EAG study carried out with the LCOHs produced by *T. citrinoviride* ITEM 4484 (hexadecanol, octadecanol, *cis*-9-octadecenol and *trans*-9-octadecenol) showed that these LCOHs compounds are not able to stimulate the antennal olfactory receptor neurons of *R. padi*. The results of single chemosensory cell recordings indicate that taste cells located on the aphid tarsomeres are involved in the perception of the tested LCOHs. In previous papers we showed that such taste cells were also involved in the perception of other or unidentified metabolites produced by *Trichoderma* species, including *T. citrinoviride,* by different aphid species (Ganassi et al., 2007; Evidente et al., 2009).

Studies on *Cydia pomonella* pheromone composition reported that the LCOH 1-tetradecanol is among the 5 components of the female effluvia (El-Sayed et al., 1999; Witzgall et al., 2001). Ebbinghaus et al. (1998) reported that some *C. pomonella* sensilla auricillica contained olfactory receptor neurons, one of which responded to minor components, including 1-tetradecanol and

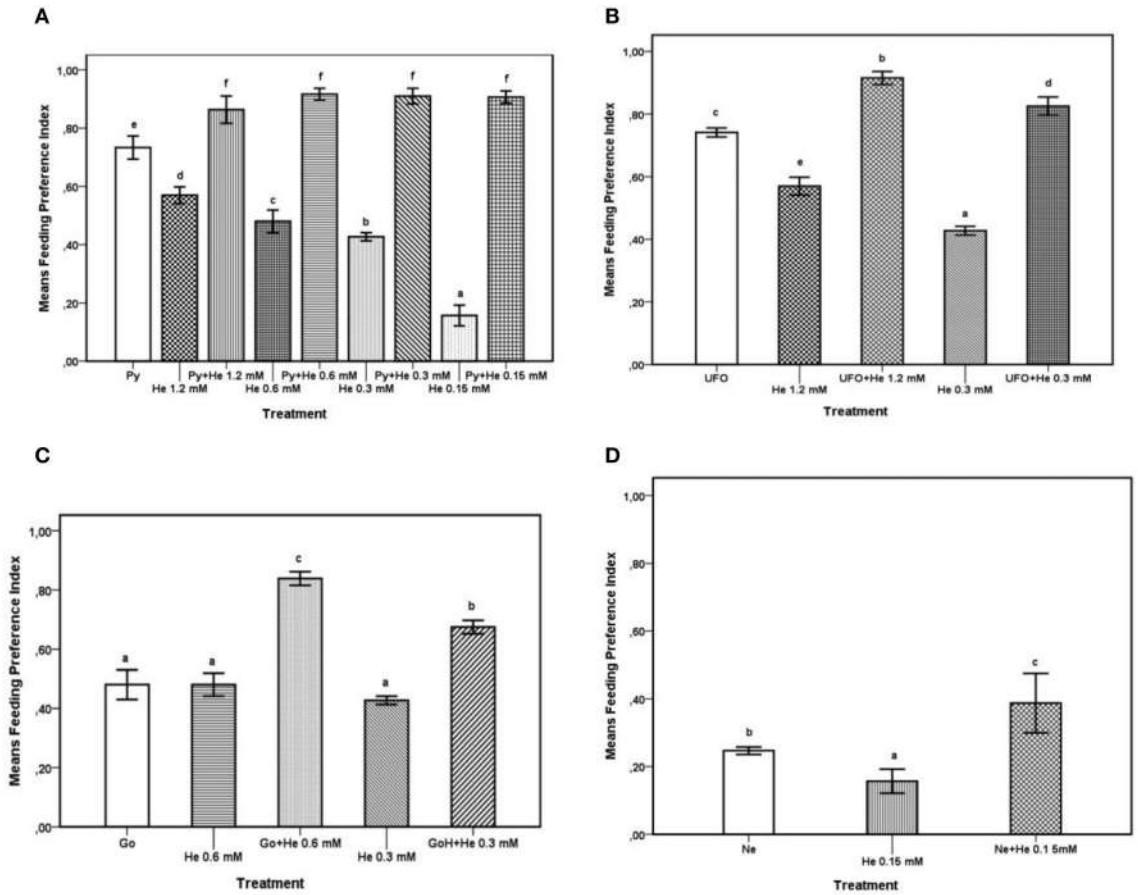

FIGURE 1 | Feeding preference test on winged morphs. For each treatment, the Feeding Preference Index (FPI) value is the mean of 8 h records. Possible values for the index range between 1 (complete phagodeterrent activity) and −1 (complete preference for treated leaves), with a value equal or close to zero indicating no effect. Bars with different letters are significantly different ($P < 0.05$) according to the SNK test. **(A)** FPI values of Pyganic® 1.4 at the dose 2.0 ml/l (Py,), 1-hexadecanol (He) 1.2, 0.6, 0.3, 015 mM, and blends of Py plus He at different concentrations (1.2, 0.6, 0.3, 015 mM). **(B)** FPI values of UFO® at the dose 1% (UFO®), 1-hexadecanol (He) 1.2 and 0.3 mM, and blends of UFO® with either 1.2 or 0.3 mM of He. **(C)** FPI values of Gondor®, applied at the dose of 2.5 ml/l (Go), 1-hexadecanol (He) 0.6 and 0.3 mM and blends of Go with 0.6 or 0.3 mM of 1-hexadecanol. **(D)** FPI values of NeemAzal®-T/S at the dose 2.5 ml/l (Ne), 1-hexadecanol (He) 0.15 mM, and a blend of Ne with 0.15 mM of hexadecanol.

1-hexadecanol. 1-Hexadecanol and 1-octadecanol are parts of *Heliothis virescens* male pheromone blend (Teal and Tumlinson, 1989) and 1-hexadecanol was discovered in *Helicoverpa armigera* sex pheromone blend along with many other components (Witzgall et al., 2010). Gas chromatography-flame ionization detection coupled with electroantennographic detection analyses have put in evidence that 1-hexadecanol in the extract of *H. armigera* female sex pheromone gland was electrophysiologically active to male antennae (Zhang et al., 2012). Hexadecanol and tetradecanol were also identified in species-specific marking pheromones, produced by males of different species of the genus *Bombus*, to attract conspecific females for mating (Urbanova et al., 2004). Keeling et al. (2003) showed that hexadecanol is a synergistic component of the honeybee queen pheromone blend to attract a retinue of workers. Jin et al. (2006) stated that *Locusta migratoria* odorant binding protein1 (LmigOBP1) displayed significant specificity to linear aliphatic alcohols and ketones of approximately 15-carbon chain length, and in particular LmigOBP1 interacted preferentially with 1-pentadecanol and 2-pentadecanone. Qiao et al. (2013) investigated the ligand-binding properties of two *Bombyx mori* chemosensory proteins (CSPs) and showed that CSP2 had a high affinity for most of aliphatic and aromatic compounds tested and the best ligands were those with 12–16 carbon atoms. Oviposition deterrence evoked by resistant cultivars of *Zea mays* toward *Chilo partellus* was attributed partly to larger quantities of 1-nonadecanol and 1-heptadecanol, compared to susceptible cultivars (Varshney et al., 2003).

Long chain aliphatic alcohols are employed for a wide variety of industrial and commercial uses, relying on their lubricating, emollient, solubilizing, or emulsifying properties. They can be found in some pharmaceutical products, agrochemical formulations, household cleaning, and personal care products (Modler et al., 2004; Veenstra et al., 2009). As far as the risk of LCOHs to human health is concerned, representative compounds from this category have been extensively tested to

TABLE 5 | Action potentials (spikes/s ± SD) recorded by single taste tarsal cells of winged ($n = 6$) and wingless ($n = 8$) morph of *Rhopalosiphum padi* on stimulation with long-chain alcohols produced by *T. citrinoviride* ITEM 4484.

Tested stimulus	Winged morphs	Wingless morphs
Resting activity	12.4 ± 2.2	18.3 ± 3.2
NaCl (100 mM)	12.6 ± 1.8	18.3 ± 3.2
NaCl (100 mM) + MeOH (5%)	12.5 ± 2.2	18.5 ± 3.4
1-Hexadecanol (1.2 mM)	28.6 ± 4.3*	31.4 ± 4.6*
1-Octadecanol (1.2 mM)	30.8 ± 4.2*	35.2 ± 4.2*
cis-9-Octadecen-1-ol (1.2 mM)	54.5 ± 6.4**	56.7 ± 7.4**
trans-9-Octadecen-1-ol (1.2 mM)	58.5 ± 8.2**	61.4 ± 9.3**

**On the same column, significant difference between resting activity and tested stimulus (t-test; *P < 0.05, **P < 0.01).*

assess their toxicity. Acute oral toxicity data in species other than rat are limited, but confirm the very low acute toxicity of these alcohols to humans (OECD, 2006). Inhalation of vapors of LCOHs in the range C6–C22 at levels up to the saturated vapor pressure is unlikely to be associated with significant toxicity; also, these chemicals are not regarded as allergy sensitizers (OECD, 2006). Given the widespread use in cosmetic industry and the relatively low numbers of reported cases of allergy, it can be concluded that LCOHs have a very low allergenic potency (Veenstra et al., 2009). The available data on eye irritation indicate that the LCOHs that may induce different levels of irritation are those with chain length comprised between C6–C11, while the eye irritation potential of LCOHs with a chain length of C12 and above is minimal (OECD, 2006). Studies on the effects of these chemicals on mammals other than humans do not show evidence of genetic toxicity or detrimental effects to the reproductive system or the developing organism (Veenstra et al., 2009).

In conclusion, the LCOHs isolated from cultures of *T. citrinoviride* ITEM 4484 and other LCOHs structurally related to them proved to function as signaling molecules that can modify the aphid feeding preferences. Based on these results, LCOHs might be useful for control of aphid pests and prevention of both direct damage caused by sap subtraction and indirect damage caused by transmission and spread of plant virus diseases. The LCOHs proved to be compatible with other agrochemicals used in organic farming, such as pyrethrum, azadirachtin A and mineral oil, and improve their efficacy. In our preliminary field trials (data not shown) the addition of 1-hexadecanol to Pyganic®1.4 showed promising results and encourages more extensive studies in the field.

The practical use of these compounds for crop protection is expected to have a low environmental impact and, due to their mode of action, not to be a threat to beneficial insects. The low toxicity and allergenicity of LCOHs let foresee a facilitated and faster evaluation process for registration purposes. An additional advantage represented by these metabolites is that they have no chiral centers and therefore can be obtained in good yields through chemical synthesis, besides than from natural sources. All together these features encourage further studies aiming at the implementation of novel agrotechnical products for aphid control and crop protection based on LCOHs. Part of the results herein presented are the subject of an international patent application (Sabatini et al., 2012) concerning "Phagodeterrent compounds of fungal origin" (PCT/IB2012/052383).

AUTHOR CONTRIBUTIONS

AC, isolation, identification, molecular characterization, and mass production of the producing strain. Experiments planning and design; SG, aphid behavioral bioassays and electrophysiological studies. Experiments planning and design; PG, Statistical analyses of feeding preference data; AC, electrophysiological studies. FF, Studies on the combined use of long shan alcohols and agrochemicals. MS, aphid behavioral studies and bioassays. AE, Chemical extraction of fungal cultures, fractionation and purification of active metabolites, characterization of long chain alcohols by spectroscopic, chemical and GC methods.

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Diversity and Activity of *Lysobacter* Species from Disease Suppressive Soils

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The genus *Lysobacter* includes several species that produce a range of extracellular enzymes and other metabolites with activity against bacteria, fungi, oomycetes, and nematodes. *Lysobacter* species were found to be more abundant in soil suppressive against the fungal root pathogen *Rhizoctonia solani*, but their actual role in disease suppression is still unclear. Here, the antifungal and plant growth-promoting activities of 18 *Lysobacter* strains, including 11 strains from *Rhizoctonia*-suppressive soils, were studied both *in vitro* and *in vivo*. Based on 16S rRNA sequencing, the *Lysobacter* strains from the *Rhizoctonia*-suppressive soil belonged to the four species *Lysobacter antibioticus*, *Lysobacter capsici, Lysobacter enzymogenes,* and *Lysobacter gummosus*. Most strains showed strong *in vitro* activity against *R. solani* and several other pathogens, including *Pythium ultimum*, *Aspergillus niger, Fusarium oxysporum,* and *Xanthomonas campestris*. When the *Lysobacter* strains were introduced into soil, however, no significant and consistent suppression of *R. solani* damping-off disease of sugar beet and cauliflower was observed. Subsequent bioassays further revealed that none of the *Lysobacter* strains was able to promote growth of sugar beet, cauliflower, onion, and *Arabidopsis thaliana*, either directly or via volatile compounds. The lack of *in vivo* activity is most likely attributed to poor colonization of the rhizosphere by the introduced *Lysobacter* strains. In conclusion, our results demonstrated that *Lysobacter* species have strong antagonistic activities against a range of pathogens, making them an important source for putative new enzymes and antimicrobial compounds. However, their potential role in *R. solani* disease suppressive soil could not be confirmed. In-depth omics'–based analyses will be needed to shed more light on the potential contribution of *Lysobacter* species to the collective activities of microbial consortia in disease suppressive soils.

Keywords: *Lysobacter*, *Rhizoctonia solani*, *Beta vulgaris*, disease suppression, plant growth promotion

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INTRODUCTION

Lysobacter are Gram-negative bacteria widely distributed in diverse ecosystems, including soil, rhizosphere, and freshwater habitats (Reichenbach, 2006). The genus *Lysobacter* was first described in 1978 by Christensen and Cook and included four species. *Lysobacter* spp. are closely related to members of the genus *Xanthomonas* and were initially misclassified as *Cytophaga*, *Sorangium,* or *Myxobacter* (Christensen and Cook, 1978). Currently, 30 *Lysobacter* species have been

taxonomically accepted (for updates see http://www.bacterio.net/lysobacter.html) and new *Lysobacter* species have been recently identified (Du et al., 2015; Lin et al., 2015; Singh et al., 2015) but are not yet included in the database. Various members of this bacterial genus have activity against a range of other (micro)organisms, including Gram-negative and Gram-positive bacteria, fungi, oomycetes, and nematodes (Reichenbach, 2006). They are well-known for the production of a variety of extracellular enzymes and antimicrobial compounds. Enzymes identified for *Lysobacter* include chitinases (Zhang and Yuen, 2000; Zhang et al., 2001), glucanases (Palumbo et al., 2005), proteases (Stepnaya et al., 2008; Gökçen et al., 2014; Vasilyeva et al., 2014), lipases (Folman et al., 2003; Ko et al., 2009) as well as elastases, keratinases, phosphatases, endonucleases, endoamylases, and esterases (Reichenbach, 2006). Antimicrobial compounds described for *Lysobacter* include lysobactin, tripopeptin, xanthobaccin, maltophilin, dihydromaltophilin, phenazine, lactivicin (Xie et al., 2012), HSAF (Li et al., 2008), and WAP-8294A2 (Zhang et al., 2011). Currently, WAP-8294A2 is in phase I/II clinical trials for controlling methicillin-resistant *Staphylococcus aureus* (anti-MRSA) (Zhang et al., 2011; Wang et al., 2013).

In terms of ecosystem services, Postma et al. (2010a) showed a correlation between the abundance of three *Lysobacter* species (*Lysobacter antibioticus*, *Lysobacter capsici,* and *Lysobacter gummosus*) in soil and the level of suppressiveness against *Rhizoctonia solani*, a devastating fungal pathogen of numerous economically important crops such as sugar beet, potato, and rice. Also in the study by Mendes et al. (2011), the Xanthomonadaceae family, to which *Lysobacter* belongs, was found more abundant in a soil suppressive against *R. solani* on sugar beet. Several studies have shown that application of *Lysobacter* spp. reduced diseases caused by different plant pathogens in several crops such as cucumber (Folman et al., 2004; Postma et al., 2009), bean (Yuen et al., 2001), rice (Ji et al., 2008), pepper (Ko et al., 2009), grapevine (Puopolo et al., 2014), sugar beet, spinach (Islam et al., 2005), and tomato (Puopolo et al., 2010). To date, however, few data are available on the frequency and diversity of *Lysobacter* species in natural habitats and little is known about the ecology and the determinative role of *Lysobacter* species in plant growth promotion and disease suppressive soils.

The work described here focused on elucidating the role of *Lysobacter* spp. in protecting plants against soil-borne diseases and in stimulating plant growth. To that end, we determined (i) the genetic and phenotypic diversity of 18 different *Lysobacter* strains obtained from soil and plant-associated environments, (ii) their activity against a range of pathogens, (iii) if these *Lysobacter* strains alone can suppress damping-off disease of sugar beet and cauliflower caused by *R. solani*, and (iv) if *Lysobacter* can promote plant growth via direct contact and/or via production of volatile compounds.

MATERIALS AND METHODS

Strains, Culture, and Storage Conditions

The *Lysobacter* strains used in this study (**Table 1**) were isolated from different Dutch soils suppressive to *R. solani*. Reference strains (**Table 1**) were obtained from the DSM strain collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). For the activity and plant growth promotion assays, *Lysobacter* strains were pre-cultured in tryptone soya broth (TSB, Oxoid) for 2–3 days at 25°C on a rotary shaker at 200 rpm and cells were washed 3 times with 0.9% NaCl unless mentioned otherwise. The fungal pathogens used in this study were mostly provided by the Institute of Sugar Beet Research (IRS). *Fusarium oxysporum* Forl1 was provided by the University of Turin, Italy (Clematis et al., 2009), *Verticillium dahliae* JR2 by B. Thomma [Wageningen University (WUR)], *Phytophthora infestans* by F. Govers (WUR), and *Aspergillus niger* was provided by L. de Graaf (WUR) (Table S1). The bacterial strains were kept in 40% (v/v) glycerol at −80°C; the fungi and oomycetes were kept in mineral oil at 10°C.

Soil Collection and Storage

The non-suppressive (conducive) soil to *R. solani* was collected from a pear orchard located in Zwaagdijk, The Netherlands (52°41′53.549″ N, 5°6′58.643″ E) in June 2012 at a depth of 10–40 cm. The soil, classified as clay soil with loam texture (29.9% of the particles are >50 μm, 26.4% of the particles are <2 μm), was air-dried, sieved (0.5 cm mesh) to remove plant/root material and stored at 8°C until use for the *in vivo* activity test of *Lysobacter* spp. against *R. solani* on cauliflower.

Genetic and Phenotypic Characterization of the *Lysobacter* Strains

BOX-PCR

To determine the genetic variation among *Lysobacter* strains, the repetitive elements in their genome were analyzed by BOX-PCR according to Rademaker et al. (2004). Amplification reactions were conducted in 25 μl volume composed of 1 μl BOX-A1R primer (10 μM), 1.25 μl dNTPs (25 mM each), 0.4 μl BSA (10 mg/ml), 2.5 μl 100% DMSO, 5 μl 5x Gitschier buffer, 0.4 μl Taq polymerase (5U/μl SuperTaq), and 14.45 μl miliQ water. DNA was added by a toothpick inoculation of bacterial cells in the reaction mix. The reaction volume was heated to 95°C for 2 min, followed by 30 cycles of 3 s at 94°C, 92°C for 30 s, 50°C for 1 min, and 65°C for 8 min. The PCR reaction was finished with an 8 min incubation at 65°C for and then kept at 8°C. Five microliter of the PCR product was loaded on an 1.5% (w/v) agarose gel and ran overnight at 40V.

Phylogenetic analyses

For each *Lysobacter* strain, the sequences of the 16S ribosomal RNA gene, the gene encoding a recombination/repair protein (*recN*) and the gene encoding the subunit C of the excinuclease ABC (*uvrC*) were amplified using primers described in **Table 2**. The markers *recN* and *uvrC* were chosen based on Zeigler (2003) who showed that these candidate genes will provide high fidelity for species prediction, and the 16S rRNA gene was included because of its broad use in taxonomic studies. Amplification reactions were conducted in 25 μl volume composed of 1 μl each of forward and reverse primer (10 μM), 1 μl dNTPs (5 mM each), 1.5 μl $MgCl_2$ (25 mM), 5 μl 5x GoTaq Flexibuffer, 0.125 μl GoTaq polymerase (5 U/μl), and 15.375 μl miliQ water. DNA was added by a toothpick inoculation of bacterial cells in the

TABLE 1 | Isolation details of the *Lysobacter* strains used in this study.

Code	Species	Strain	Soil type	Source	Crop	Origin	Location	Year	References
L02	*Lysobacter antibioticus*	3.2.10	Clay	Soil	Grass/clover	Suppressive soil	Pietersbierum, NL	2003	Postma et al., 2008
L08	*Lysobacter antibioticus*	76	Clay	Soil	Cauliflower	Suppressive soil	Zwaagdijk, NL	2003	Postma et al., 2010b
L23	*Lysobacter antibioticus*	4.1.2	Clay	Soil	Potato	Suppressive soil	Marknesse, NL	2006	Postma et al., 2008
L32	*Lysobacter antibioticus*	DSM2044	N.A.	Soil	N.A.	Type strain	Ottawa, CA	N.A.	Christensen and Cook, 1978
173	*Lysobacter antibioticus*	173	Clay	Soil	No crop	Suppressive soil	Zwaagdijk, NL	2011	(This study)
174	*Lysobacter antibioticus*	174	Clay	Soil	No crop	Suppressive soil	Zwaagdijk, NL	2011	(This study)
L12	*Lysobacter capsici*	6.2.3	Clay	Soil	Grass/clover	Suppressive soil	Hoensbroek, NL	2003	Postma et al., 2010a
L13	*Lysobacter capsici*	1.3.3	Clay	Soil	Grass/clover	Suppressive soil	Strijen, NL	2003	Postma et al., 2010a
L14	*Lysobacter capsici*	55	Clay	Soil	Cauliflower	Suppressive soil	Zwaagdijk, NL	2003	Postma et al., 2010a
L31	*Lysobacter capsici*	DSM19286	N.A.	Rhizosphere	Pepper	Type strain	South Korea	2003	Park et al., 2008
L19	*Lysobacter enzymogenes*	1.1.4	Sand	Soil	Grass	Suppressive soil	Bakel, NL	2004	Nijhuis et al., 2010
L28	*Lysobacter enzymogenes*	3.1T8	Rockwool	Root tip	Cucumber	Rockwool	Wageningen, NL	1997	Folman et al., 2003
L29	*Lysobacter enzymogenes*	C3	N.A.	Leaf	Turfgrass	Suppressive soil	Nebraska, USA	N.A.	Sullivan et al., 2003
L30	*Lysobacter enzymogenes*	DSM2043	N.A.	Soil	N.A.	Type strain	Ottawa, CA	N.A.	Christensen and Cook, 1978
L05	*Lysobacter gummosus*	2.4.7	Clay	Soil	Grass/clover	Suppressive soil	Ijzendijke, NL	2003	Postma et al., 2008
L15	*Lysobacter gummosus*	3.2.11	Clay	Soil	Grass/clover	Suppressive soil	Pietersbierum, NL	2003	Postma et al., 2008
L26	*Lysobacter gummosus*	10.1.1	Clay	Soil	pea	Suppressive soil	Ijzendijke, NL	2006	Postma et al., 2008
L33	*Lysobacter gummosus*	DSM6980	N.A.	Soil	N.A.	Type strain	Ottawa, CA	N.A.	Christensen and Cook, 1978

N.A. Not applicable/not available.
NL, The Netherlands; USA, United States of America; CA, Canada.

TABLE 2 | Primer sets used for phylogenetic analysis.

Gene target	Primer	Oligonucleotides sequence (5′ →3′)
16S rRNA	Forward	AGAGTTTGATCCTGGCTCAG
16S rRNA	Reverse	ACGGGCGGTGTGTACA
recN	Forward	CTCAAGCAATTCGCCGTC
recN	Reverse	CACCTGCACCGCGCTCTG
uvrC	Forward	CGGCAAGGCCTTCGTCAAGC
uvrC	Reverse	CGTGCAAGGCGGCGTAGAT

reaction mix. The reaction volume was heated to 95°C for 3 min, followed by 35 cycles of: 1 min at 95°C, 58°C for 1 min, 72°C for 1.4 min (for 16S rRNA), 1 min at 95°C, 57.2°C for 1 min, 72°C for 1.2 min (for *recN*), and 1 min at 95°C, 58°C for 1 min, 72°C for 2 min (for *uvrC*). The PCR reaction were finished with an 5 min incubation at 72°C for and then kept at 12°C. Five microliter of the PCR product were visualized on an 1.5% (w/v) agarose and PCR products were sequenced by Macrogen Inc. (Amsterdam, The Netherlands). Phylogenetic trees were constructed with the three markers independently or concatenated using ClustalW alignments (Thompson et al., 1994) and neighbor joining tree constructions using the Tamura 3 parameter model and discrete Gamma distribution in MEGA6 (Tamura et al., 2013).

The sequences obtained during this study are deposited in NCBI GenBank under accession numbers KT851449 to KT851466 for *uvrC*, KT851467 to KT851484 for 16S rRNA and KT851485 to KT851502 for *recN*.

Swarming Ability

Motility of the *Lysobacter* strains was assessed on soft standard succinate medium (SSM) as described in De Bruijn and Raaijmakers (2009). In brief, 5 µl of *Lysobacter* suspensions was spot-inoculated in the center of SSM agar Petri dishes [(32.8 mM K_2HPO_4, 22 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 34 mM succinic acid (w/v)), adjusted pH to 7 and 0.6% agar (w/v)]. Petri dishes were incubated for 2–12 days at 25°C.

Enzymatic Activity

Chitinase, glucanase, and protease activity of the *Lysobacter* strains were tested as described in De Bruijn et al. (in press). In brief, 2–5 µl of *Lysobacter* suspensions (of stationary phase of growth) was spot-inoculated in the center of different media containing 1.5–2% agar. For chitinase activity, R2A (Oxoid) and 1/10th strength TSB agar Petri dishes were used containing 0.2% colloidal chitin prepared from crab shell chitin (Sigma) and Petri dishes were incubated for 3–7 days at 25°C. For glucanase activity, R2A medium containing 0.5% laminarin was used and Petri dishes were incubated for 3 days at 25°C. The colonies were removed by washing with water and the medium was stained with 1% congo red. After destaining, coloration of the medium was determined. For protease activity, bacteria were inoculated on 15 g/l skimmed milk powder, 4 g/l blood agar base and 0.5 g/l yeast extract and Petri dishes were incubated for 3–7 days at 25°C.

In *Vitro* Antagonistic Activity

Lysobacter strains (**Table 1**) were grown in 5 ml TSB for 2 days at 25°C on a rotary shaker at 200 rpm. Suspensions were

washed once by centrifugation at 3800 × g for 5 min and 10x concentrated in 0.9% NaCl.

To test activity against bacterial pathogens, R2A, 1/5th potato dextrose agar (PDA, Oxoid) and Luria-Bertani (LB, Difco) agar Petri dishes were prepared with an overlay of 1% water agar cooled down to 50°C to which washed cells of a culture of the bacterial pathogens (Table S1) were added. Subsequently, 2–5 µl of the *Lysobacter* cell suspensions (of stationary phase of growth) was spot-inoculated on the medium. Petri dishes were incubated for 3–7 days at 25°C and clearing zones surrounding the colonies were monitored.

To test inhibition of mycelial growth, oomycetes, and fungal strains (Table S1) were grown on PDA at 25°C. Four 5 µl of the *Lysobacter* suspensions were spot-inoculated at the edges of Petri dishes containing 20 ml of R2A, 1/5th PDA or PDA and a fresh 5 mm agar plug with actively grown mycelium was placed in the middle of the Petri dish.

To test antagonism against fungal spores, fungi (Table S1) were grown on PDA until sporulation. To enhance spore production, *Cercospora* and *Stemphylium* were grown on vegetable juice agar Petri dishes [(vegetable juice (V8) solified with 1.5% agar)] (Beckman and Payne, 1983; Rossi et al., 2005).

Under 16 h photoperiod, and to enhance spore collection from *Verticillium* and *Aspergillus,* the spores of those two fungi were scratched from the mycelium and streaked on fresh PDA Petri dishes. Fungal spores were collected as described in Trifonova et al. (2008) with slight modifications. In brief, spores were released from the mycelium by adding 10 ml of 0.9% NaCl and scratching the surface with a sterile spatula, collected, 10-fold diluted and added to the culture media (PDA, 1/5th PDA and R2A) of 48–55°C to a final concentration of 5% (v/v). Four 5 µl of the *Lysobacter* suspensions were spot-inoculated at the edges of Petri dishes containing 20 ml of medium with spores.

For each assay, three replicates per media were used. Petri dishes without *Lysobacter* were used as controls. All Petri dishes were incubated at 25°C for 1 week and subsequent inhibitory halo formation was monitored.

In Vivo Activity of *Lysobacter* spp. Against *Rhizoctonia solani*

Spontaneous rifampicin-resistant mutants of the *Lysobacter* strains were verified by BOX-PCR. These mutants exhibited chitinase activity to the same extent as their parental strains. The rifampicin-resistant mutants were grown in 10 ml of TSB supplemented with 50 µg/ml rifampicin for 2 days at 25°C on a rotary shaker at 200 rpm. Cultures were centrifuged, washed 3 times and resuspended in 0.9% NaCl. Cell suspensions were mixed in a potting soil:river sand (1:9, w/w) mixture at an initial density of 10^7 cells/g soil and approximately 20% hydration. Rectangle shape trays (19.5 × 6 × 3.5 cm) were filled with 250 g of the potting soil:sand mixture (eight replicates per treatment) and 16 sugar beet seeds coated with thiram, hymexazol, and poncho-beta were sown in a row, 1 cm apart. Non-inoculated soil was used as a control. Trays were placed in boxes with transparent lids in a growth chamber at 24°C with a 16 h photoperiod. After 5 days, seeds germinated and a single fresh 1/5th PDA agar plug (5 mm) grown with *R. solani* AG2-2 IIIB was placed touching the first seedling, with the mycelial side toward the plant. Spread of *R. solani* was scored at regular intervals during 2 weeks by scoring the number of diseased plants as well as the distance between the inoculum and the most distal plant suffering from damping-off. In addition, the area under the disease progress curve (AUDPC) was calculated to determine the disease dispersal over time as:

$$\left(Ak = \sum_{i=1}^{Ni-1} \frac{(y_i + y_{i+1})}{2} (t_{i+1} - t_i) \right)$$

where t_i are the time points in a sequence (days) and y_i are measures of the disease dispersal (cm). Therefore, $y(0)$ is defined as the initial infection at $t = 0$ and $A(\text{tx})$ is the AUDPC (total accumulated diseased dispersal until $t = t_x$).

From each tray, the rhizospheres of two healthy sugar beet plants that were the closest to the last infected one were collected. Two replicates were pooled together in 4 ml 0.9% NaCl, vortexed for 1 min, sonicated for 1 min and vortexed for 15 s. Fifty microliter of a 10-, 100-, and 1000-fold dilution were plated on selective medium, R2A supplemented with 50 µg/ml rifampicin, 200 µg/ml ampicillin, 25 µg/ml kanamycin, and 100 µg/ml delvocid. Petri dishes were incubated at 25°C for 1 week. Colony forming units (CFU) were counted and CFU/g rhizosphere was calculated. The *in vivo* assay and the rhizosphere colonization test were done twice.

A similar experiment was performed in cauliflower using the same set up as described above with slight differences. Bacterial strains were grown in 10 ml of LB broth supplemented with 50 µg/ml rifampicin at 25°C for 3 days. The selected *Lysobacter* strains for this assay were L08, L14, L15, L19 and L29. Bacterial strains were inoculated in Zwaagdijk conducive soil at an initial density of 10^5 and 10^7 cells/g soil. Sowing, *R. solani* AG2-1/21 inoculation, growth of the plants, disease scoring, and AUDPC calculation was done as described above. The experiment was repeated twice, once with rifampicin resistant *Lysobacter* and once with non-rifampicin resistant *Lysobacter*. Statistically significant differences were determined by One-way ANOVA and *post-hoc* Dunnet's analysis ($P < 0.05$) performed in SPSS 22.0.

In Vitro Plant Growth Promotion Assay

Seed Preparation

Prior to surface sterilization, naked sugar beet (*Beta vulgaris*) seeds were soaked in 0.03 N HCl for 6 h under rotation, washed with sterile milliQ water and air-dried to enhance seed germination (Habib, 2010). Surface sterilization of sugar beet, cabbage (*Brassica oleracea*), and onion (*Allium cepa*) seeds was performed by washing the seeds in 2% sodium hypochlorite for 5 min and rinsing them with sterile milliQ water. Seeds were placed on Whatman filter paper moistened with 3 ml sterile milliQ water and pre-germinated at 25°C for 2–3 days. *Arabidopsis thaliana* (Columbia 0) seeds were sterilized in an exicator with 50 ml of commercial bleach (10% v/v) + 3% of concentrated HCl for 4 h, placed in wet Whatman filter paper and incubated at 4°C in darkness for 3 days.

Seed Inoculation

Two day-old pre-germinated sugar beet seeds were soaked in 3 ml of *Lysobacter* suspensions of 10^9 cells/ml for 30 min. Subsequently, sugar beet seeds (six seeds per container) were placed in cylinder shaped plastic containers (9 cm diameter, 8 cm height) with transparent lids containing 150 ml of 0.5 × Murashige and Skoog (MS) medium (including vitamins), and incubated in a growth chamber at 24°C with a 16 h photoperiod. Fresh and dry weight of shoots and roots were determined after 2 weeks. The experiment was done twice, with three replicates per treatment.

Root Tip Inoculation

Two days-old pre-germinated sugar beet seeds were placed in square Petri dishes (10 × 10 × 2 cm) containing 50 ml of 0.5 × MS medium (four seeds/Petri dish). Petri dishes were incubated in vertical position in a growth chamber at 24°C with a 16 h photoperiod until the roots were approximately 1 cm long and 2 μl of the *Lysobacter* suspensions of 10^9 cells/ml were, spotted onto each root tip and incubated for 1 week. Fresh and dry weight of shoots and roots was determined. The experiment was done once, with three replicates per treatment.

Volatile Assay

Two days-old pre-germinated seeds of sugar beet, cauliflower and onion were placed in containers as described above containing either 150 ml of 0.5 × MS medium or 150 g of a sterile mixture of potting soil:sand (1:9) with 20% humidity. A small Petri dish (35 mm diameter), containing 4 ml of R2A medium was placed in the middle of the container, and the *Lysobacter* strains were inoculated into the small Petri dishes at a density of 10^7 cells/Petri dish. Containers were incubated in a growth chamber at 24°C with a 16 h photoperiod for 2 weeks and fresh and dry weight of shoots and roots as well as leaf area were determined. The experiment was performed three times for sugar beet, once for cauliflower and once for onion, with five replicates per treatment. For the volatile assay in *A. thaliana*, *L. antibioticus* L08, *L. capsici* L14, *L. gummosus* L15, and *Pseudomonas fluorescens* SBW25 [known by its ability in promoting plant growth in *A. thaliana* when growing on King's B (KB) agar medium and used as a positive control (J. M. Raaijmakers, personal communication)] were used. Each bacterial strain was pre-cultured in LB broth for 2 days at 25°C, and then washed three times with 10 mM $MgSO_4$. A 10 μl drop of a bacterial suspension of 10^9 cells/ml was spotted in the small Petri dish (35 mm diameter) containing 4 ml of R2A, LB or KB agar medium and Petri dishes were incubated for 1 day at 25°C. Small Petri dishes were placed into big Petri dishes (150 mm diameter) containing 50 ml of 0.5 × MS medium and five 3-days-old pre-germinated seeds were sown per Petri dish. Petri dishes with medium but without bacteria were included as controls. Petri dishes were incubated in vertical position in a growth chamber at 21°C with a 16 h photoperiod for 21 days. After that period, fresh and dry weight of shoots and roots were determined. The experiment was repeated once with five replicates/treatment.

Seed Colonization Ability

Naked sugar beet seeds were surface sterilized as described above and soaked in 3 ml of bacterial suspensions containing 10^9 cells/ml for 30 min as described above for the seed inoculation assay (22 seeds/bacterial treatment). Six seeds from each bacterial suspension were placed in 4 ml 0.9% NaCl, vortexed 1 min, sonicated 1 min, and vortexed 15 s. Fifty microliter of both undiluted suspensions and 10, 100, 1000, and 10000x time dilutions were plated on R2A agar dishes and incubated at 25°C for 1 week. The remaining seeds were sown in squared Petri dishes containing 50 ml of 0.5 MS (four seeds/Petri dish, four replicates per treatment) and incubated as described above for the root tip inoculation assay. After 1 week, the roots of the seedlings from each Petri dish were excised and placed in 4 ml of 0.9% NaCl, vortexed 1 min, sonicated 1 min, and vortexed 15 s. Fifty microliter of both undiluted suspensions and 10, 100, 1000, and 10000x fold dilution were plated on R2A agar dishes, incubated at 25°C for 1 week and the amount of colony forming units (CFU) per seed and per root were determined by colony counting.

RESULTS

Genetic and Phenotypic Characterization of the *Lysobacter* Strains

BOX-PCR profiling of the 18 *Lysobacter* strains revealed a high genetic diversity among the different *Lysobacter* species and between strains of a given species (**Figure 1A**). *L. gummosus* strains showed the lowest intraspecific diversity whereas *L. enzymogenes* strains showed the highest diversity. Based on 16S rRNA sequences, the most phylogenetically distant species was *L. enzymogenes* (Figure S1A). When using either *recN* or *uvrC* or the three molecular markers together, however, *L. antibioticus* was the most distant of the four species (**Figure 1B** and Figures S1B,C).

The *Lysobacter* strains did not show any motility after 4 days of incubation on soft SSM agar medium. After 12 days of incubation, however, *L. capsici* (L12, L13, L14, and L31) and *L. enzymogenes* (L19, L28, L29, L30) did spread from the point of inoculation, most likely due to gliding motility (**Figure 2**). All *Lysobacter* strains used in this study showed extracellular chitinase and glucanase activities (**Figure 2**). Most strains presented proteolytic activity except for two *L. gummosus* and four *L. antibioticus* strains (**Figure 2**). Variation in these three enzymatic activities among strains belonging to the same species was observed, especially for the *L. antibioticus* strains.

The antimicrobial activity of the *Lysobacter* strains (**Table 1**) was tested on different media. Almost all *Lysobacter* strains showed a strong antagonistic activity against all pathogens tested (Table S1), except against the plant pathogenic bacterium *Pectobacterium atrosepticum*. The magnitude of the antagonistic activity of *Lysobacter* was media-dependent, with the strongest activity on R2A medium and the weakest activity on PDA medium (**Figure 2**). *L. capsici* was the most consistent species in terms of antagonistic activity, with all *L. capsici* strains showing activity on R2A against all pathogens tested except

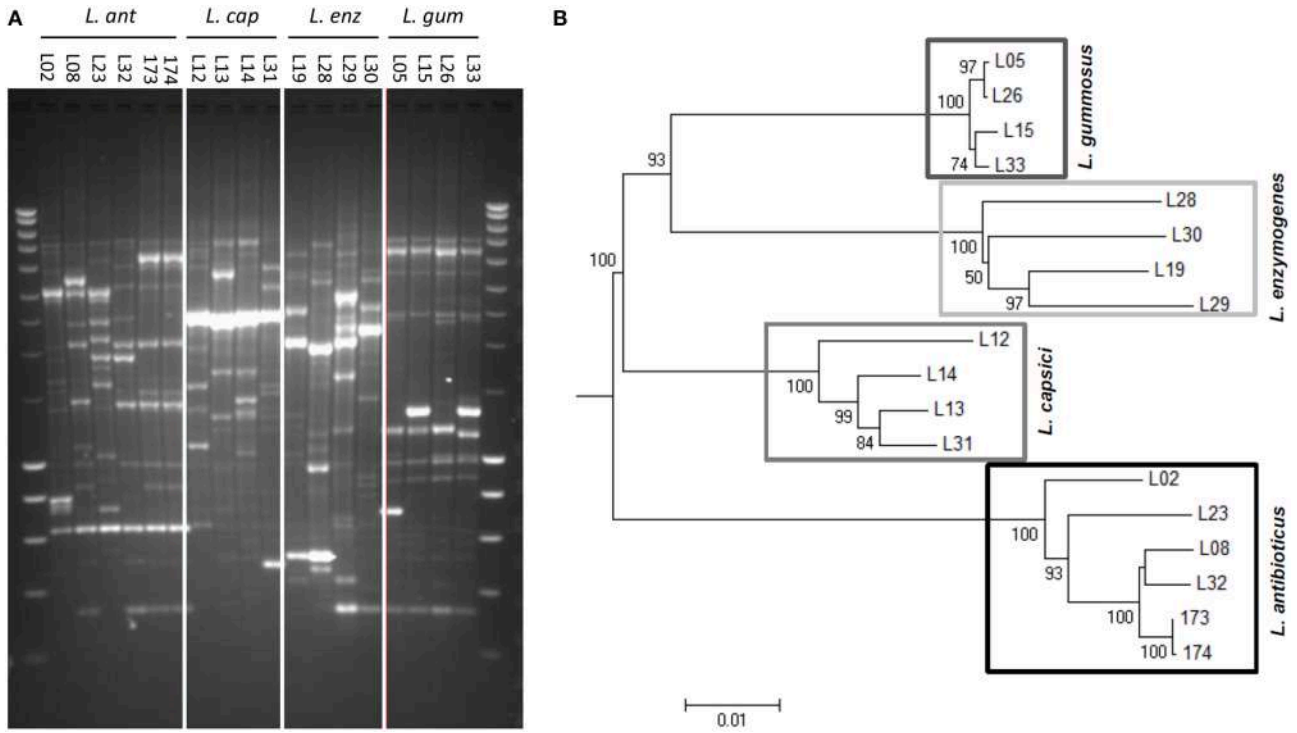

FIGURE 1 | Genetic diversity of 18 selected *Lysobacter* strains belonging to four different species. (A) Genetic profiling by BOX-PCR. Lanes on complete left and right shows Smartladder (Eurogentec) marker. **(B)** Phylogenetic tree of the *Lysobacter* strains based on the concatenated sequences of the 16S ribosomal RNA gene (16S rRNA), a gene encoding a recombination/repair protein (*recN*) and a gene encoding the subunit C of the excinuclease ABC (*uvrC*). The evolutionary relationship of the *Lysobacter* strains was inferred by alignment with ClustalW and neighbor-joining tree construction. The numbers at the nodes indicate the level of bootstrap support of 50 or higher, based on neighbor-joining analysis of 1000 resampled data sets. The bar indicates the relative number of substitutions per site.

for *X. campestris* and *L. capsici* strain L31 against *S. parasitica* (**Figure 2**). On R2A, all *L. enzymogenes* and *L. gummosus* strains, with the exception of the type strains, showed activity against all pathogens tested. The type strain of *L. enzymogenes* did show activity against *V. dahliae* JR2, *A. cochlioides* and *P. infestans,* whereas the *L. gummosus* type strain had activity against all oomycetes tested except *P. ultimum* (**Figure 2**). *L. antibioticus* strains showed the highest variation in activity, with strain L23 having the broadest antimicrobial activity (**Figure 2**).

In Vivo Activity of *Lysobacter* spp. against *Rhizoctonia solani*

The efficacy of the *Lysobacter* strains, several of which originate from *Rhizoctonia* suppressive soil, to control *Rhizoctonia* damping-off disease of sugar beet seedlings was tested in a sterilized (by autoclaving twice) sand-potting soil mixture and in a non-sterilized agricultural soil. Seed germination was not affected by the *Lysobacter* strains. In two bioassays, none of the strains was able to consistently suppress damping-off disease caused by *R. solani* after 2 weeks of plant growth (**Figure 3A**). For example, strains L19 and L05 significantly reduced damping-off disease of sugar beet in bioassay 2 but not in bioassay 1 (**Figure 3A**).

The results further showed that after an initial application of 10^7 CFU/g soil, *Lysobacter* strains established densities in the rhizosphere of sugar beet ranging from 10^3 to 10^8 CFU/g (**Figure 3B**), with substantial variation between strains and between the two bioassays. In general, *L. gummosus* strains were better rhizosphere colonizers whereas *L. antibioticus* showed the highest variation among strains. *L. antibioticus* strains L08 and 174 were only detected in the sugar beet rhizosphere in bioassay 1. *L. antibioticus* L23 was detected at high densities (10^8 CFU/g) in bioassay 1, but at 1000-fold lower densities in bioassay 2. *L. enzymogenes* L19 was only detected in bioassay 2 (**Figure 3B**).

The ability of *Lysobacter* to suppress *Rhizoctonia* damping-off disease of another host plant (cauliflower) was assessed for *Lysobacter* strains L08, L14, L15, L19, and L29 at two initial densities of 10^5 and 10^7 CFU/g of soil. Also for this crop, germination was not affected by the introduced bacterial strains and again no significant and consistent reduction in disease incidence was observed. When applied at 10^5 CFU/g of soil, strain L19 significantly reduced disease incidence but only in bioassay 2 (**Figure 3C**). For bioassay 2, colonization of cauliflower rhizosphere by the *Lysobacter* strains was determined. The results showed that the densities recovered were lower (10^1 to 10^3) than initially applied except for *L. enzymogenes* L29 and *L. gummosus* L15 when applied at 10^7 CFU/g soil (Figure S2). After an initial application of 10^5 cells/g soil, only *L. gummosus* L15 and *L enzymogenes* L19 and L29 were detected in the rhizosphere of cauliflower.

A

Phenotypic characteristics		*L. antibioticus*						*L. capsici*				*L. enzymogenes*				*L. gummosus*			
		L02	L08	L23	L32	173	174	L12	L13	L14	L31	L19	L28	L29	L30	L05	L15	L26	L33
Motility		-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
Enzymatic activity																			
Chitinase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucanase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Protease		+/-	+/-	+	+	+/-	+/-	+	+	+	+	+	+	+	+	+	-	+	-
Antagonistic activity	***Medium***																		
Fungi																			
R. sol	R2A	+/-	+/-	+/-	+	+/-	-	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-/+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	-
V. dah_J	R2A	+	+	+	+	-	-/+	+	+	+	+	+	+	+	+	+	+	+	-
	PDA	+	+	+	+	NT	NT	+	+	+	+	-	NT	+	+	+	+	+	+
	1/5 PDA	+	+	+	+	-	-/+	+	+	+	+	+	+	+	+	+	+	+	+
F. sol	R2A	-	-	-/+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	NT	NT	-	NT	-	NT	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
V. dah	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-
F. oxys	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
Forl1	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-	+	-	-	+	+	+	+	+	-	-	+	-	+	+	+	-
A. nig	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-	+/-	+/-	-
	1/5 PDA	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
C. bet	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	+	-/+	-/+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
Stem	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-/+	-/+	+	-/+	+	-/+	+	+	+	+	+	+	+	-	+	+	+	-
Oomycetes																			
A. coc	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	PDA	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-	+	+	+	+
	1/5 PDA	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
P. ult	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	-	-/+	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-/+	+	-	-	+/-	+	+	+	+	+	+	+	-	+	+	+	+
P. inf	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	PDA	+	+	+	+	NT	NT	+	+	+	+	+	+	+	+	+	+	+	-/+
	1/5 PDA	+	+	+	-	NT	NT	+	+	+	+	+	+	+	-	+	+	+	-
S. par	R2A	+	+	+	-	-	+	+	+	+	+/-	+	+	+	-	+	+	+	-/+
	PDA	+/-	+/-	+/-	-	NT	NT	+	+	+	+	-/+	-/+	-/+	-	+	+	+	+
	1/5 PDA	++	+	+	-/+	NT	NT	+	+	+	+	+	+	+	-	+	+	+	-
Bacteria																			
X. cam	R2A	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
	1/5 PDA	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+	-
P. atr	R2A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/5 PDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

B

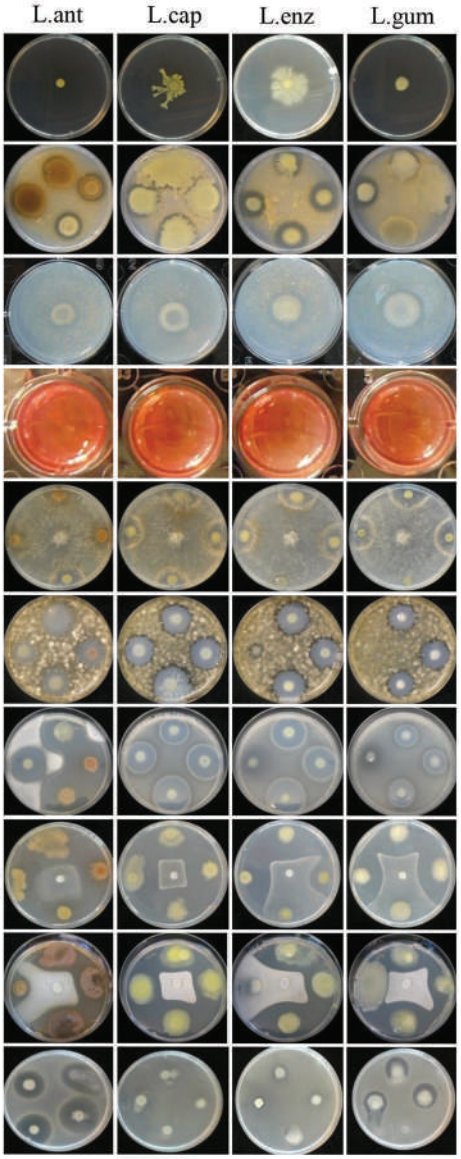

FIGURE 2 | Phenotypic characterization of the *Lysobacter* strains, including (A) motility, protease, chitinase and glucanase activities, and antagonistic activity against pathogenic fungi, oomycetes and bacteria. + indicates activity; − indicates no activity; ± indicates antagonistic activity observed after 2–3 days of incubation, but the activity disappeared upon longer incubation. For the enzymatic activity, the ± indicates weak activity; NT indicates not tested. **(B)** Pictures of phenotypic characterization of *L. antibioticus* (L. ant), *L. capsici* (L. cap), *L. enzymogenes* (L. enz), and *L. gummosus* (L. gum) for I: motility on SSM medium; II: chitinase activity; III: glucanase activity, positive glucanase activity is given by the change from red to orange color (not shown); IV: protease activity; and *in vitro* antagonistic activity on R2A (except when otherwise indicated) against V: *R. solani*; VI: *Cercospora beticola*; VII: *Verticillium dahliae*; VIII: *Pythium ultimum*; IX: *Aphanomyces cochlioides* on PDA and X: *Xanthomonas campestris* pv. *campestris* on 1/5th PDA.

Plant Growth Promotion

The ability of the *Lysobacter* strains to promote plant growth *in vitro* was tested for sugar beet, cauliflower, onion, and *A. thaliana*. For sugar beet, the 18 *Lysobacter* strains were applied to the seeds as well as to the root tips. For the first seed inoculation assay, almost all *L. antibioticus* strains negatively affected plant growth, decreasing plant biomass with 15–38% compared to the untreated control (**Figure 4**). One *L. capsici* and two *L. enzymogenes* strains negatively affected shoot biomass. In the second bioassay, no negative or positive effects on plant growth were observed for any of the strains (**Figure 4**), except for *L. gummosus* L26 which promoted root growth.

The ability of *Lysobacter* to colonize the surface of the seeds and the roots was determined for bioassay 2. Whilst bacteria were applied at an initial density of 10^8 cells/seed, bacterial recovery from the seed after 30 min of incubation ranged from approximately 10^3–10^4 cells/seed, with even lower numbers for *L. antibioticus* L32 (10^2 cells/seed; Table S2). After 1 week of plant

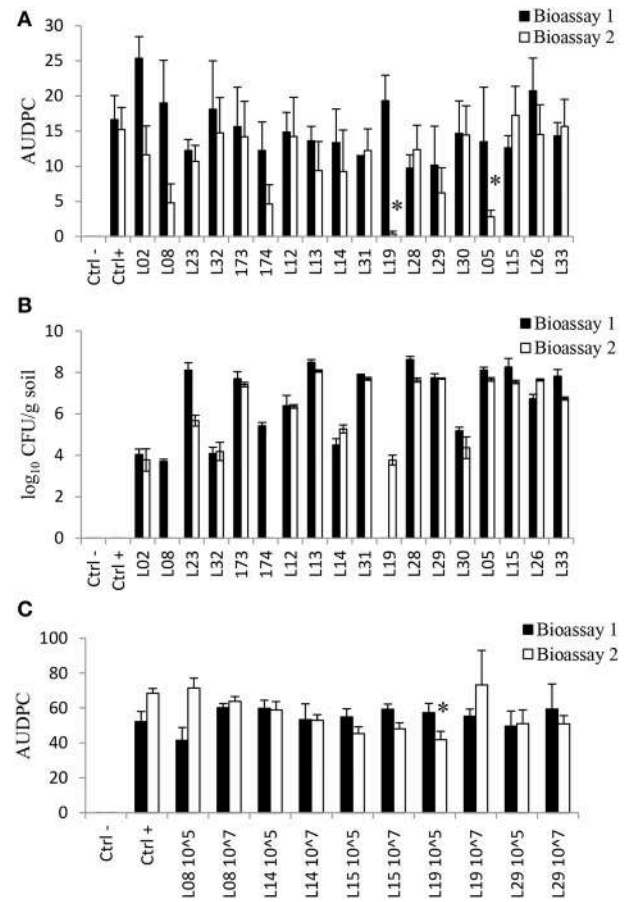

FIGURE 3 | *In vivo Rhizoctonia* disease suppression and rhizosphere colonization ability by *Lysobacter* strains. (A) Area under disease progress curve (AUDPC) of disease spread for sugar beet when *Lysobacter* strains were applied at an initial density of 10^7 CFU/g into a mixture potting soil:sand (1:9); **(B)** Colonization of the rhizosphere of sugar beet by the *Lysobacter* strains when applied at an initial density of 10^7 CFU/g into a mixture potting soil:sand (1:9). **(C)** AUDPC of disease spread for cauliflower when *Lysobacter* strains were applied into a conducive soil. 10^7 and 10^5 means an initial density of the inoculum at 10^7 and 10^5 cells/g soil, respectively; *L. antibioticus*: L02, L08, L23, L32, 173, 174; *L. capsici*: L12, L13, L14, L31; *L. enzymogenes*: L19, L28, L29, L30, and *L. gummosus*: L05, L15, L26, L33. For each of the two bioassays, an asterisk indicates a significant difference ($p < 0.05$) with the control treatment calculated by analysis of variance and Dunnett's *post-hoc* analysis.

growth, bacteria could not be detected on sugar beet roots (Table S2). Hence, *Lysobacter* appears to be a poor root colonizer under these experimental conditions.

In the root tip inoculation assay, positive effects (ranging from 17 to 28% biomass increase) were observed for dry weight of shoots by two *L. antibioticus*, two *L. capsici,* and one *L. enzymogenes* strains (Figure S3). One *L. antibioticus* and one *L. gummosus* strain increased fresh (33%) and dry (38%) root biomass respectively (Figure S3).

To determine if *Lysobacter* emits volatile compounds that promote plant growth, assays were conducted in a split Petri dish where *Lysobacter* was physically separated from sugar beet seedlings. A high variation in plant phenotypes was observed between assays. For example, *L. antibioticus* L32 increased shoot biomass with 24% and root biomass with 42% only in the first assay. *L. enzymogenes* L30 increased root biomass in the first assay whereas in the third assay it showed a negative effect on plant growth (**Figure 4**). The volatile assays were repeated in sterile potting soil:sand mixture with sugar beet, cauliflower, and onion. Also in these assays, no significant and consistent results were obtained for the *Lysobacter* strains tested (data not shown). In addition, plant growth promotion was also determined by measurement of the leaf surface and no positive or negative effects of the *Lysobacter* strains were observed (data not shown).

L. antibioticus strain L08, *L. capsici* L14, *L. gummosus* L15 were also tested for volatile-mediated growth promotion of *A. thaliana* on different media. The positive control *P. fluorescens* SBW25 significantly increased shoot and root biomass (Figure S4). However, none of the *Lysobacter* strains tested showed a plant growth promoting effect on *A. thaliana*. Furthermore, when growing on LB medium, all the three *Lysobacter* as well as *P. fluorescens* SBW25 showed a notable adverse effect on plant growth (Figure S4).

DISCUSSION

The genus *Lysobacter* is receiving substantial ecological and biotechnological interest as producers of different exoenzymes and antibiotics (Pidot et al., 2014). During the last years, several *Lysobacter* species have been isolated from Dutch soils suppressive to the fungal root pathogen *R. solani* (Postma et al., 2008, 2010b). Here, we showed that 18 *Lysobacter* strains from *Rhizoctonia* suppressive soils showed a high genetic diversity. In a recent study, comparative genomics of seven *Lysobacter* strains (five of which are included in this study) belonging to four *Lysobacter* species showed only 55% overlap in genome content (De Bruijn et al., in press). A high genetic diversity can confer an advantage under adverse environmental conditions as some members may exhibit phenotypes that allow them to survive and proliferate (Foster, 2005). Genome analysis also revealed the lack of flagellar genes (De Bruijn et al., in press), which supports our findings that none of the *Lysobacter* strains tested were motile on soft agar. Nonetheless, some dispersal was observed for *L. capsici* and *L. enzymogenes* after 12 days of incubation, most likely due to gliding motility as described previously for other *Lysobacter* species (Sullivan et al., 2003; Hayward et al., 2010).

Lysobacter is known to produce a variety of bioactive compounds, including enzymes and antimicrobial compounds. Hence, they were pointed out as an untapped source of new bioactive products (Xie et al., 2012; Pidot et al., 2014). Our results showed that the *Lysobacter* strains possess chitinase and glucanase activity, confirming and extending previous research (Zhang and Yuen, 2000; Zhang et al., 2001; Palumbo et al., 2005; De Bruijn et al., in press). Protease activity was observed for all strains belonging to *L. capsici* and *L. enzymogenes*, whereas only two out of four strains from *L. gummosus* and two out of six from *L. antibioticus* showed this activity. Chitinase, glucanase and protease activities may contribute to antimicrobial activity, since chitin, α- and β-glucans and glycoproteins are the major

A

		SUGAR BEET																			
		SEED INOC								VOLATILE											
		Assay 1				Assay 2				Assay 1				Assay 2				Assay 3			
		Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root	
Species	Strain	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D
L. antibioticus	L02		15																	34	
L. antibioticus	L08			31							28										
L. antibioticus	L23			31							23									34	
L. antibioticus	L32									24		42	27								
L. antibioticus	173			38	24						45									15	
L. antibioticus	L74	23	20								38									21	
L. capsici	L12																				
L. capsici	L13										23		22							29	
L. capsici	L14																				
L. capsici	L31		11								29										
L. enzymogenes	L19										22										
L. enzymogenes	L28																			30	
L. enzymogenes	L29	21	16																		
L. enzymogenes	L30		16									46	41							24	
L. gummosus	L05																				
L. gummosus	L15																			16	
L. gummosus	L26							30					10								
L. gummosus	L33																				

B

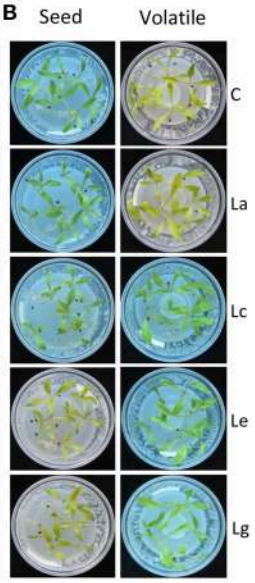

FIGURE 4 | Sugar beet plant growth promotion by *Lysobacter* strains. (A) Sugar beet seeds were grown on 0.5 MS medium and plant growth promotion was determined when *Lysobacter* strains were inoculated on seeds or by volatiles. Each assay was performed with three to five replicates. F indicates fresh weight; D indicates dry weight. Light gray boxes indicate a statistical significant negative effect in plant growth when compared to the control and dark gray boxes indicate a statistical significant positive effect. Values within the boxes, indicates the % of increase/decrease of plant weight compared to the control. **(B)** Pictures of the plant growth promotion assays. C, control; La: *L. antibioticus*; Lc: *L. capsici*; Le: *L. enzymogenes*; Lg: *L. gummosus*. Significant differences ($p < 0.05$) with the uninoculated control were calculated using analysis of variance and Dunnet's *post-hoc* analysis.

components of the cell walls of fungi (Barreto-Bergter and Figueiredo, 2014).

Most of the *Lysobacter* strains effectively inhibited the growth of oomycetes and fungi; only *L. antibioticus* and *L. gummosus* strains showed antibacterial activity. Differences in activity were observed between *Lysobacter* species and between strains of a given species, suggesting that the genus *Lysobacter* indeed may have a large reservoir of putative novel bioactive compounds. The *in vitro* antagonistic activity was media-dependent, showing stronger activity on poor medium, confirming and extending results obtained previously for the activity of *L. enzymogenes* 3.1T8 against *Pythium aphanidermatum* (Folman et al., 2004).

Due to their broad spectrum activity, *Lysobacter* members have been proposed as promising candidates for biological control of plant diseases (Hayward et al., 2010). However, none of the *Lysobacter* strains used in this study were able to consistently reduce *R. solani* infection on sugar beet and cauliflower. These results differ from those in previous studies where several *Lysobacter* strains significantly controlled plant pathogens, including *P. aphanidermatum* on cucumber (Folman et al., 2004; Postma et al., 2009), *Bipolaris sorokiniana* on tall fescue (Kilic-Ekici and Yuen, 2003), *Uromyces appendiculatus* on bean (Yuen et al., 2001), *Xanthomonas oryzae* pv. *oryzae* on rice (Ji et al., 2008), *Phytophthora capsici* on pepper (Ko et al., 2009), *Plasmopara viticola* on grapevine (Puopolo et al., 2014), *Aphanomyces cochlioides* in sugar beet and spinach (Islam et al., 2005) and *F. oxysporum* f. sp. *radicis-lycopersici* on tomato (Puopolo et al., 2010). Furthermore, *L. capsici* YS1215 was reported to have nematicidal activity, reducing root-knot caused by *Meloidogyne incognita* by inhibiting egg hatching (Lee et al., 2014).

Most of the *Lysobacter* strains tested here poorly colonized the rhizosphere of sugar beet and cauliflower. Given the importance of root colonization for biocontrol (Bull et al., 1991; Johnson, 1994; Raaijmakers et al., 1995), this suggests that the inconsistency in disease control by the *Lysobacter* strains may be due to their lack of competitiveness in the rhizosphere of sugar beet and cauliflower. The rhizosphere differs from the bulk soil by the presence of plant root exudates that create an environment rich in nutrients. Chemotaxis and active motility toward root exudates represent the first steps in rhizosphere colonization (Benizri et al., 2001; De Weert and Bloemberg, 2006). This motility may be active, through flagellar movements, or passive, through percolating water or vectors. None of the 18 *Lysobacter* strains possess flagella, what limits the capacity of the strains to effectively compete against flagellated soil bacteria for a niche in the rhizosphere. The adherence to root tissues through biofilm formation is the next step in rhizosphere colonization (Benizri et al., 2001; Ramey et al., 2004; Danhorn and Fuqua, 2007). Several traits are involved in biofilm formation including cell wall structures and extracellular polysaccharide

production (Lugtenberg et al., 2001). Biofilm production *in vitro* has been described for *L. capsici* AZ78 and appeared medium specific, (Puopolo et al., 2014). Biofilm formation was observed for *Lysobacter* sp. strain SB-K88 on roots of sugar beet (Islam et al., 2005). Biofilm formation *in situ* was not tested for our 18 *Lysobacter* strains and will be subject of future studies. The root exudate composition is plant specific (Mandimba et al., 1986) and the ability to assimilate specific amino acids, vitamin B1, carbohydrates, organic acids as well as pH tolerance and competition for limiting resources also determine the rhizosphere competence (Dekkers et al., 1999; Benizri et al., 2001; Lugtenberg and Kamilova, 2009; Ghirardi et al., 2012). In the rhizosphere there is often a limitation for soluble iron, commonly used as a cofactor in enzymes that are involved in pathways that are essential for microbial growth. Therefore, the ability to produce siderophores (small high-affinity iron chelating compounds) confers a competitive advantage. The role of competition for iron by siderophore production of *Lysobacter* sp. seems species or strain specific and not all strains, including several strains used in this study, possess iron-chelating capacity (Puopolo et al., 2010; Ko et al., 2011; De Bruijn et al., in press).

The soil type may also influence rhizosphere colonization and biocontrol activity. For example, the colonization of *Pseudomonas* sp. strain ITRI53 and *Pantoea* sp. strain BTRH79 of Italian ryegrass was higher in loamy soils compared with sandy soils (Afzal et al., 2011). The agricultural soil used in this study is a clay soil with loam texture. Several of our *Lysobacter* strains were isolated from this agricultural soil and we expected that those conditions would provide a "home-field advantage" for rhizosphere colonization of sugar beet and cauliflower. In a potting soil:sand mixture, we observed higher rhizosphere population densities on sugar beet seedlings as compared to the agricultural soil, with densities higher than the minimal dose of 10^5 CFU/g soil reported for other biocontrol strains (Xu and Gross, 1986; Leeman et al., 1995; Raaijmakers et al., 1995). Despite these densities, no significant and/or consistent biocontrol activity was observed for any of the *Lysobacter* strains tested.

Several biocontrol agents not only suppress disease but also promote plant growth (Johansson et al., 2003). None of the *Lysobacter* strains tested in this study, however, were able to significantly and consistently promote growth of 4 different crops when applied to seeds or root tips or when applied physically separated from the crop. Furthermore, volatiles produced by the *Lysobacter* strains when grown on LB medium even showed a negative effect on growth of *A. thaliana*. This may be due to the accumulation of toxic volatiles that are produced by *Lysobacter* spp. when growing in rich media. Weise et al. (2013) showed that *Serratia odorifera* inhibited the growth of *A. thaliana* plants due to the production of ammonia when grown on peptone-rich nutrient media. Iwata et al. (2010) reported that *Lysobacter* sp. E4 was able to fix nitrogen under free-living conditions and accumulated ammonia in the culture broth. Also hydrogen cyanide (HCN) produced by *Chromobacterium, Pseudomonas,* and *Serratia* have been shown to inhibit the growth of *A. thaliana* (Blom et al., 2011). More research needs to be conducted to determine if HCN or other toxic volatiles are produced by *Lysobacter*.

Overall, our results indicate that none of the 18 *Lysobacter* strains have the potential to control *Rhizoctonia* or promote plant growth of sugar beet and cauliflower, probably due to insufficient rhizosphere competence. However, the *Lysobacter* strains showed a high diversity in *in vitro* activity against 14 different pathogenic fungi, oomycetes and bacteria, suggesting that the genus *Lysobacter* constitutes an extensive source of (new) enzymes and antimicrobial compounds. Possibly *Lysobacter* needs to interact with a specific microbial community to become antagonistic to *Rhizoctonia* or to promote plant growth in natural environments. To better understand the potential contribution of *Lysobacter* species to the overall activities of the microbial communities responsible for soil suppressiveness against *R. solani*, in-depth metagenomic and metatranscriptomic analyses of the bacterial community compositions and functions will be needed to unravel the role of this genus in disease suppressiveness. Future work will include testing *Lysobacter* mixtures or mixtures with other bacterial genera abundant in soils suppressive to *R. solani*. Interactions of *Lysobacter* with other bacteria may stimulate the production of antimicrobial compounds as was shown recently for other bacterial genera (Tyc et al., 2014).

AUTHOR CONTRIBUTIONS

All authors were involved in the design of the experiments. RG and IB performed *in vitro* and *in vivo* activity bioassays, BOX-PCR and phylogenetic analyses. RG performed plant growth promotion assays. All authors contributed to the writing of the manuscript and approved submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01243

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Chitin Mixed in Potting Soil Alters Lettuce Growth, the Survival of Zoonotic Bacteria on the Leaves and Associated Rhizosphere Microbiology

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Chitin is a promising soil amendment for improving soil quality, plant growth, and plant resilience. The objectives of this study were twofold. First, to study the effect of chitin mixed in potting soil on lettuce growth and on the survival of two zoonotic bacterial pathogens, *Escherichia coli* O157:H7 and *Salmonella enterica* on the lettuce leaves. Second, to assess the related changes in the microbial lettuce rhizosphere, using phospholipid fatty acid (PLFA) analysis and amplicon sequencing of a bacterial 16S rRNA gene fragment and the fungal ITS2. As a result of chitin addition, lettuce fresh yield weight was significantly increased. *S. enterica* survival in the lettuce phyllosphere was significantly reduced. The *E. coli* O157:H7 survival was also lowered, but not significantly. Moreover, significant changes were observed in the bacterial and fungal community of the lettuce rhizosphere. PLFA analysis showed a significant increase in fungal and bacterial biomass. Amplicon sequencing showed no increase in fungal and bacterial biodiversity, but relative abundances of the bacterial phyla *Acidobacteria, Verrucomicrobia*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* and the fungal phyla *Ascomycota*, *Basidiomycota*, and *Zygomycota* were significantly changed. More specifically, a more than 10-fold increase was observed for operational taxonomic units belonging to the bacterial genera *Cellvibrio*, *Pedobacter*, *Dyadobacter,* and *Streptomyces* and to the fungal genera *Lecanicillium* and *Mortierella*. These genera include several species previously reported to be involved in biocontrol, plant growth promotion, the nitrogen cycle and chitin degradation. These results enhance the understanding of the response of the rhizosphere microbiome to chitin amendment. Moreover, this is the first study to investigate the use of soil amendments to control the survival of *S. enterica* on plant leaves.

Keywords: amplicon sequencing, chitin, *Escherichia coli* (EHEC), lettuce, phospholipid fatty acid (PLFA), rhizosphere, *Salmonella enterica*

INTRODUCTION

Utilization of organic amendments, such as chitin, is one of the most economical and practical options for improving soil and substrate quality, plant growth, and plant resilience (De Boer et al., 1999; El Hadrami et al., 2010; Sharp, 2013). Chitin is a biopolymer that is distributed among many water and soil organisms as it is a major constituent of the cell walls of fungi, the exoskeleton of arthropods and the shells of crustacean and nematode eggs. It is the second most abundant biopolymer in nature after cellulose, with an estimated natural production of 10^{10} tons per year (Jacquiod et al., 2013). Soil treatment with chitin has been shown to decrease the rate of infection of plant roots by nematodes (Sarathchandra et al., 1996; Radwan et al., 2012) and to increase disease suppressiveness against the fungal soil-borne pathogens *Verticillium dahliae* and *Rhizoctonia solani* (Cretoiu et al., 2013; Postma and Schilder, 2015). The mechanism behind this suppressiveness most often relates to a change in the microbiota in soil and rhizosphere (Cretoiu et al., 2013). Microorganisms, which are capable of hydrolyzing the chitinous cell wall of pathogenic fungi and nematodes eggs, increase their numbers and/or activities in response to the chitin added. In addition, also secondary responders to the added chitin confer overall pathogen suppression. Next to a direct effect on pathogens, changes in this rhizosphere microbiology may also affect the plant physiology and its capacity to be colonized by microorganisms, including plant and human pathogens (El Hadrami et al., 2010; Gu et al., 2013; Markland et al., 2015). Rhizosphere organisms are well-studied for their beneficial effects on plant growth and health, including nitrogen-fixing bacteria, mycorrhizal fungi, biocontrol agents, plant growth promoting rhizobacteria (PGPR), and fungi (PGPF; Berendsen et al., 2012). Studies have shown that these beneficial organisms in the rhizosphere can be increased by the utilization of chitin amendment in order to enhance plant growth and resilience to plant pathogens (Dutta and Isaac, 1979; Hallmann et al., 1999). In addition, chitin has also been shown to trigger plant immunity and acts as a pathogen-associated-molecular pattern (PAMP) triggering the plant defense against chitin-containing harmful organisms (de Jonge et al., 2010; Sharp, 2013).To date, no research has been done to investigate the indirect effect of chitin soil amendment on zoonotic bacterial human pathogens that can survive on fresh produce crops.

Authorities promote the consumption of fresh fruit and vegetables, but at the same time concerns have been raised about the food safety of leafy vegetables. Leafy vegetables, such as lettuce, are considered as high risk food, as various *Escherichia coli* O157:H7 and *Salmonella enterica* outbreaks have been related to the consumption of lettuce greenery that can carry these pathogens (Ward et al., 2002; Horby et al., 2003; Welinder-Olsson et al., 2004; Friesema et al., 2008; Nygård et al., 2008; Söderström et al., 2008). It is usually accepted that zoonotic bacterial pathogens enter the agricultural environment via animal feces. Feces may contaminate irrigation water and soil. Irrigation water is considered as the most likely key route of dispersal of zoonotic pathogens from feces to plants (Barak and Schroeder, 2012; Holvoet et al., 2014). The biology of *E. coli* and *S. enterica* on lettuce leaves under various conditions has been extensively studied (e.g., Brandl and Amundson, 2008; Oliveira et al., 2012; Van der Linden et al., 2014). A recent study showed that butterhead lettuce grown in greenhouses with a sprinkle irrigation system may present a potential health hazard when the green parts are contaminated near harvest (Van der Linden et al., 2013). Reduction in the survival of zoonotic bacterial human pathogens in the preharvest environment can help prevent spread of pathogens during post-harvest washing and packaging. A variety of direct control mechanisms such as disinfectans (including chlorine, hydrogen peroxide, organic acids, and ozons) are being used to reduce this preharvest survival, but there is a need to preserve food by natural means (Oliveira et al., 2015). Hence, bacteria isolated from the rhizosphere and leaves of leafy greens have been shown to suppress human pathogens (e.g., Markland et al., 2015; Oliveira et al., 2015) and chitin derivates have been found to have antibacterial activity against zoonotic bacterial pathogens (e.g., Jeon et al., 2014). However, no studies have investigated the indirect effect of chitin addition to the growing medium on the survival on zoonotic bacterial pathogens on the leaves. Growing media that could reduce the carrier capacity of crops for these pathogens would be an interesting strategy for sustainable control.

The objectives of this study were twofold. First, we studied the effect of chitin mixed in potting soil on lettuce growth and on the capacity of these lettuce plants to carry two zoonotic bacterial pathogens, *E. coli* O157:H7 and *S. enterica* on their leaves. Second, changes in the microbial rhizosphere of lettuce were assessed. We hypothesize that the chitin favors chitin-degrading microbiology in the soil, among which important populations of PGPR and PGPF, and the stimulation of these groups in the lettuce rhizosphere could make the plant leaves less prone to colonization by the human pathogens. To assess this colonization, we used selective platings as described by Van der Linden et al. (2013). To assess the microbial rhizosphere dynamics, two techniques were used: phospholipid fatty acid (PLFA) analysis and 16S and ITS2 rDNA amplicon sequencing. PLFA analysis is a gas chromatography-based technique widely used to monitor biodiversity in complex commodities such as soil. Specific PLFAs are markers for bacteria and fungi (Frostegård et al., 2011) and the 20 PLFA markers used in the present study discriminate six microbial groups: gram-positive bacteria, Gram-negative bacteria, bacteria (non-specific), actinomycetes (Actinomycetales), fungi and mycorrhiza (Nelissen et al., 2015). Amplicon sequencing has proven to be an efficient method to monitor changes in the relative abundance of bacterial and fungal genera or species in soil and rhizosphere (Caporaso et al., 2011; Lundberg et al., 2012).

MATERIALS AND METHODS

Chitin Soil Amendment

Chitin flakes purified from crab shell were obtained from BioLog Hepp Gmbh (lot: 90200705). An amount of 2% (dry weight chitin/dry weight potting soil) was used in each experiment.

Lettuce Growth

Pelletized butterhead lettuce seeds (*Lactuca sativa* L. var. *capitate* "Alexandria") obtained from Rijk Zwaan Distribution B.V. (De Lier, The Netherlands), were germinated on moist filter papers (Whatman filters 2) in Petri dishes. The seedlings were transplanted into a 100% peat based-potting soil with a pH of 5.5–6.0 (Universal Substrate LP2B, Peltracom, Belgium) with and without 2% chitin (one seedling per 1.5 L pot) and placed in a growth chamber with conditions set at 19°C during day and 12°C at night, a relative humidity of 70–80%, and a photoperiod of 14 h. After 55 days, five plants per treatment were sampled for PLFA analysis, five plants per treatment were sampled for amplicon sequencing and seven plants per treatment were inoculated with *S. enterica* sv. Thompson RM1987N or *E. coli* O157:H7 (see below). At the end of the experiment (8 days after pathogen inoculation, see below), the lettuce heads were harvested and weighed.

Bacterial Strains and Inoculation and Detection on Lettuce Leaves

Two bacterial strains were used: *S. enterica* sv. Thompson RM1987N and *E. coli* O157:H7 MB3885 (Van der Linden et al., 2013). Both strains were streaked from a glycerol frozen stock maintained at −70°C onto tryptone soya agar (TSA; Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h. One colony was transferred to 10 mL of tryptone soya broth (TSB, Oxoid) and incubated at 37°C for 18 h while shaken at 200 rpm. Cells of each strain were washed twice by centrifugation (6000 × *g*, 15 min) in 50 mM phosphate buffered saline (PBS, pH 7.4). The optical density (OD) was measured at 595 nm using a microplate reader and concentrations were estimated based on an OD-colony-forming-unit (CFU) mL^{-1} standard curve. The appropriate amount of cells was resuspended in PBS to a concentration of 1×10^4 CFU mL^{-1}.

The plants were inoculated at a concentration of 10^4 CFU ml^{-1} of PBS with a hand sprayer as described by Van der Linden et al. (2013). To count the pathogen concentrations on the lettuce leaves, individual leaves were placed in extraction bags with membrane filter (Bioreba) and weighed. PBS with 0.05% Tween 20 was added at a 1/1 (wt/vol) ratio and the samples were ground for ±15 s at maximum speed (Homex 6, Bioreba) until a homogenous mixture was obtained. Ten-fold dilutions of the resulting suspension were made in 0.1% peptone and 100 µl aliquots were spread-plated in duplicate on xylose lysine desoxycholate agar (XLD; Lab M, Bury, UK) overlaid with TSA for *S. enterica* (XLD-TAL) and on cefixime–tellurite sorbitol Mac Conkey agar (CT-SMAC; Lab M, Bury, UK) overlaid with TSA (CT-SMAC-TAL) for *E. coli* O157:H7 (Van der Linden et al., 2013). All plates were incubated at 37°C for 24 h. Three randomly chosen plants from each treatment were sampled at 4 and 8 days after inoculation (dai), while one plant per treatment was sampled at day 0 (= immediately after inoculation). From each plant, three middle-aged leaves were collected in a single extraction bag and analyzed for *E. coli* O157:H7 and *S. enterica* as described above. For mature lettuce, the 12th to 14th leaves in the head are considered as middle-aged. Leaf age is important factor influencing the survival of both pathogens on the leaves. Middle-aged leaves were selected because Van der Linden et al. (2013) found that the middle-aged leaves yielded the most consistent results for both pathogens, with the smallest standard deviations and smallest effect of environmental factors (which are difficult to control in the growth chamber). This was especially the case for *S. enterica*. The experiment was done twice for each pathogen. So, in total 6 leaves for 0 dai, 18 leaves for 4 dai, and 18 leaves for 8 dai were analyzed.

Phospholipid Fatty Acid (PLFA) Analysis

Soil samples (approximately 50 g) were taken from five pots per treatment and stored at −20°C until freeze-dried. Total lipids were extracted from 6 g freeze-dried soil using phosphate buffer, chloroform, and methanol at a 0.9:1:2 ratio. Neutral, glycol- and phospho-lipids were separated by solid phase extraction with respectively chloroform, acetone and methanol. Phospholipids were saponified to obtain free fatty acids, which were subsequently methylated using 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). FAMEs were analyzed with a capillary gas chromatograph-flam ionization detector (Perkin Elmer Clarus 600, Perkin Elmer, Waltham, MA, USA) with a col-elite-2560 column (100 m length × 0.25 mm ID, 0.25 µm film thickness, Perkin Elmer). The temperature program started at 75°C, followed by a heating rate of 10°C min^{-1} up to 180°C and a final heating rate of 2°C min^{-1} up to 240°C. External FAME and BAME mix (Sigma-Aldrich, St. Louis, MO, USA) were used as standard for PLFA identification and quantification. The C values were corrected using a working standard C19:0. The abundance of individual PLFAs was calculated in absolute C amounts (PLFA-C, C_x [nmol g^{-1}]) based on the concentrations in the liquid extracts using the following formula:

$$C_x[\text{nmol g}^{-1}] = \frac{A_x \cdot c_i\,[\mu g] \cdot f \cdot 1000}{A_i \cdot W\,[g] \cdot M\,[\mu g\,\mu mol^{-1}]}$$

Where C_x is the concentration of the fatty acid studied, A_x is the peak area of the fatty acid studied, A_i is the peak area of the internal standard, c_i is the absolute amount of internal standard in the vial [µg], f is the response factors of different PLFA compounds (peak area to concentration ratio compared to the internal standard; if not known, then = 1), W is the amount of soil [g], M is the molecular weight of the fatty acid [µg µmol^{-1}]. Twenty PLFAs were selected as biomarker fatty acids for six distinct microbial groups: Gram-positive bacteria, Gram-negative bacteria, bacteria (non-specific), actinomycetes (Actinomycetales), fungi and mycorrhiza (Nelissen et al., 2015, **Table 1**).

Rhizosphere Sampling and DNA Extraction

The lettuce rhizosphere was sampled according to Lundberg et al. (2012). Loose soil was manually removed from the roots by kneading and shaking. We followed the established definition

TABLE 1A | Absolute concentrations (nmol g^{-1} dry soil) ± standard error of PLFA biomarkers specific for different microbial groups in potting soil with and without 2% chitin, after 55 days of lettuce cultivation in the growth chamber.

Microbial group	PLFA biomarker	Treatment	
		Control	2% chitin
Gram positive bacteria	i-C15:0	20.22 ± 0.43	31.39 ± 2.22*
	a-C15:0	12.71 ± 0.35	19.27 ± 1.49*
	i-C16:0	7.13 ± 0.23	11.19 ± 0.77*
	i-C17:0	8.08 ± 0.20	14.91 ± 1.11*
Actinomycetales	10Me-C16:0	4.20 ± 0.20	5.83 ± 0.34*
	10Me-C17:0	4.96 ± 0.19	8.74 ± 0.56*
	10Me-C18:0	0.50 ± 0.03	3.34 ± 1.07*
Bacteria (non-specific)	C14:0	2.72 ± 0.10	3.70 ± 0.28*
	C15:0	2.05 ± 0.05	3.34 ± 0.23*
	C16:0	41.41 ± 1.64	71.72 ± 5.56*
	C17:0	1.17 ± 0.04	2.27 ± 0.16*
	C18:0	9.77 ± 0.26	16.36 ± 0.94*
Gram negative bacteria	C16:1c9	11.14 ± 0.74	25.18 ± 2.72*
	C16:1t9	4.15 ± 0.63	8.98 ± 0.65*
	C17:0cy	9.48 ± 0.53	22.56 ± 2.27*
	C18:1c11	9.77 ± 0.26	16.36 ± 0.94*
	C19:0cy	23.56 ± 0.78	49.23 ± 4.32*
Fungi	C18:1c9	15.26 ± 0.68	38.37 ± 4.62*
	C18:2n9,12	22.32 ± 3.28	31.50 ± 1.69*
Arbuscular mycorrhiza	C16:1c11	4.22 ± 0.37	8.17 ± 0.54*
Total biomass		221.82 ± 8.28	402.20 ± 29.38*

Asterisks indicate a significant increase as compared to the control ($P < 0.05$) by analysis of variance with $n = 5$.

of rhizosphere soil as extending up to 1 mm from the root surface. Subsequently, roots with the remaining soil aggregates were placed in a sterile 50 ml tube containing 25 ml phosphate buffer. Tubes were vortexed at maximum speed for 15 s, which released most of the rhizosphere soil from the roots and turned the water turbid. The turbid solution was then filtered through a 100 μm nylon mesh cell strainer to remove broken plant parts and large sediment. The turbid filtrate was centrifuged for 15 min at 3,200 *g* to form a pellet containing fine sediment and microorganisms. Most of the supernatant was removed and the pellets were stored at −20°C until DNA extraction. DNA was extracted from 250 mg of the pellet with the PowerSoil DNA isolation kit (Mo Bio, USA) according to the manufacturer's instructions. This DNA was used for bacterial 16S (V3–V4) and fungal ITS2 rDNA amplicon sequencing as described below.

16S and ITS2 Amplicon Sequencing of the Rhizosphere Samples

The bacterial V3–V4 fragment of the 16S rRNA gene was selected for amplicon sequencing. Amplification of the fragment was done using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21, described by Klindworth et al. (2013), extended with Illumina specific adaptors. Following PCR conditions were used: initial denaturation at 95°C for 3 min, followed by 25 cycles consisting of denaturation (95°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 30 s) and a final extension step at 72°C for 5 min. To amplify the fungal rDNA-ITS2 region an adapted forward primer of fITS7b is from Ihrmark et al. (2012; GTGAATCATCRAATYTTTG) and the ITS4NGSr reverse primer (Tedersoo et al., 2014) were used, both extended with Illumina specific adaptors. The ITS2-PCR conditions were as above, except for 30 cycles with an annealing time of 1 min. A second PCR was done to attach dual indices and sequencing adaptors to both the V3–V4 as the ITS2 fragments, using the Nextera XT index kit (Illumina, San Diego, CA, USA). Same PCR conditions were used as in the first PCR, but eight cycles were used instead of 25 or 30 PCR cycles. Mastermixes for all PCRs were prepared using the Kapa HiFi Hotstart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions to a total amount of 25 μl (amplification of the bacterial and fungal fragments) and 50 μl (dual indices and sequencing adaptors attachment). Each PCR step was followed by a PCR product clean-up using the Highprep PCR reagent kit (MAGBIO, Gaithersburg, MD, USA). Final libraries were quality

TABLE 1B | Relative abundance (%) ± standard error of biomarker PLFAs and PLFA groups in potting soil with and without 2% chitin after 55 days of lettuce cultivation in the growth chamber.

Microbial group	PLFA biomarker	Treatment	
		Control	2% chitin
Gram positive bacteria	i-C15:0	9.12 ± 0.19	7.77 ± 0.27*
	a-C15:0	5.73 ± 0.17	4.76 ± 0.11*
	i-C16:0	3.21 ± 0.06	2.78 ± 0.13*
	i-C17:0	3.64 ± 0.08	3.68 ± 0.05
Actinomycetales	10Me-C16:0	1.89 ± 0.05	1.45 ± 0.04*
	10Me-C17:0	2.23 ± 0.04	2.16 ± 0.06
	10Me-C18:0	0.22 ± 0.02	0.82 ± 0.25*
Non-specific bacteria	C14:0	1.22 ± 0.05	0.91 ± 0.02*
	C15:0	0.93 ± 0.03	0.83 ± 0.02*
	C16:0	18.6 ± 0.21	17.66 ± 0.12*
	C17:0	0.53 ± 0.02	0.56 ± 0.02
	C18:0	4.40 ± 0.07	4.05 ± 0.09*
Gram negative bacteria	**C16:1c9**	5.00 ± 0.21	6.15 ± 0.23*
	C16:1t9	1.85 ± 0.24	2.22 ± 0.07
	C17:0cy	4.26 ± 0.15	5.53 ± 0.17*
	C18:1c11	7.77 ± 0.23	7.30 ± 0.27
	C19:0cy	10.61 ± 0.24	12.11 ± 0.39*
	10Me-C16:0	1.89 ± 0.05	1.45 ± 0.04*
	10Me-C17:0	2.23 ± 0.04	2.16 ± 0.06
	10Me-C18:0	0.22 ± 0.02	0.82 ± 0.25*
Fungi	C18:1c9	6.88 ± 0.27	9.39 ± 0.66*
	C18:2n9,12	9.95 ± 1.32	7.83 ± 0.35
Arbuscular mycorrhiza	C16:1c11	1.91 ± 0.19	2.02 ± 0.08

*Asterisk indicates a significant difference to the control ($P < 0.05$) by analysis of variance with $n = 5$. Microbial groups and biomarkers marked **in bold** are significantly more abundant in the chitin treatment as compared to the control. Underlined microbial groups and biomarkers are significant less abundant in the chitin treatment as compared to the control.*

controlled using the Qiaxcel Advanced, with the Qiaxcel DNA High Resolution kit (QIAGEN, Germantwon, MD, USA) and concentrations were measured using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA). The final barcoded libraries of each sample were diluted to 10 nM and pooled in a 2:1 range for bacteria and fungi respectively. Resulting libraries were sequenced using Illumina MiSeq v3 technology (2 bp × 300 bp, paired-end) by Macrogen, South-Korea.

Additionally, two technical replicates for each treatment (one control and one chitin rhizosphere, so four samples in total) were done to study the reproducibility of sequencing, with a separate DNA extraction and sequencing done on the same rhizosphere of a single plant.

Sequence Reads Processing

Demultiplexing of the amplicon dataset was done by the sequencing provider. The raw sequence data is available in NCBI's Sequence Read Archive under the accession number PRJNA294362. Trimmomatic v0.32 was used for removing the primers (Bolger et al., 2014). Raw Illumina forward and reverse reads were merged using the program PEAR v.0.9.8 (Zhang et al., 2014). Length cut-off values for the merged sequences were set between 400 and 450 bp for the V3–V4 and between 200 and 480 bp for the ITS2. A minimum overlap size of 120 bp and quality score threshold of 30 were used for all sequences. An extra program ITSx v.1.0.11 was used to extract the ITS2 sequences (Bengtsson-Palme et al., 2013). In the following steps, different programs of the Usearch software v7.0.1090 were used (Edgar, 2014). Merged sequences were quality filtered with a maximum expected error of 3 with the "fastq_filter" option. Next, sequences of all samples that needed to be compared to each other were merged into one file, dereplicated and sorted by abundance. Clustering the reads into operational taxonomic units (OTUs) was done using Uparse, with an identity level of 97% for V3–V4 and 98.5% for ITS2 (Ihrmark et al., 2012; Edgar, 2014). In the case of V3–V4, chimeras were removed using Uchime with the RDP Gold database as a reference (Edgar et al., 2011). Finally, sequences of individual samples were mapped back to the representative OTUs using the "usearch_global" algorithm at 97% identity, and converted to an OTU table (McDonald et al., 2012).

Statistical Analysis and Downstream Processing of OTU Tables

Lettuce growth, zoonotic pathogens enumeration and absolute PLFA concentrations were analyzed with Statistica 12 (Statsoft) using a multi-factor analysis of variance with $P < 0.05$. Full factorial design was performed first. If all interaction terms were not significant, a *t*-test was done to compare the mean of the chitin treatment with the control treatment. For the lettuce growth, chitin (with or without) and experiment (1 and 2) were the factors with fresh weight per plant as dependent variable. For the enumeration of the zoonotic pathogens on lettuce leaves, chitin (with or without), sampling time (days 4 and 8) and experiment (1 and 2) were the factors with cfu g^{-1} lettuce leaf as dependent variable. Statistical differences in the absolute values of the PLFA's between the different treatments were determined using a MANOVA analysis.

Statistical differences of the relative abundances in PLFA were determined using ANOVA by the Statistical Analysis of Metagenomic Profiles (STAMP) program (Parks and Beiko, 2010). Correction of multiple testing was done using the Benjamini–Hochberg False Discovery Rate method. Principal coordinate analysis, in which the dissimilarity matrices were based on the Bray–Curtis index (PCoA), on the PLFA data was done using the vegan package in R (version 2.0-10; Oksanen et al., 2010) with dissimilarity matrices calculated using the Bray–Curtis index.

Operational taxonomic units tables of the V3–V4 and ITS2 amplicon sequencing were analyzed using the QIIME software package (v1.9.0; Caporaso et al., 2010b). Representative bacterial OTU sequences were aligned to the SILVA v119 97% core set (version 119) using QIIME (Caporaso et al., 2010a; Quest et al., 2012). Taxonomy assignment was done using the uclust assignment method, accepting maximum 3 hits for each query sequence and then assigning the most specific taxonomic label that is associated with at least 51% of the hits. Similarly, taxon assignments of fungal OTU sequences were done using the UNITE database (version 7.0; Kõljalg et al., 2013).

Rarefaction analysis was done using the "alpha_rarefaction.py" script of QIIME. Rarefaction curves were estimated for both bacterial as fungal OTUs (**Supplementary Figures S5** and **S7**, respectively). Convergence was reached at 50,000 sequences for the bacterial OTUs and at 10,000 sequences for the fungal OTUs. Those rarefaction depths were used to determine the number of observed OTUs representing the bacterial and fungal richness. Shannon–Wiener diversity indices were calculated using the "alpha_diversity.py" script (QIIME) and used to estimate the within sample diversity. To find significant differences among mean richness and diversity indices, ANOVA analysis was done. Tukey HSD test was used to find the mean richness and diversity indices that are significantly different from each other. Both analysis were done using the R program (version 3.1.0; R Core Team, 2015).

Multivariate analysis was done using the specific R package vegan (version 2.0-10; Oksanen et al., 2010). Dissimilarity matrices (based on the Bray–Curtis dissimilarity index) were calculated from the OTU tables of the fungal and bacterial sequences. The OTU tables were normalized by removing those OTUs with an abundance lower than 0.01% in at least one sample. Effect of chitin addition on the bacterial and fungal communities was studied by doing a PERMANOVA analysis on these dissimilarity indices. To visualize the observed differences in bacterial community composition, PCoA on the dissimilarity matrices was done.

The STAMP analysis software was used to study individual differences in the bacterial groups (Parks and Beiko, 2010). For each experiment, ANOVA analyses were done on a species table to determine the effect of chitin addition on the individual groups (phyla, species). To correct for multiple testing, we used the Benjamini–Hochberg False Discovery Rate method. The used species table was calculated by the QIIME software ("summarize_taxa_through_plots.py") and normalized by only keeping those species which were present with a minimal abundance of 0.01% in minimum one sample.

RESULTS

Effect of Chitin Soil Amendment on Lettuce Growth and Survival of Zoonotic Pathogens on the Leaves

For the lettuce growth, there was no interaction between the treatments (with chitin and without chitin) and the two independent experiments, so data were pooled. Addition of chitin significantly ($P = 0.003$) increased the fresh weight of the lettuce plants to 213.00 ± 18.76 g per plant, compared with 172.08 ± 17.75 g per plant in the control (**Supplementary Figure S1**; left = control treatment, right = chitin treatment). Moreover, the plants in the chitin treatment showed more root development as compared to the control (**Supplementary Figure S2**).

In the control without chitin, the dynamics of *E. coli* O157:H7 concentrations on the leaves were highly similar to those reported by Van der Linden et al. (2013) who grew lettuce plants in the same conditions and using the same *E. coli* isolate. There was no interaction between the two treatments (with and without chitin), the two independent experiments and the three sampling days (0, 4, and 8 dai). However, there was an interaction between the sampling days and the two experiments, so each day was analyzed separately. For day 4, there was no interaction effect between the treatments and experiments, so data could be pooled. On day 4, there was no significant effect of the chitin on the survival of *E. coli* O157:H7. On day 8 a significant reduction of *E. coli* survival in Experiment 2 ($P = 0.009$), but not in Experiment 1 was observed (interaction effect treatment-experiment; **Supplementary Figure S3**).

Also for the dynamics of *S. enterica* on the leaves in our control, we reported highly similar results as the ones obtained by Van der Linden et al. (2013). There was no interaction between the two treatments (chitin and without chitin), the two independent experiments and the

three sampling days (0, 4, 8 dai). Also no interaction was observed between the treatments and experiments, so data were pooled over the two experiments. There was an interaction between the treatments and the sampling days, so each day was analyzed separately. At day 4, no significant difference between the two treatments was found, whereas at day 8, there was significantly less survival of *S. enterica* on the leaves in the chitin treatment as compared to the control (**Figure 1**).

Effect of Chitin Soil Amendment on Lettuce Rhizosphere Microbiology Analyzed with PLFA

The soil from five individual pots of the control treatment (= without chitin) and from five individual pots of the chitin treatment were analyzed using PLFA. Both the absolute (nmol g^{-1} dry soil) and the relative abundance (%) of each biomarker were assessed per treatment. All individual PLFA biomarkers and all microbial groups were significantly increased after chitin amendment (absolute abundances), resulting in a double amount of total biomass as compared to the control (**Table 1A**). For the relative abundance, 13 of the 20 biomarkers were significantly different from the control, with a significant decrease in relative abundance for bacteria (non-specific) and Gram positive bacteria and a significant increase for the Gram negative bacteria (**Table 1B**).

To illustrate these dissimilarities in the microbial communities of the chitin supplemented soil and the control a PCoA on the PLFA data was done (**Supplementary Figure S4**). The first principal coordinate (PCo1), which represents the major variance of the dataset (94.9%) confirmed that the microbiome differed between soil with and without chitin. The second principal coordinate describes the variation between the samples in each treatment (with and without chitin) This is only a minor source of variability (2.5%), indicating a high reproducibility of the data of the five pots per treatment.

Effect of Chitin Soil Amendment on Bacterial Lettuce Rhizosphere Using 16S rDNA Based Amplicon Sequencing

The bacterial microbiomes present in rhizospheres of plants grown in potting soil with and without chitin were compared by sequencing the V3–V4 region of the 16S rDNA. The rhizospheres of five individual plants from each treatment (with and without chitin) were prepared and analyzed separately. After merging of the forward and reverse reads and quality filtering, 83.8% of the sequences were retained, resulting in an average of 92,549 sequences per sample. Rarefaction depth was reached at approximately 50,000 sequences, indicating that enough sequence reads were generated (**Supplementary Figure S5**). No differences were observed between the two technical replicates per treatment, indicating reproducibility of the sequencing. There were no significant differences in the number of observed OTUs and Shannon–Wiener diversity indices between the control and the chitin treatment (1436 ± 35 vs. 1370 ± 12 and 8.15 ± 0.03 vs. 8.17 ± 0.08, respectively), indicating that the chitin amendment did not increase the bacterial biodiversity in the rhizosphere. However, significant shifts in bacterial composition (taxonomic groups) were observed between the two treatments (PERMANOVA, $P = 0.011$) which is illustrated by a PCoA plot (**Supplementary Figure S6**). The first principal coordinate contains the major source of variability (51.8%) and refers to the different rhizospheres of the plants grown in chitin vs. non-chitin amended soil. The second principal coordinate describes the variation within the treatments. This is only a minor source of variability (17.8%), indicating a high reproducibility.

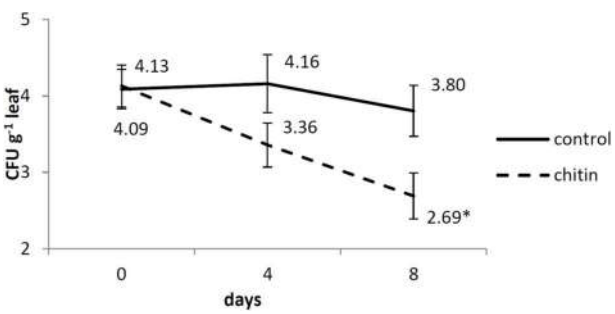

FIGURE 1 | *Salmonella enterica* sv. Thompson RM1987N dynamics on middle-aged lettuce leaves at 0, 4, and 8 days after spray inoculation analyzed by plating as described by Van der Linden et al. (2013). Full lines represent control plants, while dashed lines represent chitin treated plants. The data are calculated from the log-transformed values of the pathogen per gram tissue from two independent experiments ($n = 2$ plants or 6 leaves for day 0 and $n = 6$ plants or 18 leaves for day 4 and 8). Asterisk means significantly different between the chitin and the control treatment. Bars represent standard errors.

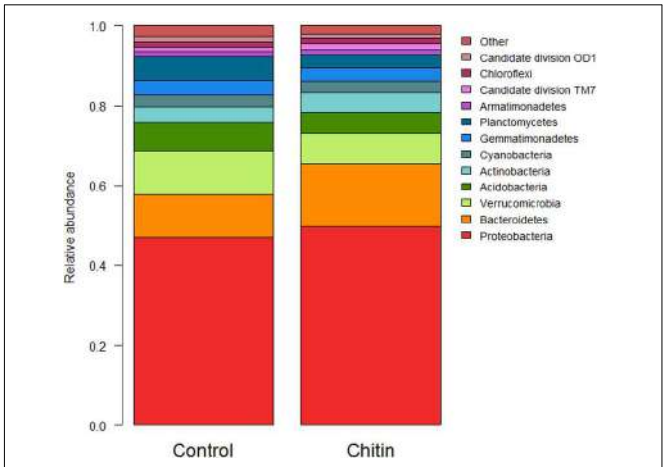

FIGURE 2 | Analysis of the bacterial composition of the lettuce rhizosphere in unamended and chitin amended potting soil. Relative abundance (percentages) of the different bacterial phyla (16S V3–V4 region) in the lettuce rhizosphere. Phyla representing less than 1% of the total community are bundled in the group "other," as their taxonomic composition may be uncertain.

In total, 28 bacterial phyla were found across all samples. Thirteen of these phyla showed a significant difference between the control and the chitin treatment, of which 10 phyla and 2 candidate divisions each represented more than 1% of the community (**Figure 2**). Most importantly, the relative abundances of the *Acidobacteria* and the *Verrucomicrobia* were significantly decreased in the chitin treatment as compared to the control, whereas the relative abundance of the *Actinobacteria*, *Bacteroidetes*, and the *Proteobacteria* was significantly increased in the chitin treatment as compared to the control (**Table 2**). Analyzing these five phyla together, it was shown that the relative abundance of the Gram negative bacteria was significantly increased in the chitin treatment. In contrast, the relative abundance of the Gram positive bacteria was not significantly different from the control.

On family level, the highest relative abundance was for the *Chitinophagaceae* and *Sphingomonadaceae*. Chitin altered the relative abundance of 40 bacterial families, in particular 11 families of the *Proteobacteria*, 8 of the *Actinobacteria*, 6 of the *Bacteroidetes*, 6 of the *Firmicutes*, 2 of the *Verrucomicrobia*, 1 of the *Acidobacteria* (unknown family of subgroup 6) and 1 of the phylum *Chlamydiae* (*Simkaniaceae*; **Figure 3**). Next to these families, which belonged to significantly altered phyla by chitin addition, two families of the *Chloroflexi* (*Anaerolineaceae* and an unknown family of the *Thermomicrobia*), two unknown families of the *Planctomycetes* and the *Spirochaetaceae* (Phylum: *Spirochaetes*) were significantly changed in relative abundance due to chitin addition (data not shown).

The relative abundance of 38 bacterial genera was significantly different between the rhizospheres of the two treatments, 18 genera represented more than 0.05% of the OTUs in one

TABLE 2 | Relative abundance (% of sequences) ± standard error of the five most dominant bacterial phyla in the lettuce rhizosphere grown for 55 days in the growth chamber in potting soil with and without 2% chitin after.

	Treatment	
	Control	**2% chitin**
Proteobacteria	47.04 ± 0.18	49.79 ± 0.36*
Bacteroidetes	10.78 ± 0.12	15.54 ± 0.19*
Verrucomicrobia	10.85 ± 0.20	7.71 ± 0.25*
Acidobacteria	7.11 ± 0.13	5.37 ± 0.13*
Actinobacteria	3.87 ± 0.04	4.82 ± 0.08*
Gram negative bacteria	68.67	73.04*
Gram positive bacteria	10.98	10.19
Total	79.65	83.24*

*Asterisk indicates a significant difference to the control ($P < 0.05$) by analysis of variance with $n = 5$. The phyla **in bold** have a significantly higher relative abundance in the chitin treatment as compared to the control, the underlined phyla have significantly lower relative abundance.*

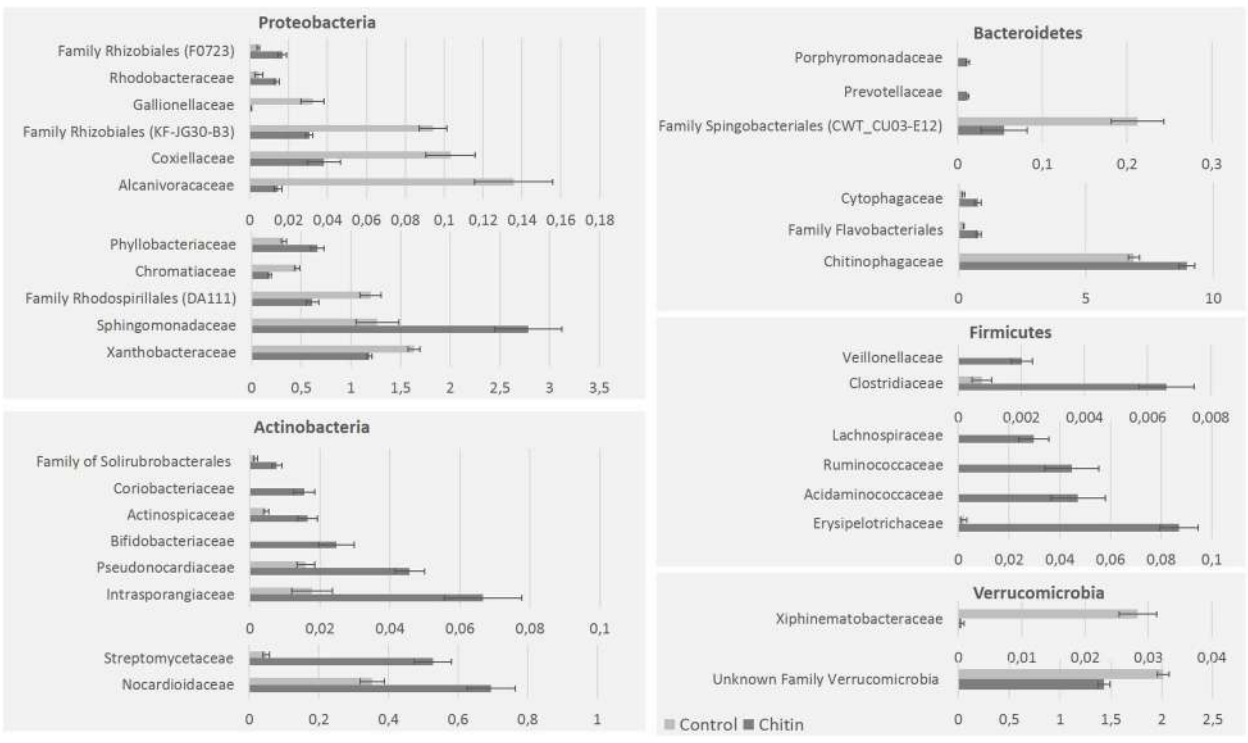

FIGURE 3 | Major bacterial taxonomical changes in the rhizosphere community after 2% chitin amendment to the potting soil. The graphics represent the significant differences (percentages) of representative families belonging to five major bacterial phyla in the lettuce rhizosphere due to the addition of chitin to the potting soil.

of the two treatments. These 18 genera are reported in **Table 3A**. Thirteen genera were significantly increased in the chitin treatment, including genera containing species that are reported to be involved in plant growth promotion, chitin degradation and biological control. Five genera, *Pseudolabrys*, *Alcanivorax, Candidatus solibacter, Nitrosococcus,* and *Aquicella* were significantly decreased.

Effect of Chitin Soil Amendment on the Fungal Lettuce Rhizosphere Using ITS2 Amplicon Sequencing

The fungal microbiomes present in rhizospheres of five plants that were grown in soil with or without chitin (10 rhizospheres of individual plants in total) were compared by ITS2 sequencing.

TABLE 3A | Significant differences in the relative abundance of bacterial genera (%) ± standard error between lettuce rhizospheres in potting soil with and without 2% chitin ($n = 5$) and the possible functions of species belonging to this genera reported in literature.

Phylum	Family	Genus	Treatment		Increase or decrease	Possible functions (reference)
			Control	2% chitin		
Proteobacteria	***Pseudomonadaceae***	***Cellvibrio***	0.09 ± 0.05	1.34 ± 0.26	**15x**	PGP, chitin degradation and N-cycle (Kolton et al., 2011; Anderson and Habiger, 2012; Suarez et al., 2014)
	Sphingomonadaceae	***Sphingomonas***	0.45 ± 0.06	1.02 ± 0.07	**2x**	PGP, chitin degradation and biocontrol (Zhu et al., 2007; Wachowska et al., 2013; van Bruggen et al., 2014)
	Sphingobacteriaceae	***Pedobacter***	0.02 ± 0.01	0.38 ± 0.09	**19x**	PGP and biocontrol (De Boer et al., 2007)
	Rhodospirillaceae	***Azospirillum***	0.03 ± 0.01	0.19 ± 0.04	**6x**	PGP and N-cycle (Saharan and Nehra, 2011)
		Dongia	0.72 ± 0.05	1.29 ± 0.06	**2x**	/
	Phyllobacteriaceae	***Nitratireductor***	0.16 ± 0.02	0.42 ± 0.05	**3x**	N-cycle (Penton et al., 2013)
	Bradyrhizobiaceae	***Afipia***	0.38 ± 0.02	0.58 ± 0.04	**2x**	/
	Coxiellaceae	*Aquicella*	0.10 ± 0.01	0.04 ± 0.01	0.4x	/
	Xanthobacteraceae	*Pseudolabrys*	1.53 ± 0.07	1.13 ± 0.03	0.7x	/
	Alcanivoracaceae	*Alcanivorax*	0.14 ± 0.02	0.01 ± 0.00	0.1x	/
	Chromatiaceae	*Nitrosococcus*	0.46 ± 0.03	0.18 ± 0.02	0.4x	N-cycle (Juretschko et al., 1998)
Bacteroidetes	***Cytophagaceae***	***Dyadobacter***	0.02 ± 0.0	0.33 ± 0.07	**16x**	/
	Chitinophagaceae	***Taibaiella***	0.30 ± 0.07	2.14 ± 0.42	**7x**	N-cycle (Zhang et al., 2013)
Nitrospirae	***Nitrospiraceae***	***Nitrospira***	0.24 ± 0.05	0.90 ± 0.10	**4x**	N-cycle (Kox and Jetten, 2015)
Actinobacteria	***Streptomycetaceae***	***Streptomyces***	0.05 ± 0.01	0.53 ± 0.06	**10x**	PGP, chitin degradation and biocontrol (Hjort et al., 2010; Saharan and Nehra, 2011)
	Nocardioidaceae	***Nocardioides***	0.11 ± 0.02	0.28 ± 0.04	**3x**	Biocontrol (Carrer et al., 2008)
Firmicutes	***Anaeroplasmataceae***	***Asteroleplasma***	0.00 ± 0.00	0.08 ± 0.01	–	/
Acidobacteria	*Solibacteraceae*	*Candidatus*	0.52 ± 0.03	0.17 ± 0.01	0.3x	/

PGP, Plant growth promotion. ***Bold*** *means a significantly higher relative abundance in the chitin treatment as compared to the control. Underlined means a significant decrease in the relative abundance in the chitin treatment as compared to the control.*

TABLE 3B | Significant differences in the relative abundance of fungal species (%) ± standard error between potting soil with and without 2% chitin ($n = 5$) and their possible functions reported in literature.

Phylum	Family	Genus	Treatment		Increase or decrease	Functions (reference)
			Control	2% chitin		
Ascomycota	**Cordycipitaceae**	***Lecanicillium***	0.09 ± 0.05	1.85 ± 0.33	**20x**	PGP, chitin degradation, biocontrol and induced resistance (Goettel et al., 2008; Hirano et al., 2008; Ownley et al., 2010; Van Nam et al., 2014; Nguyen et al., 2015)
	Pseudorotiaceae	***Pseudogymnoascus***	0.96 ± 0.30	3.46 ± 0.26	**4x**	Biocontrol (Tagawa et al., 2010)
	Pseudorotiaceae	*Pseudeurotium*	81 ± 0.42	0.12 ± 0.02	0.07x	/
Zygomycota	**Mortierellaceae**	***Mortierella***	3.21 ± 1.73	58.13 ± 2.55	**18x**	Chitin degradation (Kim et al., 2008) and biocontrol (Tagawa et al., 2010)

PGP, Plant growth promotion. Species ***in bold*** *mean a significant increase in the relative abundance in the chitin treatment as compared to the control treatment. Species underlined mean a significant decrease in the relative abundance in the chitin treatment as compared to the control treatment.*

After merging of the forward and reverse reads and quality filtering, 83.6% of the sequences were retained, resulting in an average of 50,045 sequences per sample. Rarefaction depth was reached at approximately 10,000 sequences (**Supplementary Figure S7**), indicating that enough sequence reads were generated. In total, around 21% of the sequences of the control and 17% of the sequences of the chitin amendment could not be assigned to a fungal phylum.

There were no significant differences in number of observed OTUs and Shannon–Wiener diversity indices between the control and the chitin amendment (298 ± 15 vs. 271 ± 11 and 4.81 ± 0.40 vs. 4.65 ± 0.10, respectively), indicating that the chitin treatment did not increase the fungal biodiversity. However, significant shifts in fungal composition (taxonomic groups) between the two treatments were observed (PERMANOVA, $P = 0.008$), illustrated by the OTU PCoA plot (**Supplementary Figure S8**). The first principal coordinate contains the major source of variability (64.8%) and reveals that the fungal rhizospheres of the chitin-grown plants are significantly different from the control plants. The second principal coordinate (18.8%) is highly reduced in the chitin treatment as compared to the control treatment. The fungal rhizosphere populations that developed in association with the plants grown in this chitin-amended soil cluster very tightly. It indicates that the chitin directs the fungal composition in a focused and consistent way and this is probably due to an high increase of the *Morteriella* species in the chitin treatment as compared to the control treatment (58.1% vs. 3.2%, **Table 3B**).

In total five fungal phyla were found across all samples, of which three phyla were significantly different between the two treatments: the *Ascomycota*, *Basidiomycota*, and the *Zygomycota* ($P < 0.05$, **Figure 4**). The *Zygomycota* were significantly increased in the chitin treatment, whereas the *Basidiomycota* and *Ascomycota* were significantly decreased.

On family level, chitin addition altered the relative abundance of 11 fungal families significantly, in particular seven families of the *Ascomycota*, two families of the *Zygomycota* and two families of the *Basidiomycota* (**Figure 5**). Especially the *Morteriellaceae* showed an high increase, due to the genus *Morteriella* that was strongly represented and is clearly promoted by the presence of chitin in the potting soil. Two other fungal genera of the phylum *Ascomycota* increased in relative abundance due to the chitin treatment: *Lecanicillium* and *Pseudogymnoascus*. Additionally, only one genus decreased significantly in relative abundance: *Pseudeurotium*. All genera induced by the chitin included species reported in literature to be involved in biocontrol and/or chitin degradation (**Table 3B**).

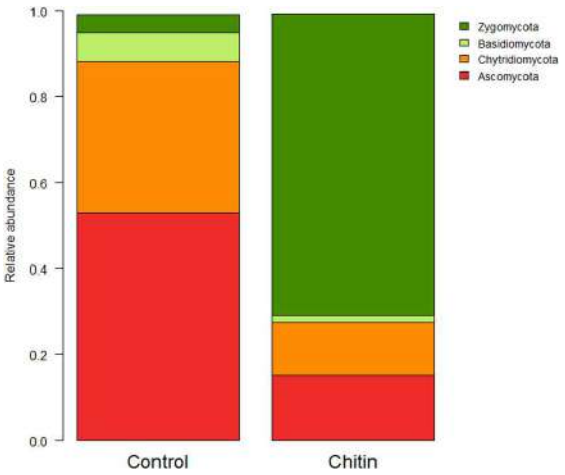

FIGURE 4 | Analysis of the fungal composition of the lettuce rhizosphere in unamended and chitin amended potting soil. Relative abundance (percentages) of the different fungal phyla (ITS2 region) in the lettuce rhizosphere. The Cercozoa group represents only a minor part of the total fungal community (control: 0.126 ± 0.076%, chitin: 0.004 ± 0.004%) and is therefore not illustrated in the bar chart.

DISCUSSION

Since farmers, consumers, and policy makers have become more aware of the impact of the use of chemical pesticides and fertilizers on human health and the environment, there is a renewed interest in the use of organic soil amendments to improve crop yield and plant resilience. It has been shown that the use of these soil amendments can have a positive influence on plant growth and development and on the suppression of plant diseases (e.g., Akhtar and Malik, 2000; Noble and Coventry, 2005; Postma and Schilder, 2015). Several studies have linked these beneficial effects to the influence of the soil amendment on the microbiome of the soil and rhizosphere of the plant. The addition of chitin for example increases the abundance of PGPR and PGPF in the soil and/or rhizosphere of the plant (Sarathchandra et al., 1996; Radwan et al., 2012; Cretoiu et al., 2013). Although chitin addition seems to control soil-borne pathogens and to enhance plant disease resistance, it was not known whether it also has an effect on the survival of human pathogens on the plant. Especially leafy vegetables are considered high risk food as they can carry human pathogens such as *E. coli* O157:H7 and *S. enterica* on their leaves (Van der Linden et al., 2013). In addition, it has been shown that biotic and abiotic stress have great influence on both the rhizosphere and phyllosphere microbiomes of lettuce (Williams et al., 2013; Williams and Marco, 2014; Erlacher et al., 2015). In the current study, we used lettuce plants grown in peat based-potting soil with and without chitin. We assessed the effect of chitin addition on (1) lettuce growth, (2) the survival of zoonotic pathogens on the lettuce leaves, and (3) rhizosphere microbial community.

Chitin addition to the soil significantly increased the fresh weight of the lettuce leaves by approximately 20%. This is in accordance to the study of Muymas et al. (2015). Chitin addition also significantly reduced the survival of *S. enterica* on the leaves. Although, not significantly in both independent experiments, also the survival of *E. coli* O157:H7 seemed to be negatively affected by the chitin amendment. The chitin soil amendment increased the absolute and relative abundance of several fungal and bacterial groups involved in plant growth promotion and

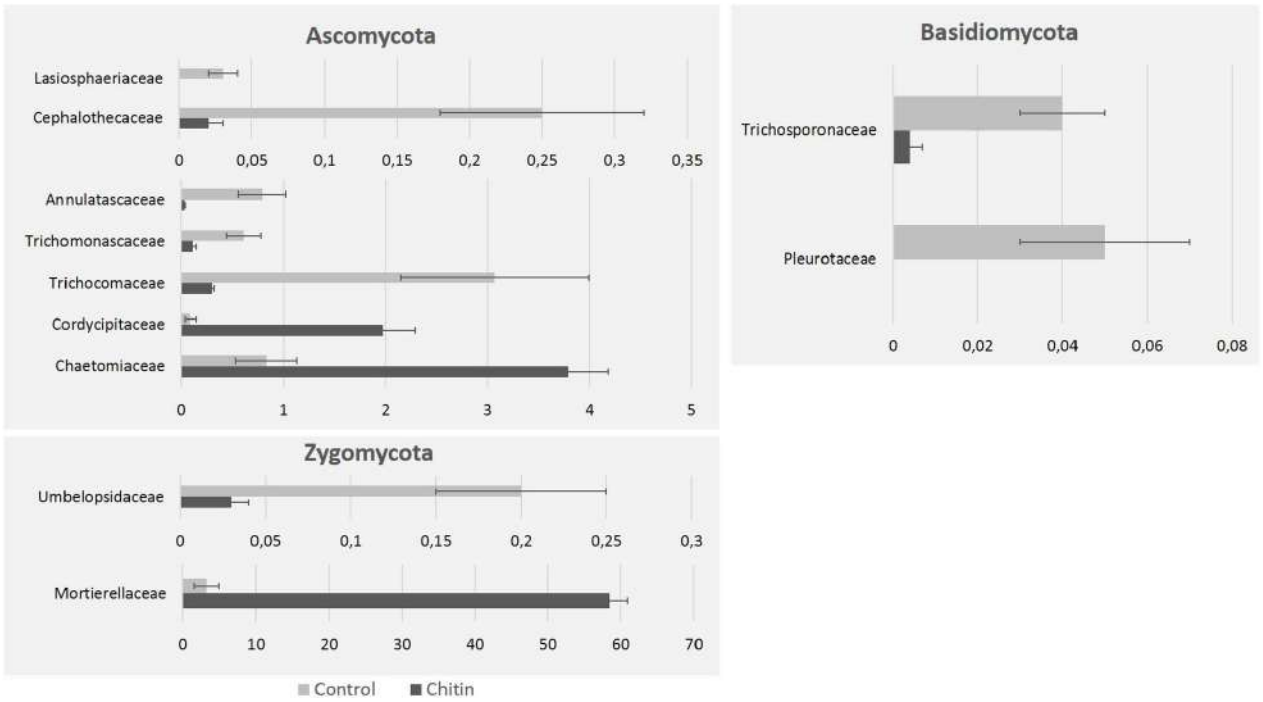

FIGURE 5 | Fungal taxonomical changes in the rhizosphere community after 2% chitin amendment to the potting soil. The graphics represent the significant differences in relative abundance of representative families in the lettuce rhizosphere, due to the addition of chitin to the potting soil. All families belong to the phyla Zygomycota, Ascomycota, and Basidiomycota.

in biological control. Since the roots are in direct contact with the soil, it is not surprising that a soil amendment such as chitin addition has an effect on the rhizosphere microbiome. It is less obvious, however, that it also has an effect on the survival of *Salmonella* on the leaves. It remains unclear what the exact mechanism is behind the decreased survival of *S. enterica* on the lettuce leaves. A range of beneficial agronomical responses can occur when chitin is added to the growing medium of plants (Sharp, 2013): (1) direct antibiosis against pests and pathogens of crops; (2) enhancement of beneficial microbes, both in plant defense and growth; (3) direct stimulation of plant defense responses against biotic stress; and (4) up-regulation of plant growth, development, nutrition and tolerance to abiotic stress. The three latter responses may explain our observed plant growth promotion effect and the reduced survival of *S. enterica* on the leaves, but only (2) has been measured in the current study using PLFA and amplicon sequencing. So, more research is needed to fully explain our observations. For example, next to an indirect effect via the rhizosphere microbiome, chitin can also act as a PAMP, directly triggering the immune system of the plant (de Jonge et al., 2010) and this may also explain the reduced colonization of *S. enterica* on the lettuce leaves. In addition, we also need to investigate which of the identified PGPR and PGPF can be responsible for the observed effects and what the underlying mechanism is. In accordance, a recent study showed that a PGPR bacterium (*Bacillus subtilis* UD1022) applied to the roots was able to influence the survival of human pathogens (*Listeria* and *Salmonella*) on leafy greens. This was correlated with an induction of the stomata closure by the *Bacillus* strain (Markland et al., 2015). *Bacillus subtilis* well-known PGPR effect is at least partly based on the production of surfactines, which induce plant immune system in a priming-like manner (Cawoy et al., 2014). In our study, no increase in the relative abundance of *Bacillus* species was seen, but other PGPR and PGPF were more than 10-fold increased after chitin addition, including bacterial species belonging the genera *Cellvibrio*, *Pedobacter*, *Dyadobacter,* and *Streptomyces* and fungal species belonging to the genera *Lecanicillium* and *Mortierella.* This confirms previous observations of De Boer et al. (1999), who showed that the rapid degradation of chitin in dune soils was most likely due to fast-growing *Mortierella* sp., whereas *Streptomyces* sp. and slow-growing fungal species (such as *Verticillium* sp, now partially re-classified as *Lecanicillium* sp.) were shown to be more involved in the degradation of chitin after prolonged incubation.

Our study addresses some limitations of previous studies and extends our knowledge about the effect of chitin on below ground microbiology because (1) rhizosphere samples were studied instead of bulk field soil; (2) both the fungal and bacterial community were assessed using Illumina sequencing; and (3) PLFA was used as an additional technique which allows quantification of microbial biomass. In our study, incorporating chitin in peat-based potting soil for almost 2 months significantly increased the relative abundance of the *Proteobacteria*, *Bacteroidetes,* and *Actinobacteria* in the rhizosphere, while those of the *Verrucomicrobia* and the

Acidobacteria were significantly decreased. This confirms previous results that describe an increase in the relative abundance of *Proteobacteria* (Jacquiod et al., 2013; Cretoiu et al., 2014), *Actinobacteria* (Jacquiod et al., 2013), and *Bacteroidetes* (Cretoiu et al., 2014) due to chitin amendment in field soil. PLFA analyses showed a twofold increase in both fungal and bacterial biomass in the rhizosphere due to chitin amendment. Cretoiu et al. (2013) however, showed a 10-fold increase in bacterial abundance, but a 10-fold decrease in fungal abundance in chitin-amended field soil compared to unamended field soil using qPCR. These comparisons show that the main trends at group or phylum level are similar, even though the experimental set-up differed (e.g., field soil vs. potting soil, soil vs. rhizosphere sampling). Based on our results and others, chitin addition thus gives reproducible shifts in microbial community even in very different soil systems. At lower taxonomic levels, differences are more common due to the specific niche of the rhizosphere, which is expected to be different from bulk soil (e.g., Lundberg et al., 2012; Peiffer et al., 2013). To the best of our knowledge, the presented study is the first study to use amplicon sequencing of the fungal ITS2 region to assess the effect of chitin soil amendment on the rhizosphere microbiome. We showed that addition of chitin to soil influenced the fungal composition of the rhizosphere, in which three major phyla shifted: an increase in the *Zygomycota* and a decrease in the relative abundance of the *Ascomycota* and *Basidiomycota.* We showed that the relative abundance of the fungal genera *Lecanicillium* and *Mortierella* was highly increased, both containing species involved in plant growth promotion, chitin degradation and biological control. Additionally, *Mortierella* sp. belonging to a the complex group of the *Mortierellales* (Wagner et al., 2013) might play an important component in the phosphorus cycling of the plant (Curlevski et al., 2010).

Both PLFA and amplicon sequencing was used for studying the rhizobiome of the lettuce plants. For both techniques: (1) different soil sampling was done and (2) different information was gathered. First, for the amplicon sequencing, 250 mg rhizosphere soil was taken as defined by Lundberg et al. (2012). Because of the amount of soil needed for PLFA analysis (6 g), it is impossible to do same soil sampling as for the amplicon sequencing. For this technique, 6 g of soil was taken from the pots. These pots were fully colonized by the lettuce roots (**Supplementary Figure S2**), so soil very close to the roots was taken and we believe that this can still be defined as rhizosphere soil. However, this is a very particular concept of rhizosphere, being very artificial. Due to this experimental restriction, the high root density could be very different from a natural situation, not only about the access of the chitin, but also about the microbiome present. Second, amplicon sequencing is known to give reliable information on microbial taxonomy, especially for higher order identification (Poretsky et al., 2014). Also information on species richness and diversity can be calculated. However, using the amplicon sequencing technique, the relative abundances are calculated and we do not have information on the real microbial biomass. PLFA analysis on the other hand provides complementary data on the total biomass and the biomass per microbial group. To make a comparison between the two techniques possible, the relative abundances of the PLFA biomarkers was also calculated, showing an increase in the relative abundance of the Gram negative-bacteria, similar as shown with the amplicon sequencing. This could not be confirmed for the Gram-positive bacteria, showing a decrease for PLFA analysis and no significant effect for the 16S rDNA amplicon sequencing.

CONCLUSION

In the current study, we demonstrated that the chitin soil amendment strategy which was previously known to be effective against plant pathogens (e.g., Dutta and Isaac, 1979; Hallmann et al., 1999; Cretoiu et al., 2013) also is able to control *Salmonella* on leafy greens. Chitin amendment in potting soil increased lettuce growth and had a decreasing effect on the survival of human pathogens on the leaves. These two effects were accompanied with changes in the rhizosphere microbiome. The observations that chitin soil amendment can increase plant yield, including lettuce, and can change soil and rhizosphere microbiology are not new, and our study confirms the results seen by other studies (e.g., Sarathchandra et al., 1996; Radwan et al., 2012; Cretoiu et al., 2013, 2014; Jacquiod et al., 2013; Muymas et al., 2015). This is, however, the first study to show that chitin soil amendment can have an effect on the survival of human pathogens on leafy vegetables. This addresses some of the knowledge gaps between food safety and plant sciences and similar studies combining both research fields are expected (Markland and Kniel, 2015; Melotto et al., 2015).

AUTHOR CONTRIBUTIONS

JD, SS, IVdL, BC, MH, and MM were involved in the design and the supervision of the experiments. JD wrote the first draft and finalized the manuscript. SS and CVM conducted the plant experiments and the bacterial counting. CDT and AH conducted the amplicon sequencing, the bio-informatics and statistical analysis of the NGS and PLFA data. All authors contributed to the writing of the manuscript and approved submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00565

FIGURE S1 |Two lettuce plants after 55 days of growth in the growth chamber (left = control, right = chitin treatment).

FIGURE S2 |Root development of two lettuce plants after 55 days of growth in the growth chamber (left = control, right = chitin treatment).

FIGURE S3 |*Escherichia coli* O157:H7 MB3885 dynamics on middle-aged lettuce leaves at 0, 4, and 8 days after spray inoculation analyzed by plating as described by Van der Linden et al. (2013). Full lines represent control plants, while dashed lines represent chitin treated plants. The data are calculated from the log-transformed values of the pathogen per gram tissue from two independent experiments ($n = 2$ plants or 6 leaves for day 0 and $n = 6$ plants or 18 leaves for day 4 and 8). Asterisk means significantly different between the chitin and the control treatment. Bars represent standard errors.

FIGURE S4 |Principal coordinate analysis (PCoA) of Bray–Curtis dissimilarity matrix calculated from the phospholipid fatty acids of chitin amended and unamended potting soil (= control) at the 55 days after planting. First PCoA axis represents 94.9% of the variability of the dataset, second axis 2.5%.

FIGURE S5 |Rarefaction curve of the 16S V3–V4 sequencing data for the rhizosphere of lettuce grown in unamended (= control) and chitin amended (= chitin) potting soil. Shown are the mean rarefaction curve for each treatment ($n = 5$) with standard error margins. Rarefaction depth for this study was set at 50,000 sequences as convergence seems to be reached for both treatments.

FIGURE S6 |Principal coordinate analysis profile of pairwise community dissimilarity (Bray–Curtis) indices of 16S sequencing data of the lettuce rhizosphere grown in chitin amended (yellow) and unamended (brown) potting soil. First and second axes represent 51.8 and 17.6% of the variance in the dataset respectively.

FIGURE S7 |Rarefaction curve of the ITS2 sequencing data for the rhizosphere of lettuce grown in unamended and chitin amended potting soil. Shown are the mean rarefaction curve for each treatment ($n = 5$) with standard error margins. Rarefaction depth for this study was set at 10,000 sequences as convergence seems to be reached for both treatments.

FIGURE S8 |Principal coordinate analysis profile of pairwise community dissimilarity (Bray–Curtis) indices of the ITS2 sequencing data of the lettuce rhizosphere grown in chitin amended (yellow) and unamended (brown) potting soil. First and second axes represent 64.8 and 18.8% of the variance in the dataset respectively.

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Microbial Profiling of a Suppressiveness-Induced Agricultural Soil Amended with Composted Almond Shells

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This study focused on the microbial profile present in an agricultural soil that becomes suppressive after the application of composted almond shells (AS) as organic amendments. For this purpose, we analyzed the functions and composition of the complex communities present in an experimental orchard of 40-year-old avocado trees, many of them historically amended with composted almond shells. The role of microbes in the suppression of *Rosellinia necatrix*, the causative agent of avocado white root rot, was determined after heat-treatment and complementation experiments with different types of soil. Bacterial and fungal profiles obtained from natural soil samples based on the 16S rRNA gene and ITS sequencing revealed slight differences among the amended (AS) and unamended (CT) soils. When the soil was under the influence of composted almond shells as organic amendments, an increase in *Proteobacteria* and *Ascomycota* groups was observed, as well as a reduction in *Acidobacteria* and *Mortierellales*. Complementary to these findings, functional analysis by GeoChip 4.6 confirmed these subtle differences, mainly present in the relative abundance of genes involved in the carbon cycle. Interestingly, a group of specific probes included in the "soil benefit" category was present only in AS-amended soils, corresponding to specific microorganisms previously described as potential biocontrol agents, such as *Pseudomonas* spp., *Burkholderia* spp., or *Actinobacteria*. Considering the results of both analyses, we determined that AS-amendments to the soil led to an increase in some orders of *Gammaproteobacteria*, *Betaproteobacteria,* and *Dothideomycetes*, as well as a reduction in the abundance of *Xylariales* fungi (where *R. necatrix* is allocated). The combination of microbial action and substrate properties of suppressiveness are discussed.

Keywords: soil, amendment, almond shells, microbial profiling, suppressiveness

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INTRODUCTION

The enhancement of soil suppressiveness using organic amendments has been widely described, especially for soil-borne diseases (Lazarovits et al., 2001; Bailey and Lazarovits, 2003; van Elsas and Postma, 2007; Bonilla et al., 2012b; Pane et al., 2013). However, this effect can be extremely variable depending on the pathosystem and the environmental conditions, and there are even some examples of the amendment application increasing disease incidence (Termorshuizen et al., 2006;

Janvier et al., 2007). The soils that become suppressive soils provide an environment in which plant disease development is reduced, even in the presence of a virulent pathogen and a susceptible host (Hadar and Papadopoulou, 2012). This phenomenon could be induced as a direct result of the activity of microorganism consortia that are naturally established on soil after application of the amendment (Weller et al., 2002).

As such, understanding the diversity, composition, structure, function and interactions of microbial communities is crucial to gain insight into the basis for suppressiveness mediated by this organic amendment (Janvier et al., 2007). Approaches for studying microbial communities in the soil are complex. Thus, employing genomic approaches to understand which changes occur in soil could be a good alternative strategy to decipher the profiling of soil microbiota (Garbeva et al., 2004).

The use of genomic techniques rely on PCR amplification of the conserved and variable regions of the microbial genome, commonly 16S ribosomal RNA (rRNA) for bacteria and 18S rRNA or internal transcribed sequences (ITS) for fungi, allowing for direct sequencing of these PCR amplicons using different high-throughput next-generation sequencing methods. Each group of PCR amplicons that shares a similar or identical variable region is considered an "operational taxonomic unit" (OTU) and is assumed to be equivalent to a microbial species or genus. The analysis of OTUs provide information about the phylogenetic diversity of the soil microbial community (van Elsas et al., 2007, 2008; Hirsch et al., 2013; Koyama et al., 2014).

Moreover, complementary techniques have arisen, such as microarrays, which have considerable potential in environmental microbial ecology, providing novel insights into how environmental factors affect microbial communities in various habitats (Hazen et al., 2010; He et al., 2012; Bai et al., 2013; Zhang et al., 2013; Tu et al., 2014). The GeoChip microarray is a comprehensive functional gene array (FGA) targeting hundreds to thousands of different gene families that play important roles in various biogeochemical processes, enabling researchers to comprehensively analyse the functional diversity, composition, and structure of microbial communities in various environments. It is a powerful FGA-based technology that can be used to survey the functional diversity, composition, structure, metabolic potential/activity, and dynamics of microbial communities, and then link them with ecosystem processes and functions (Xie et al., 2011; Xue et al., 2013; Cong et al., 2015).

Our research interest is focused on the avocado (*Persea americana* Mill.), for which southern Spain is one of the most relevant zones in the Mediterranean area for this crop. In this part of the world, one of the most limiting soilborne diseases affecting avocado trees is white root rot, caused by the fungus *R. necatrix* Prill. White root rot is considered to be an emergent threat to many woody crop plants worldwide (Pliego et al., 2009, 2012).

The role of soil microorganisms in the plant protection have been broadly reported. Thus, different microbes can contribute to the biocontrol of avocado white root rot using different weaponry such as antagonism (*Pseudomonas chlororaphis* PCL1606 or *Bacillus subtilis* PCL1608; Cazorla et al., 2006, 2007), competition for niches and nutrients (Calderón et al., 2014), or induction of systemic resistance or predation (*Trichoderma* spp.; Ruano-Rosa and López-Herrera, 2009). These microorganisms can act as single or combined with other biocontrol agents against *R. necatrix* (Ruano-Rosa et al., 2014). Other studies have reported the positive effect of the application of arbuscular mycorrhizal fungi to soil and the biocontrol activity on avocado (Hass and Menge, 1990; González-Cortés et al., 2012).

During the past decades, several approaches have been implemented to achieve an integrated management of *R. necatrix,* including physical, chemical and biological control approaches (López-Herrera et al., 1998; López-Herrera and Zea-Bonilla, 2007; González-Sánchez et al., 2013). All of these approaches seem to be effective at the experimental level, and some of them have been proven to be effective under certain conditions. However, at the same time, traditional strategies of land management have improved, and some of these strategies could be considered useful approaches to fight against diseases in avocado management, thus increasing the weaponry available against white root rot (Bonilla et al., 2012b).

One of these approaches is the use of organic amendments or mulches, which have produced beneficial effects for plants, including increasing health and yields in avocado crops (Moore-Gordon et al., 1997; Wolstenholme et al., 1997; Hermoso et al., 2011). It has been previously shown that the application of such organic matter to avocado agricultural soil can affect soil physicochemical properties and microbial communities (Bonilla et al., 2012a; López et al., 2014). Additionally, organic amendments could play a critical role in global biochemical cycles (Bonanomi et al., 2014) and could cause different effects, such as the improvement of soil fertility and the enhancement of natural suppressiveness of the soil against several phytopathogens (Cretoiu et al., 2013). Several organic amendments have shown an obvious suppressive effect against another important avocado soil-borne phytopathogen, *Phytophthora cinnamomi* (Bender et al., 1992; Downer et al., 2001).

In a previous study, it was shown that different organic matter applied as a mulch to the avocado crop exhibited suppressive effects against white root rot (Bonilla et al., 2015). Composted almond shells were one type of organic matter tested. The application of composted almond shells as a mulch led to an enhancement of the bacterial composition and activities of the soil communities in relation to the observed suppressiveness (Bonilla et al., 2015).

The objective of the present study was to gain insight into the microbial profiling present in the amended soils showing suppressive ability against the avocado soil-borne phytopathogen *R. necatrix*. The use of different microbial approaches should uncover the microbial communities potentially involved in the suppressive phenotype.

MATERIALS AND METHODS

Field of Study

Soil samples were obtained from an avocado crop field (cv. *Hass* avocado trees grafted onto cv. *Topa-Topa* seedling rootstocks) located at the Experimental Station "La Mayora"

(IHSM-UMA-CSIC, Málaga, Spain) on the coast of the Malaga Province (SE Spain). This experimental field of 2.5 km^2 (36°75′N, 4°04′O) contains 195 40-year-old avocado trees planted at 8 × 8 m. Selected avocado trees were grouped in pairs to facilitate their management. Sixteen pairs of trees were under ecological management (massive application of composted almond shells in 2002, 2007, and 2012), and another 16 pairs of trees were under conventional management (addition of mineral nutrients twice per year, as well as the application of herbicides and pesticides when necessary, López et al., 2014) and without any organic amendment.

Soil Sampling

Natural field soil samples allocated underneath of avocado trees unamended (CT) or amended with composted almond shells (AS) were taken to perform the different experiments. Soil samples were collected in April 2013, November 2013 and April 2014. Composite soil samples were taken from four different groups of paired trees with (AS) or without (CT) organic amendment and were randomly selected from throughout the avocado orchard. To obtain a composite soil sample, two sampling distal points at 1.5 m around the trunk base for each tree of a pair of trees under the same treatment were selected; the upper layer of compost was carefully removed, and 5–10 kg of soil samples (15 cm depth) were collected per pair of trees and merged. Samples were placed in cold storage and transported to the laboratory. Samples of each type of soil were sieved through a 20 mm mesh and immediately used for physicochemical and suppressiveness experiments. To perform DNA extractions, three soil samples (1 g each) from composite soil samples per each pair of trees were sieved again (2 mm diameter) and processed independently. The remaining unused soil samples were stored at −80°C.

Physicochemical Analysis of Soil Samples

Physicochemical analysis of both types of soil samples were performed at Laboratorio Caisur S.L. (Granada, Spain) using standardized methodologies. Four samples from each composite field soil sample (AS and CT) were analyzed independently.

Soil Processing

To test the potential role of soil microorganisms in suppressiveness, we prepared three types of processed soils using different treatments: Field soils (raw soils), heat-treated soils, and complemented soils (**Table 1**). We applied a moist heat treatment to the field soil samples as previously described (Weller et al., 2002), with slight modifications. Briefly, the heat treatment consisted of heating the soil in high moisture conditions at 100°C for 20 min in an autoclave. The soil was allowed to recover at 4°C overnight. Then, we performed a second treatment step, heating the soil at 100°C for 10 min in high moisture conditions. After allowing it to cool, the soil was ready to be used (**Figure 1**). Complemented soils were prepared with the purpose of observing the partial recovery of the microbial characteristics of the natural soil (Weller et al., 2002). The complemented soil consisted of heat-treated soil mixed with natural raw field soil in a 9:1 (w/w) ratio (**Table 1**).

TABLE 1 | Types of processed agricultural soils used in this study.

Soil source	Treatment code	Details of processed soils
Amended with composted almond shells	AS	Natural field soil amended with composted almond shells mulching
	ASt	AS heat-treated soil
	ASc	ASt complemented with AS in 9:1 (w/w) ratio
	ASt+CT	ASt complemented with CT in 9:1 (w/w) ratio
Unamended and under conventional management	CT	Natural field soil unamended and under conventional management
	CTt	CT heat-treated soil
	CTc	CTt complemented with CT in 9:1 (w/w) ratio
	CTt+AS	CTt complemented with AS in 9:1 (w/w) ratio

*A scheme of the processing is described in **Figure 1**.*

To evaluate changes in the culturable microbiota fraction during different times of the soil sample processing, counts of cultivable colony forming units (CFUs) of bacteria and fungi per gram of soil were performed. For this, 2 g samples of soil obtained at the different key times during the process were suspended in 20 ml of sterile saline solution (0.85% NaCl) with 0.5 g of sterile gravel and mixed at 150 rpm for 30 min on an orbital shaker at room temperature. Ten-fold serial dilutions of the obtained suspensions were plated on Luria Bertani (LB) agar with 100 mg of cycloheximide per liter, to analyse the heterotrophic bacteria group, and on potato dextrose agar (PDA) with 50 mg of chlortetracycline and 1 ml of tergitol NP-10 (Sigma) per liter (Bonilla et al., 2012a).

Suppressiveness Assays

Suppressiveness assays against white root rot caused by the virulent strain *R. necatrix* CH53 (López-Herrera and Zea-Bonilla, 2007) were conducted using two different susceptible pathosystems, avocado (Cazorla et al., 2006) and wheat (*Triticum aestivum*). The *R. necatrix* inoculum was produced on wheat seeds (Freeman et al., 1986). The seeds were soaked for 12 h in 250-ml Erlenmeyer flasks filled with distilled water. The flasks were autoclaved after excess water had been drained off. After sterilization, fungal disks of a 1-week-old culture of *R. necatrix* grown on PDA were placed aseptically in each flask. Flasks were incubated at 25°C for 2–3 weeks and were shaken every 2–3 days to avoid clustering of the seeds.

Avocado/*R. necatrix* Test System

Six-month-old commercial avocado plants were obtained from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain). The roots from the avocado plants were disinfected by immersion in 0.1% NaOCl for 20 min and then washed twice (20 min) with sterile distilled water. Then, avocado plants were placed into square plastic pots (10.5 × 10.5 × 10.5 cm) containing 0.64 L of the

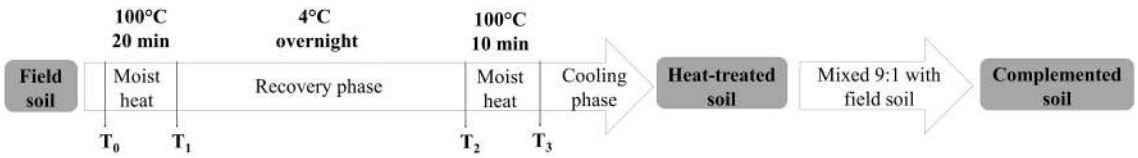

FIGURE 1 | Processing scheme of the soil heat-treatment and complementation used in this study for the agricultural field soil samples. The same procedure was followed for both unamended soil and soil amended with composted almond shells. $T_{0–3}$ indicates sampling points to perform bacterial and fungal plate counts.

sieved CT and AS types of soils. Fungal infection with *R. necatrix* was performed using wheat grains (4 holes of 2 cm depth were made per pot, 3 infected wheat grains were placed per hole) as previously described (Freeman et al., 1986). Non-infected plants were used as controls. Three sets of 15 avocado plants were tested per type of soil. The plants were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight and were watered twice per week. Aerial symptoms of avocado white root rot were recorded on a scale of 0–3, and a disease index (DI) was calculated after 5 weeks using the previously described formula (Cazorla et al., 2006).

Wheat/*R. necatrix* Test System

Wheat seeds were disinfected by immersion in 0.05% NaOCl for 10 min, washed and then placed in darkness between pieces of moist filter paper in a growth chamber for 2–3 days at 25°C to induce germination. Then, germinated seedlings were disinfected again by immersion in 0.1% NaOCl for 20 min and washed (20 min) with sterile distilled water. Seedlings were placed into plastic seedling trays (5 cm diameter × 5.5 cm) containing 0.08 L of different types of soils and either infected with *R. necatrix* using wheat grains (three grains per slot) or not infected to be used as controls. Three sets of 50 wheat seedlings were tested per type of soil. The seedlings were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight and were watered twice per week. Aerial symptoms were evaluated, and the disease index percentage was calculated as previously described for the avocado/*R. necatrix* system (Cazorla et al., 2006). Disease index percentage was recorded after evaluation of symptoms, with values ranging between 0 (healthy plant), 1 (yellowing stem base), 2 (drying stem base), and 3 (dead plant). The number of diseased seedlings was determined 7 weeks after beginning the assay, and the disease index was calculated as previously described (Cazorla et al., 2006).

Soil DNA Extraction

Soil DNA extraction was performed using 1.0 g of soil samples and a PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc, Carlsbad, CA, USA). DNA was extracted from three independent soil samples per pair of trees for amended and unamended soil (AS and CT) and checked for quality. To test the DNA quality we performed a DNA digestion using the restriction enzyme *Eco*RI (New England BioLabs®, Inc., Ipswich, MA, UK) and PCR amplification of the variable region of the bacterial 16S rDNA with the universal bacterial primers 341F and 907R as described by Muyzer et al. (2004). Digestion and PCR products were analyzed for size by agarose gel electrophoresis and ethidium bromide staining. Suitable samples were mixed and DNA quantity and quality ($A_{260}/A_{230} > 1.8$ and $A_{260}/A_{280} > 1.7$) were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Three independent DNA extractions were performed per each pair of trees, and then merged to create a composite DNA sample. Three of these composite DNA extractions were independently analyzed for each type of field soil (AS and CT). DNA was stored at −20°C for further analyses.

Analysis of 16S rRNA and its Gene Sequence

Two composite DNA samples from each soil type were sent for sequencing by STAB VIDA (NGS Laboratories, Caparica, Portugal) and sent to ChunLab (Seoul, Korea) to obtain the microbial DNA sequences of the 16S rRNA gene and ITS hypervariable regions. Sequences were analyzed using QIIME software (Caporaso et al., 2010) and CLcommunity™ software (ChunLab). Sequences of a length less than 200 nt were excluded from the analysis. The data were filtered for noisy sequences, checked for the presence of chimeras, and binned into OTUs (Peiffer et al., 2013) at the 97% sequence similarity level. A representative sequence of each OTU was taxonomically classified. The relative abundance of microbial clades at different taxonomic levels was calculated as the average value from two independent analyses and was used to perform the comparative distribution analysis.

Geochip Analysis

Three of the composite samples of purified test DNA (800 ng per sample) from the two different types of soils studied (AS and CT) were sent to Glomics Inc (Norman, Oklahoma) for the sequencing analysis (Tu et al., 2014). Briefly, after the hybridization steps, the arrays were washing, dried and then scanned. The images obtained were analyzed by NimbleScan software (Roche NimbleGen Inc., Madison, WI) using the gridding file containing GeoChip 4.6 probes and NimbleGen control probes to determine the intensity of each spot and to identify low quality spots, which were removed prior to statistical analysis (probe spots with coefficient of variance > 0.8 were removed). Extracted data were then loaded into the GeoChip data analysis pipeline at the Institute for Environmental Genomics (Microarray Data Manager, http://ieg.ou.edu/microarray/; Liang et al., 2010; Deng and Zhou, 2013). First, the average signal

intensity of the common oligo reference standard (CORS) was calculated for each array, and the maximum average value was applied to normalize the signal intensity of samples in each array. Second, the sum of the signal intensity of the samples was calculated for each array, and the maximum sum value was applied to normalize the signal intensity of all of the spots on an array, which produced a normalized value for each spot in each array. Spots were scored as positive based on a floating signal-to-noise ratio [SNR = (signal mean–background mean)/background standard deviation] so that hyperthermophile control probes accounted for 5% of positive probes. Spots that were detected in less than two samples were also removed. Before statistical analysis, logarithmic transformation was carried out for the remaining spots, and the signals of all spots were transformed into relative abundances (the sum of the number of hybridized probes for each gene category or gene function between the number of total detected probes).

Data processing was used for further analyses. Genes that overlapped between treatments (AS and CT) were calculated by dividing the number of overlapped genes between the treatments by the number of all genes detected in both treatments. Gene function diversity was calculated using the Shannon-Weiner index (H', alpha diversity) and Simpson's index (1/D, beta diversity). We performed a detrended correspondence analysis (DCA) to measure the differences of community functional gene structure between treatments. For comparing the different gene function communities, a hierarchical clustering analysis using Bray-Curtis distances was also performed. To analyse the unique detected probes in the AS samples, we performed a Venn diagram analysis using an on-line tool (http://bioinfogp.cnb.csic.es/tools/venny/). Previously, we prepared two databases by selecting genes (probes) that hybridize exclusively in each type of soil and compared them. This website provided us with a list of 2766 AS unique detected sequences from suppressive soil, which were selected to perform specific comparative analysis.

Statistical Methods

For suppressive analysis, the data were statistically analyzed using an analysis of variance (Sokal and Rohlf, 1986), followed by Fisher's least significant difference test ($P = 0.05$) using SPSS 22 software (SPSS Inc., Chicago). For GeoChip 4.6 analysis, significant differences in relative abundances of the microbial gene diversity between different soils were analyzed by an unpaired Student's *t*-test. A significance level of $P < 0.1$ was adopted for all comparisons. Based on the standard error, the 95% confident interval for each response variable was obtained and the significant differences between the soils were estimated.

RESULTS

Characteristics of Avocado Field Soils

The soil samples were taken from the same avocado orchard but from trees under different soil management (AS-amended or unamended). Soil characteristics of the experimental avocado field revealed sandy-loam textures for the amended (AS) and unamended (CT) soils. The pH was not substantially different among these samples and ranged from 7.20 to 7.55 (nearly neutral pH). Some macro- and micro-nutrients, such as potassium, iron and manganese, were also increased in the AS-amended soil (data not shown).

White Root Rot Suppressiveness Assay

Suppressiveness assays against white root rot were performed using the avocado/*R. necatrix* and the wheat/*R. necatrix* experimental plant test systems. AS-amended and unamended avocado agricultural soils, after different experimental heat treatments an complementantions were used (**Figure 1**; **Table 1**).

Bacterial and fungal counts of AS-amended and CT soil were very similar, with values of 6.5 and 6.6 $\log_{10}$ bacterial cfu/g, respectively, and 5.0 and 5.1 $\log_{10}$ fungal cfu/g, respectively. After the heat treatment of the soil, bacterial counts decreased and stabilized, without any further changes after a second heat treatment in any type of soil (**Table 2**). There were no differences in the results obtained for fungal count (**Table 2**).

For avocado/*Rosellinia* test system, the disease incidence was evaluated after 5 weeks and at the end of the assay, and the disease index (DI) was calculated (**Figure 2A**). In these studies, AS field soil samples displayed better suppressive ability than CT field soil samples. Plants growing in the presence of AS-amended soil samples displayed a significantly lower DI than plants cultivated in the presence of CT soil samples at the end of the experiment (**Figure 2A**). The disease suppressiveness activity was reduced when AS soil samples were heat-treated (ASt) but showed no changes in CTt soil. Moreover, suppressiveness was complemented by soils ASc and CTt+AS, when incorporating AS soil samples. Complemented soil ASt+CT and CTc did not have a disease-suppressive ability, with levels resembling those for the heat-treated unamended soil (**Figure 2A**).

For the wheat/*R. necatrix* plant test system, disease incidence was tested 7 weeks after inoculation when the disease index (DI) was calculated (**Figure 2B**). Similar to the results shown by the avocado/*R. necatrix* test system, the AS-amended soil exhibited better suppressive ability than CT soil. The suppressiveness phenotype was significantly lost in heat-treated soils (ASt and CTt) and was partially recovered when we used amended field soil to complement (ASc and CTt+AS). The soils complemented with unamended soil, CTt and ASt+CT, had a disease-suppressive ability similar to that of heat-treated unamended soil (**Figure 2B**).

TABLE 2 | Plate counts of total heterotrophic bacteria and fungi during the soil heat-treatment of the unamended and amended with composted almond shells.

Plate counts of	Soil source sample	Sampling points during the heat-treatment process			
		T_0	T_1	T_2	T_3
Heterotrophic bacteria	AS	6.5 ± 0.48	5.9 ± 0.76	6.0 ± 0.42	5.9 ± 0.59
	CT	6.6 ± 0.30	5.9 ± 0.64	5.9 ± 0.30	5.7 ± 0.64
Heterotrophic fungi	AS	5.0 ± 0.90	4.7 ± 0.67	4.9 ± 0.57	4.7 ± 0.60
	CT	5.1 ± 0.98	4.9 ± 0.55	5.0 ± 0.67	4.8 ± 0.87

$T_{0–3}$ indicates sampling points used along the process. Microbial counts data are presented as log10 cfu/g soil ± standard deviation.

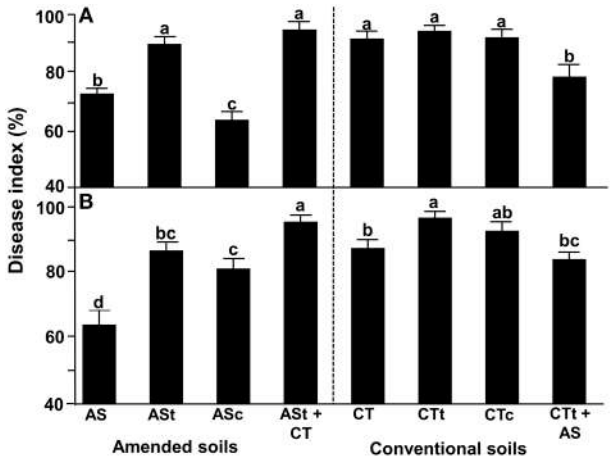

FIGURE 2 | Suppressiveness assays using the avocado/*R. necatrix* (A) and wheat/*R. necatrix* (B) test systems. AS, agricultural field soil amended with composted almond shells; ASt, AS heat-treated soil; ASc, ASt complemented with AS in 9:1 (w/w) ratio; ASt+CT, ASt complemented with CT in 9:1 (w/w) ratio; CT, Agricultural field soil under conventional management; CTt, CT heat-treated soil; CTc, CTt complemented with CT in 9:1 (w/w) ratio; CTt+AS, CTt complemented with AS in 9:1 (w/w) ratio. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant difference test ($P = 0.05$). Values of bars with different letters indications denote a statistically significant difference.

Characterization of the Soil Microbial Community Based on 16S rRNA Gene and its Sequencing

DNA profiling approaches and the sequencing of 16S rRNA and the ITS variable regions of extracted and mixed DNA revealed the relative abundances of microbial clades at different taxonomic levels. However, only the most abundant OTUs were quantified with a level of precision sufficient to perform the comparative distribution analysis due to the high level of OTU richness.

In both samples, *Archaea* were found in a very low relative abundance (<0.1%). Thus, the bacterial 16S rRNA gene sequences allowed us to identify 33 different representative phyla in AS soil samples and 26 phyla in CT soil samples, from which 5 and 7 phyla comprise more than 1% of the community in AS and CT, respectively (**Figure 3**).

In AS soil samples, the 5 most abundant phyla (above 89% of relative abundance) were *Proteobacteria* (50.08%), *Acidobacteria* (22.64%), *Bacteroidetes* (8.05%), *Planctomycetes* (4.27%), and *Actinobacteria* (4.09%). In contrast, the analysis of CT soil samples revealed that the most abundant (representing above 95%) phyla were *Proteobacteria* (45.48%), *Acidobacteria* (27.39%), *Bacteroidetes* (8.79%), *Planctomycetes* (60.99%), *Actinobacteria* (3.19%), *Nitrospirae* (1.70%), and *Gemmatimonadetes* (1.63%).

At the class level, the AS soils presented a high abundance of uncultured bacteria from the groups of *Acidobacteria* (EU686603, 18.44%), *Gammaproteobacteria* (17.85%), *Alphaproteobacteria* (15.28%), and *Betaproteobacteria* (11.4%) (**Figure 3**). In CT soil samples, the class analysis resulted in a similar representation of class abundance, including uncultured bacteria EU686603 (22.99%), *Alphaproteobacteria* (17.7%), and *Gammaproteobacteria* (10.7%).

In both soil samples, the phylum *Proteobacteria* is the most abundant (50.08 and 45.48%). Differences in this group have been shown between the two soil samples. In general, diversity is higher in AS soil samples that exhibit a predominance of the classes *Gammaproteobacteria* (36%) and *Alphaproteobacteria* (30%) and a low percentage of *Deltaproteobacteria*. In CT soil samples, a clear predominance of *Alphaproteobacteria* can be observed (39%). Remarkably, we observed an increase in AS soil samples (almost 2x) of the orders *Steroidobacter* (28%) and *Burkholderiales* (13%) and the decrease of *Rhodospirales* (from 18% in CT to 8% in AS) (Figure S1A).

We observed 76 different classes in AS soil samples and 65 classes in CT soil samples. We detected 24 and 13 specific bacterial classes in AS and CT, respectively, and a slightly higher richness in AS samples (Figure S2A).

The analysis of ITS sequences to reveal the abundance of eukaryotic microbes allowed us to identify a high abundance of fungal microbes. Eukaryotic microbes different from fungi ranged from 7.97% (AS) to 9.52 (CT). Among the fungi detected, the unclassified fungi comprises 8.04% (AS) and 4.28% (CT), and those below 1% represent 2.9% in CT soil samples and 3.4% in AS soil samples.

The most abundant fungal groups (approximately 70%) that are in both soil samples are of the phyla *Ascomycota* and *Basidiomycota* and of the group *Mortierellales*. In AS soil samples, an increase in the relative abundance of *Ascomycota* can be observed (**Figure 4**), (35.37% in CT and 45.79% in AS), as well as a reduction in the group of *Mortierellales* (18.37 in CT and 9.92% in AS).

The analysis of the most abundant group of microorganisms (*Ascomycota*) revealed that in AS soil samples an increase of the class of *Dothideomycetes* (from 40% in CT to 54% in AS) was observed. Additionally, a reduction of the class of *Sordariomycetes* (from 38% in CT to 29% in AS) was observed. Also of note in reference to fungal order in AS soil samples, a huge increase of *Pleosporales* (from 16% in CT to 48% in AS) was observed. Remarkably, one of the fungal order that decreased in AS soil samples was the order *Xylariales* (from 8% in CT to 3% in AS), where the pathogen *R. necatrix* is allocated (Figure S1B).

We observed 39 different classes in AS soil and 50 classes in CT soil. We detected 7 and 18 specific bacterial classes in AS and CT soil, respectively, and observed a slightly higher richness in CT samples (Figure S2B).

GeoChip Analysis in Soil Samples

The number of total genes detected by GeoChip analysis and overlapping genes between treatments were measured to understand the functional diversity and structure of the microbial communities. The number of total genes detected ranged from 27348 to 28491 and from 29311 to 33526 in AS and CT samples, respectively. An unpaired Student's *t*-test showed that these values were significantly different. The percentage of overlapping genes between samples ranged from 77.18% for AS (77.41, 75.25, and 78.88%) to 73.16% for CT (76.25, 65.70, and 77.52%) (Figure

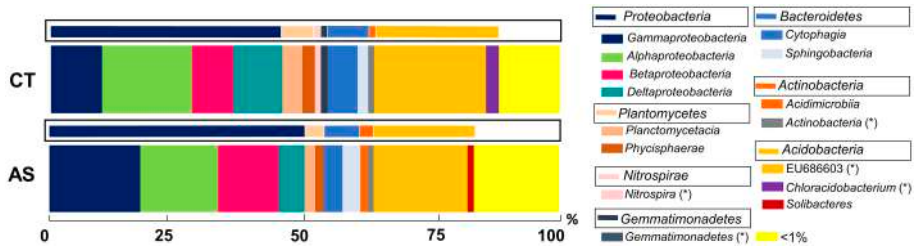

FIGURE 3 | Analysis of microbial communities present in field soil samples unamended (CT) and amended with composted almond shells (AS). Relative abundance (percentage) of different prokaryotic groups detected by 16S rRNA gene sequencing analysis of soil DNA. Analysis of microbial groups are marked at the class level (thick bars) and the phylum level (boxed thin bars). < 1%, sum of all detected groups with a relative abundance less than 1%. *Taxonomic characteristics of these groups are uncertain.

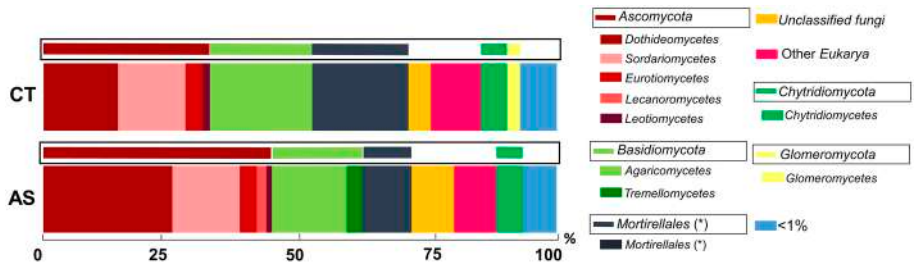

FIGURE 4 | Analysis of microbial communities present in field soil samples unamended (CT) and amended with composted almond shells (AS). Relative abundance (percentage) of different eukaryotic groups detected by ITS region sequence analysis of soil DNA. Analysis of microbial groups are marked at the class level (thick bars) and at phylum level (boxed thin bars). < 1%, sum of all detected groups with a relative abundance less than 1%. *Taxonomic characteristics of these groups are uncertain.

S3). This value fell to 65.43% when we compared overlapping genes between treatments (AS_{1-3} and CT_{1-3}). DCA (detrended correspondence analysis) and hierarchical clustering (with Bray-Curtis distance) were performed (Figure S3) using all of the detected genes, showing that functional structure of the microbial community was similar in the replicates but different among the soils (AS and CT).

To understand the effects of composted almond shells on the microbial communities and the acquired suppressive capacity, microbial functional genes categorized as participating in biogeochemical cycles and other important soil processes were examined (**Figure 5**). Gene functions related to the carbon cycle were the gene category most represented in all samples. C cycling probes were significantly more abundant than other categories in AS samples (36.65% in AS and 34.54% in CT), whereas genes related to organic contaminant degradation (12.42% in AS and 12.81% in CT), metal resistance (14.58% in AS and 16.32 in CT) and virulence (1.59% in AS and 1.61% in CT) were significantly more abundant in CT samples. There were no significant differences in N, P, and S cycle genes and other gene categories such as stress, fungi functions, soil benefit and soil borne pathogens (**Figure 5**).

Key genes for acetogenesis, C degradation, C fixation, methane metabolism, and other genes related to the C cycle were detected in the two types of soils (Figure S4A). The relative abundance of genes related to the C degradation category were the highest and exhibited significant differences between the AS samples and the CT samples. In this category, we found the presence of degradative genes of the most abundant C sources derived from plant and animal sources that could be present in soil ecosystems, such as starch, hemicellulose, cellulose, chitin, and lignin. There were few significant differences between samples in these categories of detected genes (Figure S4A).

Of the nitrogen cycle category, only the ammonification subcategory had a higher significant difference for amended soil (Figure S4B). In this subcategory, there are genes that function in the decomposition of organic matter and cycling of accumulated N.

Related to the sulfur cycle, the analyses performed exhibited a higher significant difference ($P < 0.1$) in only the sulphite reductase genes of AS samples compared to CT samples. These genes encode enzymes that catalyze the reduction of sulphite to sulfide, using iron as cofactor, and provide a source of S to microbiota. The CT samples exhibited a higher significant difference in sulfate reductase, a protein involved in sulfur reduction by anaerobic respiration (Figure S4C).

Statistical analyses showed no significant differences in the relative abundance of genes involved in the phosphorous cycle for these samples.

The analysis of genes in the category of environmental adaptability showed significant differences ($P < 0.1$) in the subcategories, as shown in Figures S4D–F. Genes involved in

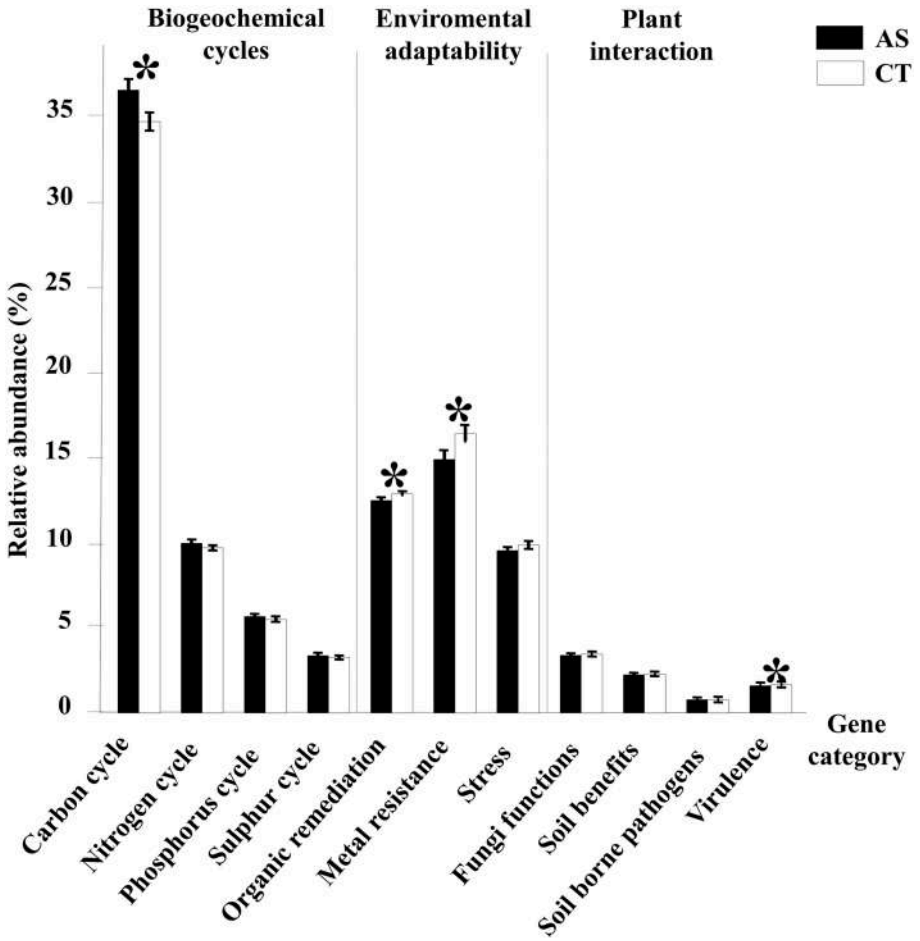

FIGURE 5 | GeoChip analysis of functional gene categories. Relative abundance of all detected genes from different gene categories analyzed in this study. *Indicates significant statistical differences ($p < 0.1$) between the two types of soil samples, amended soil (AS) and conventionally managed soil (CT). Standard error bars are shown.

the organic degradation of aromatics, such as chlorinated and pesticide-related compounds, had a higher significant relative abundance for amended soil than conventional managed soil. Similar results were obtained for genes related to osmotic and oxygen stress, from the stress category, and metal resistance to cobalt and lead, which had slightly higher significant relative abundance for AS samples than CT samples. On the other hand, unamended soils exhibited significantly higher values of relative abundance for genes related with stress induced by glucose limitation and metal resistance to cadmium and other metals.

The category of plant interaction covers a wide range of different functional genes involved in microbial interactions with plants, including genes related to fungal function, soil benefit, soil borne pathogens, and virulence. The analyses performed showed significant differences ($P < 0.1$) in some subcategories, as shown in Figures S4G–J. There were not any significant differences in the genes in the categories of soil benefit or fungi function. Nevertheless, CT samples exhibited a higher significant relative abundance of detected genes from the oomycetes subcategory (soil borne pathogen), which included different genes from this pathogenic group. Genes related to virulence processes such as iron oxidation or secretion had a higher significant relative abundance for amended soils; whereas unamended soils exhibited significantly higher values for genes involved in virulence actions such as iron uptake (aerobactin genes) and pilin formation.

Unique DNA Probes Detected in as Suppressive Soil Samples

Results of the GeoChip analysis and the Venn diagram representation allowed us to determine microbial specific gene functions detected exclusively in each treatment and the number of commonly detected probes (27364) (**Figure 6A**). We found 6674 unique detected probes in CT samples and 2766 unique detected probes in AS samples (approximately 10% of the total AS detected genes) from the gene categories analyzed. Approximately 34.49% of the unique hybridizations were related to the Carbon cycle category (**Figure 6B**), mainly to starch and chitin degradation (Table S1). The Organic remediation gene category exhibited 14.53% unique hybridizations of genes related

to the degradation of aromatic compounds. The Stress category had 13.38% unique hybridized probes and the Metal resistance category had 11.86% unique hybridized probes. The Nitrogen cycle category exhibited 8.57% unique hybridized probes, mostly in genes related with denitrification. The remaining gene categories had lower percentages: Sulfur cycle 5.60%, Fungi function 3.69%, Soil benefit 2.64% [approximately 44% of unique detected probes in this category correspond mainly with antimicrobial genes such as *cat* (catalase), *phzF* (phenazine), or *pcbC* (isopenicillin)], Phosphorus cycle 2.28%, Virulence 1.88%, and Soil borne pathogen 1.08% (**Figure 6B**). This analysis allowed us to relate different gene functions implicated in the metabolism of different soil compounds with bacterial or fungal classes present in the AS soil (Table S1).

DISCUSSION

The application of organic amendments to agricultural soils is a longstanding practice, and examples of organic-amendment-mediated suppression of soilborne diseases were reported as early as the late nineteenth century (Stone et al., 2004). Growers have observed that different types of organic materials suppress root rot for varying lengths of time. At present, nursery and greenhouse growers successfully use compost-amended potting mixes to suppress soilborne diseases, such as *Pythium* and *Phytophthora* root rots, in container systems (Hoitink et al., 1991). However, limited field studies have been conducted to determine the impact of soil amendments on microbial communities in actual organic and conventional production systems (Drinkwater et al., 1995; Gunapala and Scrow, 1998; Bulluck and Ristaino, 2001). In the case of avocado orchards, organic matter-mediated disease suppression against *Phytophthora cinnamomi* has been observed in avocado agricultural fields organically managed in Australia. Organic amendments (barley straw, sorghum residues, and native grass) were added to the soil under the trees as a mulch layer resulting in the suppression of *Phytophthora* root rot of avocado (Malajczuk, 1979, 1983). Additionally, our previous studies also demonstrated that different organic amendments can influence the composition and diversity of soil bacterial communities in avocado plants growing in microcosms after DGGE analysis, showing enhancement of specific populations such as *Burkholderia* and *Frateuria* (Bonilla et al., 2012a, 2015). Among different organic matter tested on avocado crops, composted almond shells (AS; commercial almond shells derived from the almond industry were piled and traditionally composted) exhibited enhancement of soil suppressiveness against *R. necatrix* (Bonilla et al., 2012a, 2015), the causal agent of avocado white root rot (Pliego et al., 2012). Even when soil suppressiveness against *R. necatrix* is improved after the addition of AS, only subtle changes in the bacterial community and composition and specific enzymatic activities have been reported using DGGE analysis (Bonilla et al., 2015). It must be considered that a wide range of factors can affect soil microorganism communities (van Veen et al., 1997). The soil samples used in our study came from the same orchard (same type of soil, environmental conditions, plant age, and cultivar, etc.), but were under different management, and this was assumed to be the only difference between the samples. The soil influenced by the amendment of AS showed some characteristics that differed from the conventional unamended soil. The almond shells are a high dry matter-containing substrate, composed of approximately 95% organic matter, with poor values of glucose, fructose, or sucrose. The characteristics and composition of AS makes this substrate an acceptable growing media for soilless culture (Valverde et al., 2013). Moreover, it must be taken into account that the avocado is a shallow rooted tree, with most of the feeder roots allocated in the top 15 cm, which needs good aeration. Roots are helped by the presence of a rich surface of organic mulch, as shown by the tendency of healthy feeder roots to grow into any decomposing litter layer (Chanderbali et al., 2013).

In this work, a metagenomic approach to the community composition of amended and unamended avocado soils have been performed for the first time. The use of metabarcoding and GeoChip techniques allowed a better knowledge on the community composition and their potential activities. In first place, an attempt to identify key factors involved in this enhanced suppressivity after the addition of organic amendments revealed the crucial role of the microbiota present in the organic amended soil. The microbiota evolved in the composted almond shells and was crucial for suppressiveness because the reduction of the bacterial population after a heat treatment in the organic amendment resulted in a more conducive phenotype (heat-treated soil samples harbor 10^5 cfu/g, most likely composed mainly by sporulated bacterial and fungal microorganisms). Moreover, total or partial suppressiveness was recovered when these heat-treated soil samples were complemented with a portion of soil influenced by AS, but it remained conducive when complemented with a portion of conventional soil (CT). This effect has been previously described for different suppressive soils, where sterilization by autoclaving, steam pasteurization, and irradiation rendered soils conducive to the pathogen studied (Malajczuk, 1983; Weller et al., 2002; Mendes et al., 2011). Suppressiveness experiments performed do not excluded the possibility that the disinfected avocado root used could harbor endophytic microorganisms, but our results significantly pointed out the role of the composted almond shells in the plant protection against *R. necatrix*. Thus, our results support the crucial role of microbes present in AS for turning the conducive CT soil into a more suppressive soil against *R. necatrix*.

To gain insights into the microbial diversity present in the soil samples, we used several different approaches. Phylogenetic marker analysis based on the sequencing of 16S rDNA and ITSs revealed a relatively similar array of prokaryotic and eukaryotic populations present in the AS and CT soil samples; however, a different response has been described in the literature for other types of organic matter from different sources, such as composted municipal waste (Zaccardelli et al., 2013). It is remarkable that in our model system, the group of fast-growing, easily cultivable *Proteobacteria* is the dominant group of prokaryotes in both soil samples. These data are similar to those previously observed for other soil and rhizosphere

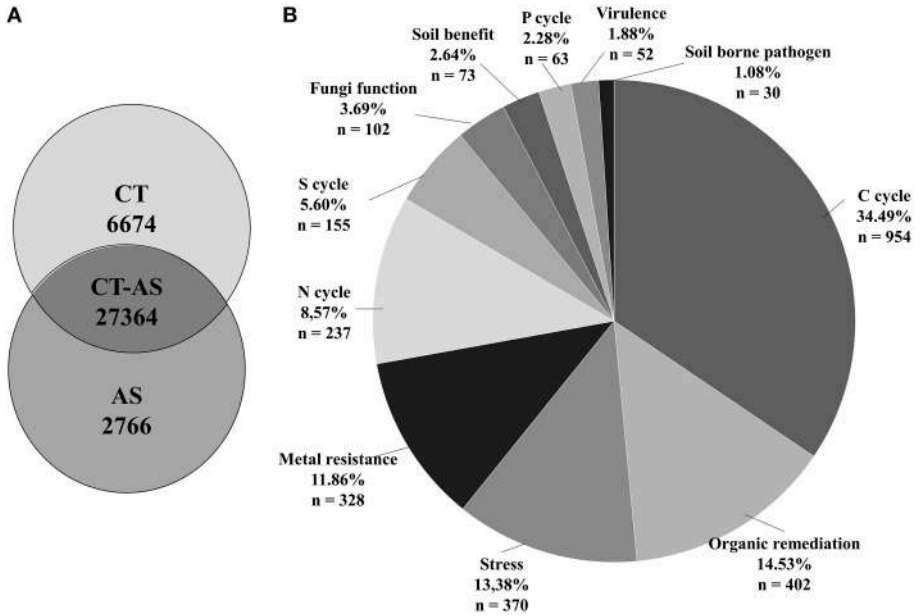

FIGURE 6 | GeoChip analysis of unique detected genes. (A) Number of core and unique detected genes (different gene ID) of amended soil (AS) and conventionally managed soil (CT). **(B)** Assigned functions of the unique genes detected in the AS sample.

samples with a high presence of organic matter (Lynch and Whipps, 1990; Paul and Clark, 1996; Hawkes et al., 2007; Mendes et al., 2011). Moreover, the representation of the other phyla different than *Proteobacteria* were quite similar among AS-amended and unamended soils, thus contradicting the idea that a highly specific community is stimulated by the addition of AS. Diversity analysis confirmed the previously obtained results (Bonilla et al., 2015), highlighting the enhancement of specific microbial populations in AS-amended samples, such as *Betaproteobacteria* (*Burkholderiales*) and the class of *Gammaproteobacteria*, which have been reported to protect plants from fungal infections in other suppressive soils (Mendes et al., 2011). It is important to note the clear enhancement in AS-amended soil of the order *Steroidobacter*, previously reported to play an essential role in the positive interactions with plants; for example, controlling seed germination, stem, and root elongation or stress protection in plants (Zarraonaindia et al., 2015).

In contrast, analysis of eukaryotic ITS revealed a different abundance distribution of microbes among the two types of soil samples. Fungal clones were the most common and dominant microbial eukaryotes in the soil. AS-amended soil samples had an increased relative abundance of *Ascomycota*. This fact is not surprising considering that *Ascomycetes* are the largest group on true fungi (Larena et al., 1999). Moreover, the dominance of *Ascomycota* has been observed during different composting processes (De Gannes et al., 2013; Neher et al., 2013), where most of them are saprophytic and live on dead organic material that they help decompose (Agrios, 1997; Viebahn et al., 2005). This behavior easily explains their higher abundance when composted almond shells are added to the soil as mulch. Within *Ascomycota*, the group that exhibited the most apparent and highest increase of abundance in AS-amended soil samples was the fungal class of *Dothideomycetes*. A high abundance of *Dothideomycetes* in soils with at high hydrocarbon concentrations has been previously reported (Ferrari et al., 2011), suggesting its preference for those habitats with a high concentration of organic matter where it participates in biomass conversion (Shrestha et al., 2011). Moreover, the large increase of the phylum *Pleosporales* (*Dothideomycetes*) is also not surprising because this group is very well-known to contain species that chlorinate lignin as a first step of biomass conversion during plant litter degradation (Ortíz-Bermúdez et al., 2007). Interestingly, it has been shown that several genera of *Dothideomycetes* exhibit an increased presence in suppressive soils because they harbor endohyphal bacteria from groups that are capable of hydrocarbon biodegradation, such as the *Xanthomonadales*, *Pseudomonadales*, *Burkholderiales*, and *Sphingomonadales* (Hoffman and Arnold, 2010). *Dothideomycetes* have also been shown to increase slightly in AS-amended soils. However, the group that shows an apparent decrease in AS-amended soils is *Mortierellales*. This group has a complex phylogeny (Wagner et al., 2013) and is considered to be ubiquitous in the bulk and rhizospheric soil, implying that it could play a role in maintenance of the micro-ecological balance (Miao et al., in press). Interestingly, the group of *Glomeromycota*, which contains different groups of symbiotic fungi previously detected in avocado (Hass and Menge, 1990; González-Cortés et al., 2012), it is clearly detected in unamended soils, but decreased in the amended ones (below 1%). A possible explanation could be that in the AS amended soils, take place a strong competition with other decomposing fungi, such as the *Dothideomycetes*, more adapted to an environment with high amount of decomposing organic matter. Finally, it should be noted that members of *Xylariaceae*, to which *R. necatrix* belongs

(Pliego et al., 2012), are less abundant in AS-amended soils, thus revealing a negative effect on this fungal group. These results indicate that the soil fungal community was affected by the soil amendment with AS.

Phylogenetic markers such as the prokaryotic 16S and eukaryotic ITS region do not carry explicit functional information. For this, the use of GeoChip-based analysis allowed for the analysis of microbial functional genes encoding key enzymes involved in major biogeochemical processes that facilitate linking microbial community structure to potential ecological functions (Torsvik and Ovreas, 2002). Using this technique, we screened potential functional gene diversity among unamended and AS-amended soil samples.

Probe signals and DCA analysis indicated that the microbial community functional structures differed between CT and AS soil samples. The sample sites are very close together, so the differences observed in the microbial communities are thought to be the result of amendment with organic matter.

Generally, similar abundance patterns of functional genes involved in nutrient cycling processes such a nitrogen, phosphorous or sulfur cycling, were found in both types of samples. However, AS-amended samples had higher signal intensities for C degradation (carbon cycle) genes than CT, with some differences being statistically significant. Substrates for this group of genes ranged from labile C to more recalcitrant C (e.g., starch, hemicelluloses, cellulose, chitin, and lignin). These results suggest that AS-amended microbial have a greater capacity for C degradation than CT communities. This suggests, as expected, an important role of carbon cycling in response to the addition of organic matter to the soil. However, no differences in gene abundance for N, P, or S cycling was observed. This can be explained because almond shells are a lignin-rich waste resulting from the almond industry, mostly composed of approximately 27% lignin and 73% holocellulose (Caballero et al., 1996), and those cycles were not compromised. However, statistical differences in the abundance of genes related to organic remediation and metal resistance were observed in AS-amended soil displaying lower levels than CT. This observation may be due to a decrease in the available compounds due to the high sorption ability of the composted almond shells and derivate compounds from its degradation, which have been previously reported to be able to remove such substances from the soil (Pehlivan et al., 2009).

Interestingly, both soil samples shared a core of probes corresponding to approximately 90% of the assayed sequences (27364 probes). However, approximately 10% of the total probes analyzed were unique for AS-amended samples (2766 probes). When the sequence of these probes were analyzed, they resulted in a very similar distribution to that previously shown for the whole GeoChip analysis, with above 34.5% corresponding to C cycling, followed by probes related to organic remediation (14.5%), stress (13.4%), metal resistance (11.9%), or the N cycle (8.6%). These results support the following previously described results: systems associated with organic matter-mediated general suppression; suppression typically occurs as a result of the activation of the indigenous microbial community (Lockwood, 1990); and suppressive activities can be generated by one to few populations of organisms (Gerlagh, 1968; Cook and Baker, 1983; Hoitink and Boehm, 1999; Weller et al., 2002). Postma et al. (2000) found that qualitative rather than quantitative shifts in the bacterial community correlate with disease suppressiveness, and several studies indicated that mechanisms within the microbial activity of the soil are responsible for the suppression of pathogens (Rovira and Wildermuth, 1981; Nitta, 1991; Workneh and van Bruggen, 1994; van Os and van Ginkel, 2001).

Among the specific taxa stimulated, *Pseudomonadaceae*, *Burkholderiaceae*, *Xanthomonadales*, and *Actinobacteria*, harbor genera and species with activity against plant pathogenic fungi (Postma et al., 2010). Additionally, it is important to note that *Pseudomonas, Rhizobium, Bacillus, Variovorax, Phyllobacterium,* and *Azospirillum*, are considered the most efficient plant growth-promoting bacteria (Bertrand et al., 2001).

Sequencing of specific probes present in AS-amended soils revealed the presence in such soil samples of genes for bacterial and fungal catalases, phenazine biosynthetic genes (from *Proteobacteria*) or the presence of potential antibiotics produced by *Actinobacteria* (data not shown). Nearly all of these probes corresponded to the GeoChip category "soil benefit," where the antimicrobials from different groups were analyzed. To the best of our knowledge, no probes from *Bacilli* were used, so the role of antimicrobials such as iturin or fengicins, produced by *Bacillus* spp., cannot be discussed based on our results.

It is important to note that the genus *Pseudomonas* (class *Gammaproteobacteria*) and *Bacillus* (class *Bacilli*) are two of the most prominent bacteria that can be isolated from avocado soil and rhizosphere displaying antifungal activity and plant protection against soil-borne pathogens (Cazorla et al., 2006, 2007; González-Sánchez et al., 2010). Our results reinforce the importance of such microorganisms in the soil and root ecology of the avocado crop. These groups of microorganisms can produce metabolites, such as siderophores and antibiotics, with specific suppressive activity against soilborne pathogens. Antagonistic pseudomonads, including *Pseudomonas chlororaphis*, play a role in white root rot suppressiveness (Cazorla et al., 2006; Calderón et al., 2014). However, other types of rhizobacterial taxa may differ in prevalence between suppressive and conducive soils, suggesting that the microbial basis of white root rot could be far more complex than solely a *Pseudomonas* property; it has also been observed for other pathosystems such as *Thielaviospsis basicola*-mediated black root rot of tobacco (Almario et al., 2014).

In conclusion, and taking together the results obtained in this work and in previous works related, a theoretical model about the role of the microorganisms in enhancing suppressiveness after amendment with composted almond shells can be proposed (Figure S5). Soil amendments with composted almond shells resulted in an extra input of organic matter rich in lignin that could be initially degraded by fungal members of the community (such as *Dothideomycetes*) and Actinobacterias. Lignin degradation from composting almond shells would produce a progressive release to the soil of more simple compounds. Those compounds, together with others also present in the almond shells, could lead to an increase in carbon

sources available, such as cellulose, hemicellulose, and aromatic compounds. At this point, some *Proteobacteria* already present in the soil (such as *Gammaproteobacteria* and *Betaproteobacteria*) could take advantage metabolizing that available organic matter, thus slightly enhancing their population. These groups of microorganisms could harbor, among other, genes involved in antifungal enzymatic activities and production of antimicrobial compounds that could have an effect on the interaction with other microbes. The resulting modified microbiota after addition of composted almond shells could be more active against some groups of phytopathogenic fungi (as *Xilariales,* where *R. necatrix* is included) finally showing a phenotype of induced suppressiveness effect.

AUTHOR CONTRIBUTIONS

FC and AD designed and corrected the manuscript. NB contributed to data management. CV performed date management and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00004

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Characterization of Soil Suppressiveness to Root-Knot Nematodes in Organic Horticulture in Plastic Greenhouse

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The fluctuation of *Meloidogyne* population density and the percentage of fungal egg parasitism were determined from July 2011 to July 2013 in two commercial organic vegetable production sites (M10.23 and M10.55) in plastic greenhouses, located in northeastern Spain, in order to know the level of soil suppressiveness. Fungal parasites were identified by molecular methods. In parallel, pot tests characterized the level of soil suppressiveness and the fungal species growing from the eggs. In addition, the egg parasitic ability of 10 fungal isolates per site was also assessed. The genetic profiles of fungal and bacterial populations from M10.23 and M10.55 soils were obtained by Denaturing Gradient Gel Electrophoresis (DGGE), and compared with a non-suppressive soil (M10.33). In M10.23, *Meloidogyne* population in soil decreased progressively throughout the rotation zucchini, tomato, and radish or spinach. The percentage of egg parasitism was 54.7% in zucchini crop, the only one in which eggs were detected. *Pochonia chlamydosporia* was the only fungal species isolated. In M10.55, nematode densities peaked at the end of the spring-summer crops (tomato, zucchini, and cucumber), but disease severity was lower than expected (0.2–6.3). The percentage of fungal egg parasitism ranged from 3 to 84.5% in these crops. The results in pot tests confirmed the suppressiveness of the M10.23 and M10.55 soils against *Meloidogyne.* The number of eggs per plant and the reproduction factor of the population were reduced ($P < 0.05$) in both non-sterilized soils compared to the sterilized ones after one nematode generation. *P. chlamydosporia* was the only fungus isolated from *Meloidogyne* eggs. In *in vitro* tests, *P. chlamydosporia* isolates were able to parasitize *Meloidogyne* eggs from 50 to 97% irrespective of the site. DGGE fingerprints revealed a high diversity in the microbial populations analyzed. Furthermore, both bacterial and fungal genetic patterns differentiated suppressive from non-suppressive soils, but the former showed a higher degree of similarity between both suppressive soils than the later.

Keywords: antagonistic potential of soil, biological control, biodiversity, DGGE fingerprints, *Meloidogyne* spp., *Pochonia chlamydosporia*, vegetable crops

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INTRODUCTION

Root-knot nematode (RKN), *Meloidogyne* spp., is the most harmful plant-parasitic nematode on vegetable crops in the world (Sasser and Freckman, 1987). In Spain, RKN are present in all horticulture production areas (Melgarejo et al., 2010) causing economical losses. Estimation of the maximum yield losses on important vegetable crops include: 88% for cucumber, 60% for tomato, 39% for zucchini, 37% for watermelon, and 30% for lettuce (Verdejo-Lucas et al., 1994; Sorribas et al., 2005; Talavera et al., 2009; Giné et al., 2014; López-Gómez et al., 2014; Vela et al., 2014).

Soil fumigants and nematicides are the most popular control methods (Talavera et al., 2012). However, the Directive 2009/128/EC from the European Commission promotes the use of non-chemical methods based on integrated pest management strategies in order to reduce the use of pesticides.

A sustainable production system uses environmentally friendly alternatives to preserve and enhance beneficial organisms, which represents the antagonistic potential. Soils with high antagonistic potential lead to suppression of soil borne pathogens. In a suppressive soil, pathogens do not establish, persist, or establish but cause little or no damage (Baker and Cook, 1974). Suppressive soils have already been described for many soil pathogens (Weller et al., 2002) including plant parasitic nematodes (Timper, 2011). Suppressive soils to cyst nematodes and RKN have been intensively studied. In such soils, fungal parasites were responsible of suppression in cereal (Kerry, 1980), sugar beet (Westphal and Becker, 1999, 2001), and soybean cyst nematodes (Chen, 2007), as well as RKN (Pyrowolakis et al., 2002; Adam et al., 2014). However, the suppression mechanisms are not well understood. Janvier et al. (2007) summarized the biotic and abiotic factors related to soil suppressiveness. Among them, the soil microbiota plays an important role (Weller et al., 2002), being essential to sustain biological productivity (Garbeva et al., 2004). Soil microbial diversity changes depending on the type of plant, soil, and management, and the interaction of microorganisms with those other factors can lead to the soil's disease suppressiveness (Garbeva et al., 2004). The study of microbial communities can be done by culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 2004), which allows the analysis of the total microbial structure of the soil, including the microorganisms that cannot be recovered by cultivation (Smalla and Heuer, 2006).

In Spain there are no reports of soils suppressive to RKN, despite the occurrence of antagonists of the nematode (Verdejo-Lucas et al., 1997, 2002, 2013; Olivares-Bernabeu and López-Llorca, 2002). In 2010, 10 commercial organic production sites were sampled in the northeastern horticultural growing area of Spain to assess the occurrence of fungal egg parasites of RKN and the percentage of parasitized eggs. Fungal egg parasites occurred in all sampled sites, and mean percent of parasitized eggs was 36.2 (Giné et al., 2012). In some of those sites, growers did not use any specific control measures against *Meloidogyne*, although attenuated disease symptoms were observed, mainly at the end of the spring-summer crops. Then, could be considered that some of those soils could be suppressive to RKN despite be intensively perturbed agrosystems, this is, with several crops per season and favorable climatic conditions that enable nematodes' development. Furthermore, as far as we know, there is little knowledge about the fluctuation of soil suppressiveness during the cropping sequences in commercial farms or the microbial profiles of RKN suppressive soils. Thus, two sites were selected in order to (i) determine the fluctuation of the RKN population's densities and the percentage of fungal egg parasitism along 2 years, (ii) assess soil suppressiveness in pot test, (iii) know the parasitic ability to RKN eggs of the fungal isolates from each soil, and (iv) compare microbial profiles between these two soils and a conducive one.

MATERIALS AND METHODS

Sites

Two commercial organic horticultural production sites, M10.23 and M10.55, cropped in plastic greenhouses were selected from a previous study (Giné et al., 2012) considering that the percentage of fungal egg parasitism was similar to the average obtained from all organic production sites sampled (36.2%). Both sites are located at the Tarragona province (northeastern Spain). Physicochemical properties and enzymatic activity of soils are presented in **Table 1**, and the rotation sequences conducted for both sites appear in **Table 2**. Soil at M10.23 was infested with *Meloidogyne javanica*; fertilization was done using a mixture of composted sheep and chicken manure at a rate of 2 kg m^{-2} that was incorporated into the soil just before transplanting each crop. Weed management was done by flaming and mechanically. Soil at M10.55 was infested with *M. javanica* and *Meloidogyne incognita* at a rate 10:1; fertilization was done with composted sheep manure at a rate of 1.7 kg m^{-2} that was also incorporated just before transplanting each crop. Mustard was grown as a cover crop planted in summer, just at the end of the spring crop, and incorporated as green manure 2 weeks before transplanting the autumn crop. Weeds were managed mechanically.

The commercial production site M10.33 was selected as non-suppressive soil due to its history on *Meloidogyne* infestation and disease severity on cucumber, pea, and tomato. At the end of those crops, fungal egg parasites were recovered at low percentage, 4.1% after cucumber crop (Giné et al., 2012), and 0% after the pea and tomato crops (data not shown). The grower managed RKN by biosolarization after the spring-summer crop. The site was conducted under integrated production in plastic greenhouse located in the province of Barcelona (northeastern Spain). Physicochemical properties and enzymatic activity of soils are also presented in **Table 1**. Fertilization was based on pellets of composted manure combined with chemical fertilizers. Weeds were managed mechanically. The soil of this site was used in the DGGE analysis for comparison between microbial communities of soils.

Fluctuation of RKN Population Densities

Composite soil samples were collected at the beginning and at the end of each crop to determine initial (*Pi*) and final

TABLE 1 | Physicochemical properties and enzymatic activity of soil of two vegetable production sites managed organically (M10.23 and M10.55) and an integrated production site (M10.33) in plastic greenhouses at the beginning of the study.

Variable	Sites		
	M10.23	M10.55	M10.33
Sand (%)	45	68	50
Silt (%)	40	0	20
Clay (%)	15	32	30
Soil texture (USDA)	Loam	Sandy clay loam	Sandy clay loam
pH	8.3	8.1	7.9
Organic matter (w/w)	5.8	2.5	1.6
Electric conductivity (μS/cm)	276	1069	2030
B (ppm)	2.8	1.1	4.6
Exchangeable Ca (meq 100 g^{-1})	17.3	18.2	8.8
Available Ca (meq 100 g^{-1})	17.2	19.0	14.6
Lime	3.8	4.1	4.4
Cation exchange capacity (meq 100 g^{-1})	41.2	25.7	13.7
Cu (ppm)	3.6	2.5	3.5
Available P (ppm)	379.4	75.8	107.6
Fe (ppm)	11.4	5.0	5.0
Exchangeable Mg (meq 100 g^{-1})	4.0	3.0	2.3
Available Mg (meq 100 g^{-1})	5.0	3.7	4.7
Mn (ppm)	64.0	2.5	148.0
N (ppm)	2329	1498	865
Exchangeable K (meq 100 g^{-1})	1.2	0.7	0.5
Available K (meq 100 g^{-1})	1.9	0.7	1.0
C/N	14.4	9.7	10.5
Exchangeable Na (meq 100 g^{-1})	0.3	0.5	0.3
Available Na (meq 100 g^{-1})	1.0	3.2	3.0
Zn (ppm)	20.6	2.5	81.0
Ca + Mg/K	18.0	31.5	21.4
P/N	0.2	0.1	0.1
Fluorescein diacetate hydrolysis (μg fluorescein h^{-1} × g soil)	5.5	1.0	2.0
b-glucosaminidase (μmols *p*-nitrophenol h^{-1} × g soil)	0.4	0.1	0.1
Urease (μmols N–NH_4 h^{-1} × g soil)	1.6	0.9	0.1
Protease (μg tyrosine h^{-1} × g soil)	4.5	12.4	8.7

(*Pf*) nematode population densities. Each plastic greenhouse was divided in four plots of 75 and 82 m^2 at the M10.23 and the M10.55 sites, respectively. Individual samples consisting of 20 soil cores were taken from the first 30 cm of soil with a soil auger (2.5 cm diameter) from each plot. Soil cores were mixed thoroughly and sieved through a 4-mm aperture screen to remove stones and separate roots from soil. RKN juveniles (J2) were extracted from two 250-cm^3 soil subsamples using the sieving and centrifugation-flotation method (Jenkins, 1964). J2 were counted and expressed as J2 per 250 cm^3 of soil. The reproduction rate of RKN in each crop was calculated as *Pf*/*Pi* ratio. At the end of each crop, eight plants per plot were randomly collected and removed from the ground with a pitchfork; damage caused by RKN in the root system was rated for galling based on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971). Roots were carefully washed free of soil, mixed, chopped, and root-knot nematode eggs extracted from two10 g-subsamples by macerating them for 10 min in a blender containing a 1% NaOCl solution (Hussey and Barker, 1973). Eggs were counted and expressed per g of root.

Soil temperature and soil water content from each site were recorded at 60 min intervals with temperature probes (5TM, Decagon devices, Inc., Pullman, WA, USA) placed at 15 cm depth.

Fungal Egg Parasitism

At the end of each crop, fungal egg parasites of RKN were isolated according to the de Leij and Kerry (1991) procedure modified by Verdejo-Lucas et al. (2002). Briefly, per each plot, 10–20 egg masses were handpicked from roots and placed in a watchglass containing sterile distilled water. The outer part of the gelatinous matrix was removed from the egg masses with tweezers to eliminate potential surface colonizers. Egg masses were then placed in an Eppendorf microcentrifuge tube containing 1 ml of sterile distilled water. Eggs were dispersed from the egg masses using a pestle and 333 μl-aliquots of the eggs' suspension were spread onto each of three replicated Petri dishes (9-cm diameter) containing a growth restricting medium (streptomycin, 50 mg l^{-1}; chloramphenicol, 50 mg l^{-1}; chlortetracycline, 50 mg l^{-1}; Rose Bengal, 50 mg l^{-1}; triton, 1 ml l^{-1}; and 1% agar) (Lopez-Llorca and Duncan, 1986). Plates were incubated at 25 ± 0.5°C. Number of parasitized eggs was recorded after 24 and 48 h under a dissecting microscope and percentage of parasitism was then calculated as the number of parasitized eggs per plate/number of eggs per plate. Eggs were considered parasitized if fungal hyphae grew from inside. At least, 20 parasitized eggs per plot and crop were individually transferred to corn meal agar (CMA) to establish pure cultures of the fungi. Fungal isolates were stored in 1% (w/v) water-agar slants, as well as lyophilized and stored at 4°C.

Fungal Parasites Characterization

Identification of fungal species isolated at the end of the first crop was carried out by PCR amplification and sequencing of the internal transcribed spacers (ITSs) of the rDNA regions. DNA was extracted from 50 mg of mycelium collected from single spore cultures on potato dextrose agar (PDA) using the E.Z.N.A kit® Plant MiniPrep (Omega Bio-Tek) according to the protocol described by the manufacturer. The PCR reaction was performed in 25 μl mix that contained 1 μl of the DNA extraction, 10.5 μl MiliQ water (Qiagen), 12.5 μl Taq PCR Master Mix (Qiagen) and 0.5 μl of each primer (5 pmol), ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). PCR conditions were the same as those described in the original studies (White et al., 1990). PCR products were cleaned using MinElute PCR Purification Kit (Qiagen) and sequenced by Secugen (Madrid, Spain). DNA sequences were analyzed using the BLAST database (July 2013) and assigned to the reference isolate sequences with the highest bit score. Identification of fungal isolates from eggs produced on the rest

TABLE 2 | *Meloidogyne* population densities in soil at planting (*Pi*) and at the end of the crop (*Pf*), galling index, number of eggs on roots, and percentage of egg parasitism in two commercial organic vegetable productions sites in plastic greenhouse during two consecutive years (2011–2013).

Site	Crop[a]	Date	N° of juveniles 250 cm^{-3} soil		Galling index	N° of eggs g $root^{-1}$	Parasitized eggs (%)
			Pi	*Pf*			
M10.23	Zucchini cv. Dundoo	07/2011–11/2011	2951 ± 487	61 ± 15	1.6 ± 0.3	1301 ± 530	54.7 ± 13.9
	Tomato cv. Royesta (R)	01/2012–07/2012	61 ± 15	0 ± 0	0 ± 0	0 ± 0	Nem
	Radish cv. Saxa	11/2012–02/2013	9 ± 9	3 ± 3	0 ± 0	0 ± 0	Nem
	Spinach cv. Gigante de invierno	11/2012–02/2013	4 ± 4	9 ± 3	0 ± 0	0 ± 0	Nem
	Fallow	02/2013–07/2013	6 ± 3	15 ± 12	Na	Na	Na
M10.55	Tomato cv. Lladó (R)	02/2011–07/2011	238 ± 62	1013 ± 883	0.2 ± 0.1	41 ± 26	3.0 ± 0.04
	Zucchini cv. Dundoo	07/2011–11/2011	1013 ± 883	1351 ± 238	3.0 ± 0.4	1870 ± 478	84.5 ± 3.6
	Lettuce cv. Maravilla	11/2011–03/2012	1351 ± 238	81 ± 17	0.1 ± 0.1	0 ± 0	Nem
	Cucumber cv. Dasher II	03/2012–06/2012	81 ± 17	1329 ± 505	6.3 ± 1.0	6026 ± 1165	71.7 ± 2.7
	Mustard cv. Caliente 109	06/2012–09/2012	1329 ± 505	40 ± 18	Na	Na	Na
	Lettuce cv. Maravilla	09/2012–11/2012	40 ± 18	56 ± 5	2.2 ± 0.2	999 ± 645	0.2 ± 0.2
	Tomato cv. Caramba (R)	02/2013–07/2013	19 ± 6	126 ± 30	1.3 ± 0.8	206 ± 115	16.0 ± 10.6

Data are mean ± standard error of four replications. Galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971).
[a]R, Resistant cultivar; Na, Not available; Nem, Not egg masses.

of crops was carried out according morphological characters (Gams, 1988).

Soil Suppressiveness against RKN in Pot Tests

Two experiments were carried out in 2012. Experiment 1 was conducted from March 27 to June 10 [907 degree-day (DD), 10°C basal temperature and thermal constant between 600 and 700 DD over the basal temperature; (Ferris et al., 1985)] with soil samples taken in January 2012. Experiment 2 was carried out from August 9 to October 23 (1092 DD, 10°C basal temperature and thermal constant between 600 and 700 DD over the basal temperature) with soil samples taken in July 2012. Both experiments were conducted using the same procedure. A soil sample was taken from the first 30 cm of soil with a hoe. Sample consisted of 48 soil cores (12 per plot). Soil was mixed thoroughly and passed through a 5-mesh sieve to remove stones and separate roots from soil. A part of soil was sterilized at 121°C during 1 h and the procedure was repeated after 1 day. The rest of soil was stored at 4°C until the experiment was carried out. Sterilized soil was mixed with steam-sterilized sand at a ratio 1:1 (dry w: dry w) to avoid soil compaction and improve plant growth. The same procedure was carried out with non-sterilized soil. After that, RKN juveniles were extracted from two 500-cm^3 subsamples of both sterilized and non-sterilized soil mixtures using Baermann trays (Whitehead and Hemming, 1965) maintained at 27 ± 2°C for a week to determine the level of nematode inoculum at the beginning of the experiments. Thereafter, soil was placed in 3-L pots and a susceptible tomato cv. Durinta was transplanted into each pot at three true developed leaves stage. Nematode inoculum consisted of juveniles emerged from eggs that were extracted from tomato roots by the Hussey and Barker (1973) procedure and placed in Baermann trays (Whitehead and Hemming, 1965) for a week at 27 ± 2°C. Soil was inoculated with *M. incognita* J2 to achieve a total of 3000 J2 per plant, which was added in two opposite holes, 3 cm deep, made in the soil at 2 cm from the stem of the plants.

Ten replicate pots were prepared per each soil mixture, site, and experiment. Plants were arranged at random on a greenhouse bench, were irrigated as needed and fertilized with a slow-release fertilizer (15N + 10P + 12K + 2MgO + microelements). Soil temperatures and soil water content at 8 cm depth was recorded at 30 min interval during the experiments.

At the end of the experiments, plants were removed from pots. Roots were washed with tap water to remove soil particles and gently dry before determine fresh weight. Galling index was estimated according to Zeck scale (1971). To determine percentage of egg parasitism, three egg masses were handpicked from individual plants growth in both sterilized and non-sterilized soils and processed according to the method described previously. Fungi growing from eggs were isolated and identified as previously described. Eggs were extracted from roots by Hussey and Barker (1973) method, and reproduction factor was calculated considering *Pi* as number of juveniles inoculated, and *Pf* number of non-parasitized eggs per plant (Sorribas et al., 2003).

Parasitism of Fungal Isolates against RKN Eggs

Five single-spore culture isolates of *Pochonia chlamydosporia* coming from each pot test and site were assessed for fungal egg parasitism. Single 5 mm-diameter plugs from the margin of the colony growing on PDA were transferred to the center of plates containing 1% water agar (WA) and incubated at 25°C in the dark for 2 weeks. Sterilized RKN eggs used as inoculum were obtained according to the procedure of Verdejo et al. (1988) modified. Briefly, 30 *M. incognita* eggs masses coming from tomato roots were handpicked and placed in a sterile conical

centrifuge tube containing 1 ml of 4% NaOCl solution. The egg suspension was shaken during 4 min at 30 s intervals, and finally diluted 10 times with sterile distilled water. Egg suspension was left undisturbed for 30 min to allow deposition. After that, sterilized nematode eggs were spread axenically around 1-cm apart from the margin of the colony using a Pasteur pipette. Plates were incubated at 25°C in the dark for 1 week. Eggs surrounded by a dense fungal colony were considered as parasitized and validated by observation under the light microscope (Lopez-Llorca et al., 2002). Percentage of egg parasitism was calculated as described previously. Three replicate plates were prepared per each fungal isolate and experiment.

DGGE Analysis of Fungal and Bacterial Soil Community DNA

Fungal and bacterial profiles from M10.23 and M10.55 soils were obtained by DGGE, and compared to a commercial vegetable production site, M10.33, managed under integrated production, but with low percentage of fungal egg parasitism (4.1%) (Giné et al., 2012). Soil samples used for this study were taken in February 2013.

DNA extraction of soil samples was carried out using the Ultraclean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol, using 0.25 g of soil. DNA extractions were performed from each composite soil sample. The quantity and quality of the extracted DNA was checked by agarose gel electrophoresis and by spectrophotometer measurement at wavelength 260 and 280 nm. All DNA samples were stored at −20°C for further analyses.

Fungal DNA was amplified using a nested approach, where primers EF4/ITS4 (Gardes and Bruns, 1993) amplifies 18S rDNA and ribosomal ITS regions in a first PCR, and this product is then used as template in a second PCR applying primers ITS1f-GC/ITS2 (White et al., 1990). Bacterial DNA was amplified using the universal bacterial primers 341F-GC and 907R (Muyzer et al., 2004). The PCR mixture and conditions were the same as those described in the original studies. PCR products were analyzed for size and quantity by agarose gel electrophoresis and stained with ethidium bromide. DGGE analyses were carried out using a D-Code Universal Detection System (Bio-Rad Laboratories, Richmond, CA, USA).

Nine hundred nanograms of PCR product were loaded onto 8% (w/v) polyacrylamide gels (40% acrylamide/bis solution, 37.5:1, Bio-Rad) with denaturing gradients ranging from 10 to 50% for the fungal DNA and 20 to 70% for the bacterial DNA (100% denaturants defined like 7 M urea and 40% v/v deionized formamide) (Schäfer and Muyzer, 2001). Electrophoresis was performed in 1× Tris-acetate-EDTA (TAE) buffer, at 60°C. The gel with fungal DNA was run for 16 h at 75 V, while the gel with bacterial DNA was run for 16 h at 80 V. Gels were stained with ethidium bromide (0.5 μg/ml), and inspected under UV illumination and photographed. Prominent bands were excised from the gels, reamplified, and then purified using the PCR Clean up Kit (MoBio Laboratories) for subsequent sequencing.

Sequencing reactions were performed by Macrogen (South Korea) using the Big Dye Terminator v3.1 sequencing kit; reactions were run in an automatic capillary type ABI 3730XL analyzer-96. Sequences were first screened to detect potential chimeric artifacts using the Chimera.uchime program in Mothur 1.33.3 (http://www.mothur.org/wiki/Download_mothur) (Edgar et al., 2011) and then compared to those deposited in the GenBank nucleotide database using the BLAST program (Tatusova and Madden, 1999; Maidak et al., 2001).

Statistical Analyses

Statistical analyses were carried out with the SAS system software V9.2 (SAS Institute Inc., Cary, NC, USA). Variables were transformed when required to $\log_{10}$ (x + 1) or arcsine square root (x + 0.5). Data from pot experiments to assess soil suppressiveness were compared between experiments and site by *t*-Student test, using the *t*-test procedure, and were pooled together as replications of a single experiment because no differences ($P > 0.05$) were found. Then, data were submitted to *t*-Student test to compare between sterilized and non-sterilized mixture soil per each site. Data from experiments conducted to determine the ability of fungal isolates to parasitize RKN eggs were submitted to analysis of variance using the general linear model (proc glm) to compare the parasitic capability between isolates per site. When the analyses were significant ($P \leq 0.05$), the means were separated according to the least significant difference (LSD) test.

DGGE images were analyzed using the InfoQuest™FP 4.5 software (Bio-Rad Laboratories, Richmond, CA, USA). Similarities of the DGGE profiles were calculated based on the Dice coefficient and dendrograms were obtained using the UPGMA clustering algorithm. A band position tolerance of 0.5% was used. Band patterns were normalized using the marker lanes as reference, allowing the comparison among samples loaded on different DGGE gels. The number of DGGE bands in each fingerprint was used as a measure of the apparent fungal and bacterial richness (S). Shannon Index was used as a measure of genetic diversity, and was calculated as H = pi ln pi, where pi is the relative intensity of each DGGE band. Evenness (E) was calculated as E = ln (S). Diversity variables were submitted to nonparametric analysis of variance (proc npar1way) using Wilcoxon rank sum test.

RESULTS

Fluctuation of RKN Population Densities and Percentage of Fungal Egg Parasitism

Daily soil temperature and water content of soil, as well as crop rotation sequences in sites M10.23 and M10.55, are presented in **Figures 1**, **2**, respectively.

In M10.23, nematode population in soil decreased progressively from 2951 J2 250 cm^{-3} of soil in July 2011 to 15 J2 250 cm^{-3} of soil in July 2013, all throughout the rotation zucchini-resistant tomato-fallow-radish/spinach-fallow. At planting the winter crops (radish and spinach), nematode densities were below 10 J2 250 cm^{-3} of soil, and did not increase at the end of the crop. For these two crops, no galls were observed in the roots and no eggs were recovered (**Table 2**). Maximum densities of eggs per g root were recovered from the

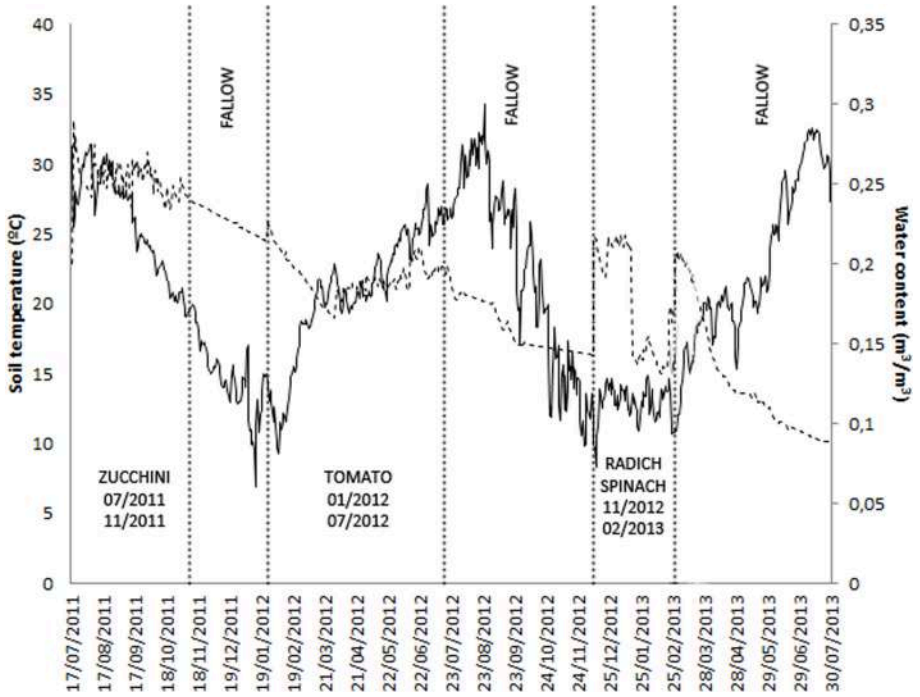

FIGURE 1 | Fluctuation of mean daily soil temperature, soil water content, and crop rotation sequence in site M10.23.

RKN susceptible zucchini cv. Dundoo cropped during summer, but not from the resistant tomato cv. Royesta. Disease severity in zucchini ranged from 0 to 4, while no galls were observed on the tomato roots. Fungal egg parasitism was only detected in zucchini (54.7%) in which root infection occurred and egg masses were produced (**Table 2**). *P. chlamydosporia* was the only fungal species isolated.

In M10.55, densities of nematodes in soil peaked at the end of spring-summer crops. Population densities at planting of the susceptible cucumber cv. Dasher II and the zucchini cv. Dundoo crops were 81 and 1013 J2 250 cm^{-3} of soil, respectively, but disease severity was less than expected [galling index (GI) of 6.3 and 3.0, respectively (**Table 2**)]. High percentage of fungal egg parasitism was recorded after cultivation of the susceptible cucurbit crops (84.5 and 71.7%), it was low after the cropping of resistant tomatoes (16%), and not apparent in the winter lettuce crop (0%), in which no egg masses were produced (**Table 2**). Nematode densities decreased after incorporation of the mustard cover crop into the soil, but survivors were able to infect roots (GI = 2.0) of the following crop of lettuce cultivated from September to November 2013, and to produce eggs, some of which were parasitized (0.2%). Again, *P. chlamydosporia* was the only fungal egg parasite recovered.

Soil Suppressiveness against RKN in Pot Test

Minimum and maximum soil temperatures ranged from 18.6 to 25.7°C (21.9 ± 1.8°C, mean ± standard deviation) in experiment 1, and from 17.9 to 30.7°C (24.7°C ± 3.4°C) in experiment 2. Water content of soil ranged from 0.14 to 0.31 w^3/w^3 (0.22 ± 0.04 w^3/w^3) and from 0.15 to 0.26 w^3/w^3 (0.21 ± 0.03 w^3/w^3) in experiment 1 and 2, respectively.

Fungal egg parasites were recovered only from non-sterilized soils, being *P. chlamydosporia* the only fungal species identified. Eggs were parasitized at a rate of 24.8% in non-sterilized soil from site M10.23, and 70.9% from site M10.55 (**Table 3**). In non-sterilized M10.23 soil, fewer ($P < 0.05$) eggs per plant (73.30%), lower reproduction factor (73.91%), and less disease severity (17.07%) were recorded compared to the sterilized soil according to the Abbott's formula. In addition, less ($P < 0.05$) tomato fresh root weight (61.96%) was also recorded. Similar results were obtained with the non-sterilized M10.55 soil, in which the number of eggs per plant, reproduction factor and tomato fresh root weight were 61.43, 66.67, and 45.07% less ($P < 0.05$) than in the sterilized one, although disease severity did not differ ($P > 0.05$) (**Table 3**).

Parasitism of Fungal Isolates against RKN Eggs

P. chlamydosporia isolates from site M10.23 parasitized between 55.5 and 97.4% of the RKN eggs, and those from site M10.55 between 56.5 and 93.7%. In both sites, 3 out 10 fungal isolates parasitized more than 90% of the RKN eggs (**Table 4**).

DGGE Analysis of Fungal and Bacterial Communities

Band profiles obtained by the DGGE of bacterial and fungal rDNA amplified fragments and the DGGE fingerprints cluster

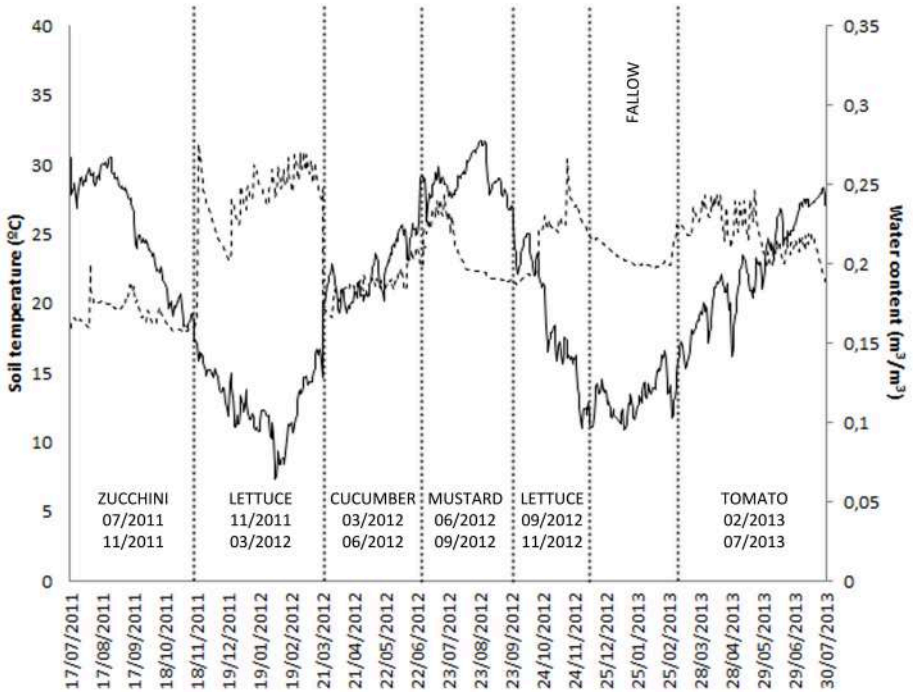

FIGURE 2 | Fluctuation of mean daily soil temperature, soil water content, and crop rotation sequence in site M10.55.

TABLE 3 | Effect of soil sterilization or not- sterilization of sites M10.23 and M10.55 on *Meloidogyne* densities on roots, reproduction factor, galling index, percentage of egg parasitism and fresh root weight of tomato cv. Durinta inoculated with 3000 juveniles per pot after completion of one nematode generation.

Site	Soil mixture[a]	Fresh root weight (g)	N° of eggs (× 10^3)/plant[b]	Reproduction factor[c]	Galling index[d]	Parasitized eggs (%)
M10.23	Sterilized	16.3 ± 1.3*	41.2 ± 13.4*	13.8 ± 4.5*	4.1 ± 0.2*	0
	Non-sterilized	6.2 ± 0.5	11.0 ± 3.0	3.6 ± 1.0	3.4 ± 0.2	24.8 ± 1.7
M10.55	Sterilized	14.2 ± 1.5*	40.7 ± 8.8*	12.6 ± 2.9*	4.3 ± 0.2	0
	Non-sterilized	7.8 ± 0.6	15.7 ± 2.8	4.2 ± 0.7	3.8 ± 0.1	70.9 ± 2.0

*Data are mean ± standard error of 20 replications. Data within the same column and site followed by *indicates a significant difference between soil treatment at $P < 0.05$ according to the Student's t -test.*

[a] *Sterilized soil mixture, 50% sterilized soil + 50% sterilized sand; Non-sterilized soil mixture, 50% non-sterilized soil + 50% sterilized sand.*

[b] *Parasitized eggs excluded.*

[c] *Number of non-parasitized eggs per plant/initial population density.*

[d] *Galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971).*

analysis are shown in **Figure 3**. The 16S rRNA-DGGE analysis (**Figure 3A**) revealed composite banding patterns reflecting a high microbial diversity. Conversely, the ITS rDNA-DGGE analysis (**Figure 3C**) showed a lower diversity in the fungal communities. Two first-order clusters were clearly differentiated by the UPGMA analysis of the DGGE fingerprints, both in the bacterial and the fungal communities of the soils. These first-order clusters were identified at a similarity score of 53 and 65% for the fungal and the bacterial communities, respectively. Regarding the bacterial community, the first-order cluster differentiated non-suppressive M10.33 soil from M10.23 and M10.55 suppressive soils, and the second-order subcluster (75.5% similarity) differentiated between both suppressive soils (**Figure 3B**). Concerning the fungal communities, M10.23 soil was clearly differentiated from the rest in the first-order clusters, and M10.55 and M10.33 soils were grouped in a second-order subcluster (56.5% similarity) (**Figure 3D**). The bacterial and fungal genetic diversity was evaluated based on the number of DGGE bands and their relative intensity. Diversity variables for the bacterial communities did not differ between soils (Shannon–Wiener $P = 0.12$; richness $P = 0.73$; evenness $P = 0.09$), but some of them did for the fungal communities. The Shannon–Wiener index and the evenness in soil M10.55 differed ($P = 0.05$ and $P = 0.03$, respectively) from M10.33 but not from M10.23 soils. However, richness was similar between soils ($P = 0.45$).

TABLE 4 | Percentage of parasitized eggs of *Meloidogyne* spp. by isolates of *P. chlamydosporia* in *in vitro* test.

Site	Assay. Isolate[a]	Parasitized eggs (%)
M10.23	C1.1	55.5 ± 1.0 d
	C1.2	66.5 ± 18.7 bcd
	C1.3	83.7 ± 7.3 abcd
	C1.4	60.6 ± 10.7 cd
	C1.5	86.5 ± 3.9 abc
	C2.1	82.9 ± 2.7 abcd
	C2.2	97.4 ± 1.8 a
	C2.3	88.0 ± 4.8 abc
	C2.4	90.7 ± 5.6 ab
	C2.5	93.9 ± 2.1 ab
M10.55	H1.1	56.5 ± 6.5 f
	H1.2	64.0 ± 5.6 ef
	H1.3	65.1 ± 5.9 ef
	H1.4	76.7 ± 1.7 de
	H1.5	82.6 ± 4.0 cd
	H2.1	93.7 ± 1.7 ab
	H2.2	89.0 ± 0.7 abc
	H2.3	91.1 ± 0.3 abc
	H2.2	93.3 ± 4.3 abc
	H2.5	86.3 ± 2.4 bcd

Data are mean ± standard error of three replications. Data within the same column and site followed by the same letter did not differ ($P < 0.05$) according to the LSD test.
[a]Single-spore isolates of P. chlamydosporia isolated at the end of both pot assay, in June 10 (1), and October 23 (2) 2012.

In **Figures 3A,C**, the bands marked with numbers correspond to the dominant bands that were extracted from the DGGE gels and sequenced. **Tables 5**, **6** show the sequenced bands, their similarity values compared to the closest related GenBank sequences, and their phylogenetic affiliations. Sequence similarity values compared to previously reported sequences were more than 93.5% in all cases. The majority of the 23 bacterial sequences belonged to the phylum Bacteroidetes (65.2%) followed by Proteobacteria (17.4%) (**Table 5**). Regarding fungi, the 41 sequences fell into three taxonomic groups. On average, Ascomycota (56.1%) was the most abundant phylogenetic groups, followed by Basidiomycota (31.7%) (**Table 6**).

The bacterial and fungal rRNA sequences determined in this study are available at the GenBank under accession numbers KT991569 through KT991632. Each band designation includes a code specifying its origin (ASS, Agricultural Soil Suppressiveness) followed by a number indicating the order in which the sequence was isolated from the gel.

DISCUSSION

In this study, two suppressive soils to RKN were identified, increasing the list of previous studies reporting this kind of agricultural soil (Stirling et al., 1979; Gaspard et al., 1990; Pyrowolakis et al., 2002; Timper, 2011; Adam et al., 2014). However, as far as the authors know, this is the first report of suppressive soils to RKN in which vegetables are cultivated organically in plastic greenhouses. In addition, despite several studies to identify suppressive soils to RKN, none reported the fluctuation of both nematode densities and fungal egg parasites during the rotation sequences. This is the first comparison of microbial profiles of both suppressive and non-suppressive soils to be published.

The antagonistic potential of agricultural soils, defined as its capacity to prevent or reduce the spread of pathogens by biotic factors, is a product of the capacity of the microbial antagonists to survive the agronomic practices and their ability to limit the damage caused by the pathogens (Sikora, 1992). It is widely accepted that high levels of suppressiveness to plant parasitic nematodes are only achieved under perennial crops or monoculture in which soil perturbation practices are low (Baker and Cook, 1974). However, this study shows that high levels of soil suppressiveness can be also achieved in highly perturbed crop systems, probably due to the confluence of favorable interactions between plant-RKN-antagonists, cultural practices and abiotic factors. Both sites, M10.23 and M10.55, were located in the same cropping area, with similar agro-climatic conditions, but differing in crop management. In both sites, RKN were detected in soil and in roots at the beginning of the study. However, nematode densities decreased to near and below detectable levels in soil and roots, respectively, at the end of the study in site M10.23, but not in site M10.55, in which RKN was always detected. Agricultural practices such as crop rotation, tillage and organic amendments have been proved to influence the antagonistic potential of soil (Sikora, 1992; Kerry and Bourne, 1996; Westphal and Becker, 2001; Janvier et al., 2007; Timper, 2011), and could be the reason for the results of this study. For instance, site M10.23 was fertilized with a mixture of sheep and chicken manure but only sheep manure was used at M10.55. Chicken manure has been reported to suppress RKN infection and reproduction on several crops (Kaplan and Noe, 1993; Riegel and Noe, 2007), but there is still limited information about the suppressive capacity of sheep manure.

In site M10.23, the nematode was able to reproduce on susceptible crops cultivated during spring-summer, in which fungal RKN egg parasites were isolated, mainly *P. chlamydosporia.* The highest percentage of fungal egg parasitism was recorded on zucchini, which ranged from 30 to 78% in the four plots (mean of 54.7%). At the end of this crop, galling index ranged from 0 to 4, less than expected considering a *Pi* of 2951 J2 250 cm^{-3} of soil, and in which the nematode completed three generations according to thermal requirements of *M. incognita* (Vela et al., 2014). Vela et al. (2014) recorded galling indexes of 2.6 and 5.1 on zucchini cultivated in plastic greenhouse, with *Pi* of 222 and 594 J2 250 cm^{-3} of soil, respectively, and in which nematodes completed two generations. *P. chlamydosporia* is a fungal egg parasite that affects the increase of nematode inoculum (J2) and consequently reduces disease severity when more than one generation occurs, because emerged J2 from non-parasitized eggs are able to invade roots (Bailey et al., 2008). Results from the pot test conducted for just one nematode generation showed differences in disease severity between sterilized and non-sterilized soils, indicating that other microorganisms could

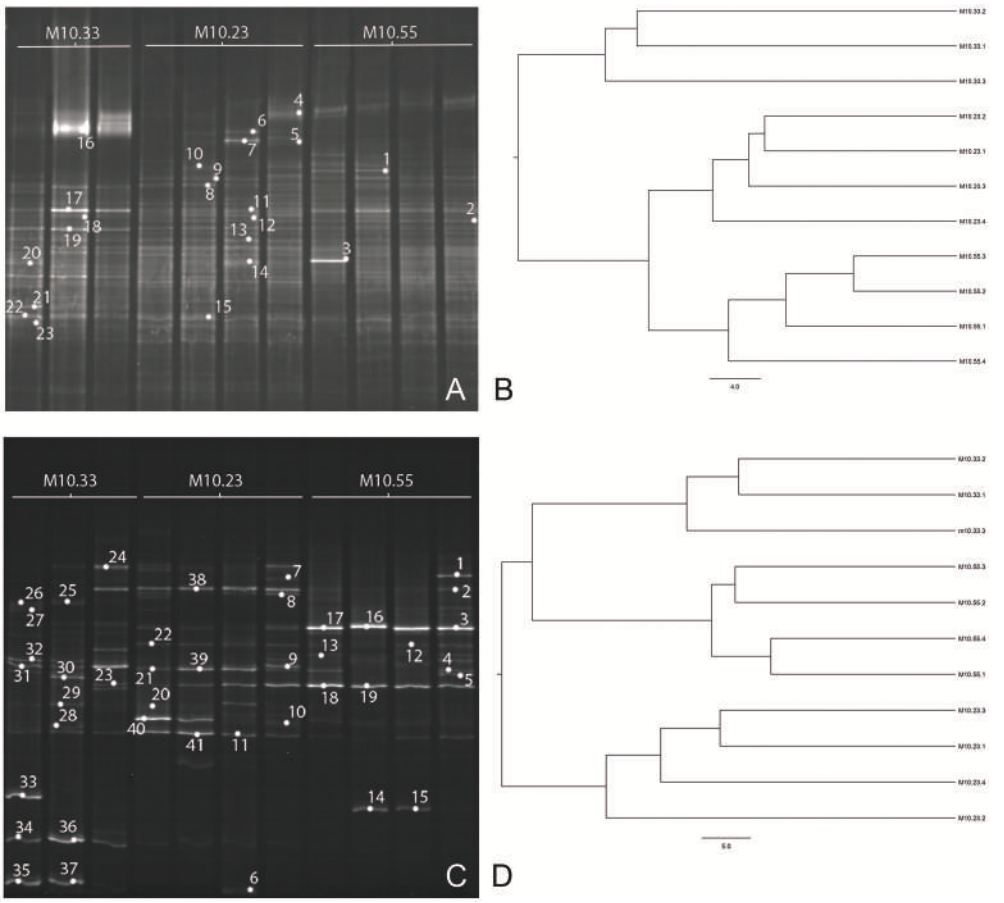

FIGURE 3 | DGGE profiles of 16S rRNA fragments of bacteria (A) and partial fungal ITS sequences (C) from DNA sample replicates of one non-suppressive (M10.33) and two suppressive (M10.23 and M10.55) soils. Bands marked with numbers correspond to the bands excised from the gel and sequenced. Cluster dendrograms based on UPGMA algorithm show similarity among DGGE band patterns of bacteria **(B)** and fungi **(D)**. Bar indicates percentage of divergence.

be involved in soil suppressiveness. Fungal and bacterial DNA sequenced from soil DGGE revealed the presence of several species that can affect nematodes by the production of active toxins against RKN J2 such *Stachybotrys* spp. *Cladosporium* spp. (Qureshi et al., 2012), and *Flavobacterium* spp. (McClure, 1989); by inducing the activity of other nematode antagonists, such *Chryseobacterium* spp. that induce trap formation in *Arthobotrys oligospora* (Li et al., 2011); by suppressing disease severity, such *Chryseobacterium* spp. (Liu et al., 2014); or by parasitizing RKN eggs, such *Fusarium equiseti* and *Cladosporium* spp. (Giné et al., 2012). The growing media used in this study did not allow bacterial isolation. Thus, the use of culture independent methods is necessary to complement the information obtained by traditional culture dependent ones in order to know the composition and function of microbial communities and their putative contribution to soil suppressiveness.

At the end of autumn-winter crops, no galls were observed and no eggs were extracted from roots. Soil temperatures influence the movement of J2 in soil, root penetration and infection, development and reproduction of RKN. Minimum and maximum soil temperatures at planting autumn-winter crops were 8.3 and 17.3°C (mean 12.8°C), temperatures below the minimum activity threshold of J2 (Roberts et al., 1981). Thus, roots could escape infection.

In site M10.55, *Meloidogyne* was detected in soil and roots of each crop. Nematode densities fluctuated during the cropping season as well as *P. chlamydosporia,* the only fungal species isolated from eggs. Highest nematode densities and levels of egg parasitism were recorded at the end of cucumber and zucchini cultivated in spring-summer and summer-winter, respectively. *Meloidogyne* completed two generations on cucumber and three on zucchini according to RKN thermal requirements on these crops (Giné et al., 2014; Vela et al., 2014). However, disease severity was less than expected, as occurred in M10.23. Soil microbial profiles showed the occurrence of Cyanobacteria, able to suppress RKN densities and disease severity (Khan et al., 2007), and the fungi, *F. equiseti*, and *Preussia* spp, which have been reported as egg parasite of *Heterodera schachtii* (Saleh and Qadri, 1990). Results of pot experiments suggest that the only active antagonist of RKN

TABLE 5 | DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of bacterial populations.

Phylotype			Phylogenetic affiliation		
Band code[a]	Sequence length (bp)	Accession code	Taxonomic linage[b] (Phylum, Class, Order, Family, Genus)	Closest match[c] (accession no.)	Similarity (%)[d]
B1	336	KT991569	Bacteroidetes (100), Sphingobcteria (83), Sphingobacteriales (83)	Uncultured bacterium clone (KM155241)	94.4
B2	368	KT991570	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100)	Uncultured *Sphingobacterium* sp clone (KM155241)	98.9
B3	536	KT991571	Cyanobacteria/Chloroplast (100)	Uncultured Streptophyta clone (JF703638)	99.8
B4	551	KT991572	Bacteroidetes (100), Flavobacteria (100), Flavobacteriales (100), Flavobacteriaceae (100), *Flavobacterium* (100)	*Flavobacterium* sp. (JN650574)	100
B5	519	KT991573	Bacteroidetes (100), Flavobacteria (100), Flavobacteriales (100), Flavobacteriaceae (100), *Chryseobacterium* (100)	*Chryseobacterium* sp. (KJ482798)	100
B6	570	KT991574	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), KD3-93 (100)	Uncultured Bacteroidetes (AM116744)	98.5
B7	556	KT991575	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), KD3-93 (100)	Uncultured bacterium clone (JF176318)	97.9
B8	547	KT991576	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (100)	Uncultured bacterium clone (KJ909017)	99.6
B9	541	KT991577	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (100)	Uncultured Flexibacteriaceae bacterium (FM209167)	99.4
B10	442	KT991578	Bacteroidetes, Sphingobcteria, Sphingobacteriales, Cytophagaceae (100), *Flexibacter* (100)	Uncultured Bacteroidetes bacterium (KJ024617)	98.0
B11	510	KT991579	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (96), *Flexibacter* (89)	Uncultured Bacteroidetes bacterium (HF564268)	99.0
B12	511	KT991580	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (94)	Uncultured Bacteroidetes bacterium (HF564268)	99.8
B13	449	KT991581	Proteobacteria (100), Gammaproteobacteria (100), Xanthomonadales (98). Sinobacteriaceae (98), *Steroidobacter* (96)	Uncultured bacterium clone (GQ263704)	95.8
B14	519	KT991582	Proteobacteria (100), Gammaproteobacteria (100), Xanthomonadales (100), Xanthomonadaceae (100), *Lysobacter* (99)	*Lysobacter* sp. MHS036 (DQ993327)	97.5
B15	485	KT991583	Proteobacteria (100), Alphaproteobacteria (100), Rhizobiales (100), Methylobacteriaceae (100), *Methylobacterium* (100)	*Methylobacterium radiotolerans* (LC026013)	100
B16	490	KT991584	Bacteriodetes (100), Flavobacteria (100), Flavobacteriales (100); Flavobacteriaceae (100)	*Winogradskyella rapida* (KF009869)	93.5
B17	518	KT991585	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (100)	Uncultured Sphingobacteriales bacterium (KF733506)	99.6
B18	484	KT991586	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (99), *Flexibacter* (99)	Uncultured Bacteroidetes bacterium (HF564295)	98.6
B19	513	KT991587	Bacteroidetes (100), Sphingobcteria (97), Sphingobacteriales (97), Cytophagaceae (94)	Uncultured Sphingobacteriales bacterium (AM936482)	99.8
B20	514	KT991588	Firmicutes (100), Bacilli (100), Bacillales (96), Bacillaceae (82)	*Marinococcus halophilus* (HF678777)	99.8
B21	472	KT991589	Unclassified Chloroflexi	Uncultured bacterium clone (HQ697759)	100
B22	494	KT991590	Proteobacteria (100), Alphaproteobacteria (100), Rhizobiales (100), Methylobacteriaceae (100), *Methylobacterium* (100)	*Methylobacterium mesophilicum* (KP293855)	100
B23	487	KT991591	Acidobacteria (100), Acidobacteria (100), Acidobacteriales (100), Acidobacteriaceae (100), Candidatus *Solibacter* (100)	Uncultured bacterium clone (JQ654947)	99.6

[a] *Band numbers correspond to those presented in* ***Figure 3A*** *for bacterial samples.*

[b] *Taxonomic string with bootstrap values (in parentheses), generated in mothur using SILVA database reference file release 119.*

[c] *Closest relative according to INSA (International Nucleotide Sequence Database).*

[d] *Percentage sequence similarity with closest INSA using BLAST tool.*

was *P. chlamydosporia* because despite high percentage of egg parasitism was recorded, there was no reduction on disease severity after completion of one nematode generation, but it did in field conditions in which the nematode completed more than one.

Despite resistant tomato cultivars suppressed nematode densities and disease severity, as previously reported in plastic greenhouses in Spain (Sorribas et al., 2005; Talavera et al., 2009), *P. chlamydosporia* was also isolated, but the percentage of egg parasitism decreased compared to those on susceptible crops.

TABLE 6 | DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of fungal populations.

Phylotype			Phylogenetic affiliation		
Band code[a]	**Sequence length (bp)**	**Accession code**	**Taxonomic linage[b](Phylum, Class, Order, Family, Genus)**	**Closest match[c](accession no.)**	**Similarity (%)[d]**
F1	231	KT991592	Ascomycota (100), Pezizomycetes (100), Pezizales (100), Pyronemataceae (100), *Pseudaleuria* (100)	Uncultured fungus clone (JF432996)	100
F2	219	KT991593	Ascomycota (100), Pezizomycetes (98), unclassified_Pezizomycetes_order (69)	Uncultured fungus clone (JX323746)	97.72
F3	173	KT991594	Ascomycota (100), Pezizomycetes (99), unclassified_Pezizomycetes_order (75)	Uncultured fungus clone (JX323746)	97.70
F4	189	KT991595	Ascomycota (100), Sordariomycetes (100), Hypocreales (99), Hypocreales_family_incertae_sedis (60), *Fusarium* (60)	*Fusarium equiseti* (KM246255)	98.4
F5	165	KT991596	Ascomycota (100), Dothideomycetes (89), Pleosporales (89), Sporormiaceae (84), *Preussia* (74)	Uncultured fungus clone (JX340328)	100
F6	251	KT991597	Ascomycota (100), Eurotiomycetes (100), Onigenales (100), Arthrodermataceae (100), *Ctenomyces* (100)	Uncultured fungus clone (JX349691)	99.2
F7	186	KT991598	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100), Mortierellales (87), Mortierellaceae (85), *Mortierella* (74)	Uncultured fungus clone (JX377362)	97.85
F8	192	KT991599	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100), Mortierellales (99), Mortierellaceae (98), *Mortierella* (98)	Uncultured fungus clone (JX345268)	99.48
F9	169	KT991600	Ascomycota (100), Dothideomycetes (100), Capnodiales (100), Capnodiales_family_incertae_sedis (100), *Cladosporium* (100)	*Graphiopsis chlorocephala* (JN116693)	100
F10	180	KT991601	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100), Mortierellales (99), Mortierellaceae (94), *Mortierella* (94)	Uncultured fungus clone (JX387233)	98.9
F11	210	KT991602	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (100), *Stachybotrys* (100)	Uncultured *Stachybotrys* clone (KF493978)	100
F12	201	KT991603	Ascomycota (99), Sordariomycetes (96), Microascales (73), Microascaceae (73), *Pseudallescheria* (69)	Uncultured *Pseudallescheria* clone (KM108739)	99.5
F13	173	KT991604	Ascomycota (100), Sordariomycetes (98), Microascales (74), Microascaceae (74), *Pseudallescheria* (66)	Uncultured *Pseudallescheria* clone (KM108739)	100
F14	290	KT991605	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (94), *Psathyrella* (60)	Uncultured fungus clone (GQ225128)	100
F15	292	KT991606	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Psathyrella* (64)	Uncultured fungus clone (GQ225128)	100
F16	249	KT991607	Ascomycota (87), Pezizomycetes (78), Pezizales (76), Pyronemataceae (74), *Heydenia* (50)	Pezizaceae sp (JQ775581)	98.35
F17	220	KT991608	Ascomycota (100), Pezizomycetes (96), Unclassified Pezizomycetes genus (53)	Uncultured fungus clone (JX323746)	97.27
F18	193	KT991609	Ascomycota (100), Sordariomycetes (100), Microascales (75), Microascaceae (75), *Pseudallescheria* (71)	Uncultured *Pseudallescheria* clone (KM108739)	100
F19	193	KT991610	Ascomycota (100), Sordariomycetes (97), Microascales (81), Microascaceae (80), *Pseudallescheria* (79)	Uncultured *Pseudallescheria* clone (KM108739)	100
F20	212	KT991611	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (100), *Stachybotrys* (100)	Uncultured fungus clone (JX348029)	100
F21	202	KT991612	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (93), *Fusarium* (93)	*Fusarium equiseti* isolate (KM246255)	100
F22	194	KT991613	Ascomycota (100), Sordariomycetes (99), Hypocreales (99) Hypocreales_family_incertae_sedis (84), *Fusarium* (83)	Uncultured *Fusarium* clone (KP235758)	97.94
F23	193	KT991614	Ascomycota (100), Sordariomycetes (98), Microascales (85), Microascaceae (85), *Pseudallescheria* (82)	Uncultured *Pseudallescheria* clone (KM108739)	100
F24	221	KT991615	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100), Mortierellales (100), Mortierellaceae (99), *Mortierella* (99)	Uncultured fungus clone (GQ866183)	97.26

(Continued)

TABLE 6 | Continued

Phylotype			Phylogenetic affiliation		
Band code[a]	**Sequence length (bp)**	**Accession code**	**Taxonomic linage[b](Phylum, Class, Order, Family, Genus)**	**Closest match[c](accession no.)**	**Similarity (%)[d]**
F25	305	KT991616	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100
F26	304	KT991617	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	Uncultured Coprinopsis clone (GQ219811)	100
F27	298	KT991618	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (99), *Psathyrella* (61)	Uncultured fungus clone (GQ225128)	100
F28	287	KT991619	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	Uncultured *Coprinopsis* clone (GQ219811)	100
F29	307	KT991620	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100
F30	204	KT991621	Ascomycota (100) Sordariomycetes (100), Microascales (99), Microascaceae (99), *Pseudallescheria* (99)	Uncultured fungus clone (JX383001)	94.2
F31	204	KT991622	Ascomycota (100), Dothideomycetes (100), Capnodiales (100), Capnodiales_family_incertae_sedis (100), *Davidiella* (53)	*Cladosporium sphaerospermum* (KP174687)	100
F32	245	KT991623	Ascomycota (96), Sordariomycetes (90), Microascales (61), Microascaceae (60), *Scedosporium* (47)	Uncultured fungus clone (JQ989314)	99.59
F33	298	KT991624	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (99), *Psathyrella* (58)	Uncultured fungus clone (GQ225128)	100
F34	297	KT991625	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100
F35	308	KT991626	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	Uncultured *Coprinopsis* clone (GQ219811)	100
F36	305	KT991627	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100
F37	279	KT991628	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Coprinopsis* sp. (AB499044)	100
F38	206	KT991629	Fungi_phylum_incertae_sedis (99), Fungi_class_incertae_sedis (99), Mortierellales (99), Mortierellaceae (95), *Mortierella* (95)	Uncultured soil fungus clone (JX489813)	100
F39	209	KT991630	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (95), *Fusarium* (95)	*Fusarium equiseti* isolate (KM246255)	100
F40	313	KT991631	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinellus* (100)	Uncultured fungus clone (JX353314)	100
F41	211	KT991632	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (100), *Stachybotrys* (100)	Uncultured *Stachybotrys* clone (KF493978)	100

[a] *Band numbers correspond to those presented in* ***Figure 3C*** *for fungal samples.*
[b] *Taxonomic string with bootstrap values (in parentheses), generated in mothur using Findley database.*
[c] *Closest relative according to INSA (International Nucleotide Sequence Database).*
[d] *Percentage sequence similarity with closest INSA using BLAST tool.*

A positive relation ($r = 0.89$, $P = 0.042$) between egg density on roots (logarithm) and percentage of egg parasitism was found demonstrating the density dependent relationship, as previously stated (Bourne and Kerry, 1998).

Lettuce cultivated from November to March or September to November reduced or maintained nematode densities in soil, but the number of eggs on roots was fewer when planted in November than in September. Absolute minimum and maximum soil temperatures from November to March were 5.1°C and 21.0°C, and 16.5°C and 29.1°C from September to November. Thus, in lettuce planted in September, RKN was able to accumulate enough degree days to complete its life cycle, to produce more eggs and to maintain densities in soil than when cultivated from November to March in which no eggs were produced and fewer nematodes were recovered from soil. The date of planting has an important repercussion in the life cycle of *Meloidogyne* because after root penetration, the nematode needs to accumulate a minimum number of degree days over a specific temperature threshold to complete its life cycle, otherwise, the crop will act as a trap crop. Some crops as lettuce, radish, and arugula have been used as trap crops (Cuadra et al., 2000; Melakeberhan et al., 2006). In north-eastern Spain, lettuce acted as a trap crop when it was transplanted in middle October or November, but not in September when the nematode was able

to accumulate enough degree days to produce eggs (Ornat and Sorribas, 2008).

The cover crop of mustard cv. Caliente 119 (a blend of white mustard, *Sinapis alba,* and Indian mustard, *Brassica juncea*) was used as green manure. After its incorporation into the soil, nematode densities dropped considerably as well as percentage of fungal egg parasitism at the end of the following lettuce crop. Mustard cv. Caliente 119 has been shown effective against plant-parasitic nematodes and soil-borne fungi (Potter et al., 1998; Charron and Sams, 1999; Friberg et al., 2009). Nevertheless, *P. chlamydosporia* survived, being recovered after the following resistant tomato crop in 2013.

DGGE fingerprints revealed the occurrence of fungal and bacterial species that have been reported associated with the cuticle of RKN J2 or egg masses, or *Heterodera glycines* cysts (Nour et al., 2003; Adam et al., 2014; Cao et al., 2015), but the effect of the majority of them on viability of the nematode is unknown. Some of them such as *Mortierella* spp., Sphingobacteriales, and *Methylobacterium* spp. (reported associated with the J2 cuticle), and *Flexibacter* spp. (associated with the cysts of *H. glycines*) were identified in non-suppressive and one or both suppressive soils. *Davidiella* spp. reported associated with the J2 cuticle was only identified in non-suppressive soil M10.33; *Sphingobacterium* spp., reported associated with *H. glycines* cysts was only identified in M10.55. *Steroidobacter* spp. and *Lysobacter* spp. were reported associated with RKN egg masses and were only identified in M10.23. Several species of *Lysobacter* spp., affect egg hatching of *Meloidogyne* sp. (Chen et al., 2006; Lee et al., 2014) and reduced disease severity in pot tests (Lee et al., 2013).

Diversity indices were similar for both suppressive and non-suppressive soils. In fact, suppressiveness is more related to microbial functionality than diversity. In both suppressive soils, *P. chlamydosporia* was the only and most prevalent fungal egg parasites recovered from RKN eggs throughout the study and deemed to be one of the factors responsible for soil suppressiveness in M10.23, and the most responsible in M10.55. In this study, a density dependent relationship between percent of egg parasitism and density of eggs in roots was found, according to that reported by Bourne and Kerry (1998). Moreover, great variability in virulence of several isolates coming from the same soil was also found. It is known that isolates of *P. chlamydosporia* from the same or different soils differ greatly in growth, development and virulence, in their saprophytic and parasitic ability, and in their ability to colonize the plant rhizosphere (Kerry and Hirsch, 2011). Thus, the environmental plasticity and variability in the virulence showed in this study could be a strategy to persist in a given site, even at low densities. *P. chlamydosporia* was fully adapted to these soil environments and agronomical management practices. It was recovered from eggs in field and pot experiments in site M10.55, or in pot experiments from non-sterilized soils despite no eggs being produced in the majority of crops in field conditions in site M10.23. This plasticity could explain why *P. chlamydosporia* has been found more frequently in the last years in north-eastern Spain, since integrated and organic production systems have been increasingly implemented by growers (Verdejo-Lucas et al., 2002; Giné et al., 2014).

This research provides new information about the antagonistic potential of soils against RKN in two sites used for commercial production of vegetables under organic standards in plastic greenhouse during two growing seasons. *P. chlamydosporia* was the main biotic factor responsible of suppressiveness in site M10.55, because it was the only fungal species recovered from RKN eggs in the field study and pot experiments, and no other antagonist species or effects on RKN were identified by DNA sequencing from DGGE or in pot experiments. However, in M10.23, RKN suppressiveness could be attributed to a combination of microbes, because despite *P. chlamydosporia* was isolated from eggs, some other microorganism with antagonistic effect against the nematode were identified by DGGE and results from pot test agree with their mode of action. Besides the biotic factors identified in both sites, a combination of several agronomic practices such as crop rotation, including RKN resistant cultivars and cover crop as green manure, the addition of organic amendments, and date of planting, can contribute to prevent nematode build-up. These findings will lead to further studies deep in the knowledge of the relations between microbial communities and crop management that achieve soil suppressiveness, in order to design strategies to improve the antagonistic potential of soil.

AUTHOR CONTRIBUTIONS

This work is a part of the Ph.D. thesis of AG, directed by FS, AG, and FS were involved in field studies, pot tests, isolation and identification of fungal egg parasites, and pathogenicity tests. MC, MM, and NG we involved in DGGE analysis. All authors contributed to the writing of the manuscript and approved submission.

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Concilience in Entomopathogenic Nematode Responses to Water Potential and Their Geospatial Patterns in Florida

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The geospatial patterns of four species of native entomopathogenic nematodes in Florida were previously shown to be related to soil properties that affect soil water potential. Here we compared the responses to water potential of third stage, infective juvenile (IJ), *Steinernema* sp. (Sx), and *Steinernema diaprepesi* (Sd) in controlled conditions. The two species were selected because they are closely related (*Steinernema glaseri*-group), but tend to occupy different habitats. In columns of sandy soil with moisture gradients ranging from field capacity (6% w:w) to saturated (18%), Sx migrated toward wetter soil whereas Sd migrated toward drier soil. Survival of two isolates each of Sx and Sd for 7 days in the absence of food was greatest at 18 and 6% soil moisture, respectively. After three cycles of migration through soil to infect insect larvae 10 cm distant, Sd dominated EPN communities when soil columns were maintained at 6% moisture, whereas Sx was dominant in soil maintained at 18% moisture. When rehydrated after 24 h on filter paper at 90% RH, 50% of Sd survived compared to no Sx. Two isolates of Sd also survived better than two isolates of Sx during up to 24 h in a hypertonic solution (30% glycerol). The behavioral responses of both species to water potential and osmotic gradients were consistent with surveys in which Sx was recovered only from flatwoods ecoregions with shallow water tables and poorly drained soils, whereas Sd most frequently inhabited the central ridge ecoregion comprising well-drained soils and deeper water tables. Comparative proteomic analysis revealed differential expression of proteins involved in thermo-sensation (guanylyl cyclase and F13E6-4) and mechano-sensation and movement (paramyosin, Actin 3, LET-99, CCT-2), depending on whether Sd was in soil at 6 or 18% moisture. Proteins involved in metabolism, lectin detoxification, gene regulation, and cell division also differed between the two conditions. Our data suggest the plausibility of modifying soil moisture conditions in flatwoods orchards in ways that favor more desirable (effective) EPN species. Similarly, these particular behavioral traits are likely to be useful in guiding the selection or engineering of EPN species for use in different ecoregions.

Keywords: conservation biological control, entomopathogenic nematodes, soil water potential, proteins

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INTRODUCTION

Florida citrus orchards are inhabited by diverse and abundant communities of native entomopathogenic nematodes (EPNs) that contribute significantly to the biological control of soilborne insect pests (Beavers et al., 1983; McCoy et al., 2000; Duncan et al., 2003, 2007). These nematodes, in the genera *Steinernema* and *Heterorhabditis*, infect insect larvae and then release symbiotic bacterial entomopathogens that kill the insect. Of nine EPN species reported to inhabit citrus orchards and adjacent natural areas in Florida, four are encountered most frequently. *Heterorhabditis indica* Poinar, Kanunakar, and David is virtually ubiquitous in Florida orchards, while other species are more restricted in the habitats they occupy (Duncan et al., 2003, 2007, 2013; Campos-Herrera et al., 2013a,c). Orchards on the central ridge usually support populations of *Steinernema diaprepesi* Nguyen and Duncan and often *Heterorhabditis zealandica* Poinar. An undescribed *Steinernema* sp. *glaseri*-group is found occasionally in orchards in the various flatwoods regions where *S. diaprepesi* and *H. zealandica* are infrequently encountered. The central ridge is an ecoregion characterized by higher elevation and deep, well-drained sandy soil in contrast to flatwoods regions which are at lower elevation and have shallow water tables and finer textured sandy soils that tend to be less well-drained than those of the central ridge.

The abundance of the root weevil pest *Diaprepes abbreviatus* L. is generally greatest in poorly drained areas of flatwoods orchards and least in well-drained soils on the central ridge (McCoy, 1999). The potential role of EPNs in modulating weevil population density is poorly understood. EPN communities tend to be less species rich and diverse in habitats that favor large *D. abbreviatus* populations, compared to habitats with fewer weevils, but EPN population size does not appear to differ greatly between those habitats (Campos-Herrera et al., 2013a). Mechanisms other than EPN population density that might enhance EPN regulation of weevils on the central ridge compared to the flatwoods include better efficacy in the more porous, central ridge soils (Campos-Herrera and Gutiérrez, 2009), greater EPN species diversity and richness (Jabbour et al., 2011), and/or the occurrence of more effective EPN species (Gaugler, 1999). Greenhouse experiments indicated that *S. diaprepesi* and *Steinernema* sp. reduced weevil feeding damage to citrus roots more than did either *H. indica* or *H. zealandica* (El-Borai and Duncan, 2007; El-Borai et al., 2012). Widespread occurrence of *S. diaprepesi* in central ridge orchards is consistent with lower weevil numbers there. However, the infrequent occurrence of *Steinernema* sp. in flatwoods orchards may favor larger weevil populations in EPN-depauperate areas.

The abundant, but regionally distinctive EPN communities in Florida orchards suggest the possibility of developing conservation biological control tactics to modify less conducive habitats in ways that make them more favorable to certain EPN species. Moreover, the difference in habitats occupied by two closely related species in the *S. glaseri*-group V (Spiridonov et al., 2004; Campos-Herrera et al., 2011), suggests the potential utility of *S. diaprepesi* and *Steinernema* sp. as models to study habitat adaptation. Campos-Herrera et al. (2013a) analyzed the spatial patterns of EPNs and more than 30 biotic and abiotic properties of soils in citrus orchards across the Florida peninsula. Four soil properties that affect soil water potential (water holding capacity, organic matter, clay content and depth to ground water) explained the most variability in EPN communities. Accordingly, in the present study we compared the responses of *S. diaprepesi* and *Steinernema* sp. to a variety of conditions with water potentials similar to those encountered in the central ridge (from dry to field capacity) and flatwoods (from dry to saturated) soil habitats in Florida. Our hypothesis was that *S. diaprepesi* and *Steinernema* sp. would respond positively to drier and wetter conditions, respectively. We also characterized differences in protein expression by *S. diaprepesi* in wetter and drier soil conditions in order to identify proteins that are potentially involved in the adaptation to soil water potential.

MATERIALS AND METHODS

Entomopathogenic Nematode Cultures

Steinernema diaprepesi (Sd), *Steinernema* sp. *glaseri*-group (Sx), *Heterorhabditis zealandica* (Hz), and *H. indica* (Hi) were originally isolated from caged *D. abbreviatus* larvae buried for several days in commercial citrus orchards in Florida. Morphological and molecular analyses were performed for identification upon collection (Nguyen, 2007; Campos-Herrera et al., 2011). Several geographic isolates of all EPN species were maintained in pure cultures in the laboratory using the last instar larvae of the greater wax moth, *Galleria mellonella* L. (Woodring and Kaya, 1988). Infective juveniles (IJs) that emerged from insect cadavers into White traps were harvested in tap water and stored at 15°C for 1–5 days before use. The isolates Sd Hancock (GPS coordinates and Genbank accession number; 28.293232, −82.257191, GU173996) and Sx Webber (28.2526, −82.4741; not submitted) were used in all experiments. Two additional isolates, Sd Bartow (27.885035, −81.756878, GU173994) and Sx Arcadia (27.2273, −81.9649, GU174002), were used to further test and validate species differences in some experiments described below. The *S. diaprepesi* isolates were collected from sites 80 km distant from each other and those of Sx were separated by 122 km.

EPN Migration in Moisture Gradients

Sand columns were constructed as described by El-Borai et al. (2011) with the modification of using a 3.5 cm length of Tygon® tubing to tightly connect two glass jars (17 cm^3 volume each; BTL, sample type 111, CLR, SNAPC; Wheaton Science Products, Millville, NJ.). One jar was filled with sand at 6% moisture (wt water/dry wt soil) and the other jar was filled with sand at 18% moisture. The connecting tube was filled with sand at 10% moisture. All parts were connected together and 200 IJs of Sd or Sx in 0.2 ml water were introduced into the center of the column through a small hole in the Tygon tubing. The sand used in all experiments was obtained by washing and autoclaving a sandy soil through a 40 mesh sieve (0.425 mm openings) onto a 100 mesh sieve (0.150 mm openings) where it was washed repeatedly. The resulting clean sand facilitated recovery of test nematodes by rinsing the substrate from jars into 100 mL water, swirling the suspension, permitting the soil to settle for 3–4 s, and

decanting the nematodes for counting (Abou-Setta and Duncan, 1998). Soil columns were arranged in a plastic box lined with moist paper with equal numbers of jars containing 6 or 18% soil moisture pointing in the same cardinal direction. After 24 h the columns were disassembled and nematodes from each jar were recovered and counted. Two experiments were conducted, each with 15 replications. Paired *t*-tests were used to determine whether the number of recovered IJs differed significantly from one soil moisture to the other.

EPN Survival and Soil Moisture in the Absence of Host Insects

The experimental units consisted of sterile Petri dishes (60 × 15 mm) filled with soil obtained from two treatments in an ongoing field experiment near Auburndale on the Florida central ridge (81:48:44.65W, 28:06:53.98N, 49 m elevation). Soil from an "advanced production system" (APS) treatment designed to grow citrus trees more quickly was fertigated daily for 2 years via drip irrigation (Schumann et al., 2012). Soil from a conventional citriculture treatment was fertilized with dry fertilizer four times annually and irrigated as needed by microsprinklers. The APS treatment changed several soil properties compared to those under conventional citriculture and reduced the abundance of naturally occurring Sd in the field plots (Campos-Herrera et al., 2013c, 2014). The soil was either oven dried (70°C) in one trial and autoclaved and air dried before used in a second trial. The moisture content (g water per 100 g dry soil) of the soil was adjusted to 2% (very dry), 6% (field capacity), and 18% (saturated) by mixing with tap water. Approximately 1300–1400 IJs in 0.5 ml of tap water were pipetted onto the soil surface of each plate. The moisture level adjustments were calculated to include the water in the nematode inoculum. Twenty replications for each EPN species/soil moisture/soil type were prepared. All plates were sealed with parafilm and incubated at 27°C. After 7 days, soil and inner dish surfaces of each plate were rinsed into Baermann funnels and IJs were recovered after 1 week. In a second trial, the nematode extraction was done directly using sucrose centrifugation (Jenkins, 1964). All live IJs were counted with the aid of an inverted compound microscope (20–400×). Treatment effects within each species were determined by two-way ANOVA (cultural practice x moisture level) followed by Tukey's HSD-test ($P = 0.05$). The experiment was conducted twice.

Sandy soil from a citrus orchard at the Citrus Research and Education Center in Lake Alfred was autoclaved, air-dried, and used to test the survival of two isolates each of Sd and Sx at soil moistures of 6 and 18%. The experiment was conducted as described previously with Baermann funnels used to recover IJs.

EPN Community Structure and Soil Moisture in Presence of Host Insects

Petrie dish assays at soil moistures of 6 and 18% were conducted as described in the previous section except that air dried Candler sand (97:2:1, sand:silt:clay) soil was used and a mixture of 500 IJs each of Sd, Sx, Hi, and Hz were added to each dish. Treatments were replicated 30 times. After 7 days, IJs were extracted from half of the assay dishes per treatment as described above. The other half were inverted and placed on the top of soil columns contained in a PVC tube (10 × 5-cm-diameter). The soil moisture in each column was the same (6 or 18%) as that of the soil in the inverted Petrie dish. The columns were wrapped in aluminum foil and maintained at room temperature (23 ± 3°C) for 14 days, after which they were secured with duct tape to the top of a second, identical soil column containing four *G. mellonella* larvae. Fly screen (2 mm openings) was fastened with glue to the bottom of each column section to secure the larvae within the bottom section. The sand columns were again covered with aluminum foil and maintained for two additional weeks after which they were disassembled. The bottom section was secured to the top of an identical column containing four *G. mellonella* larvae and the IJs in the top section were recovered using sucrose centrifugation (Jenkins, 1964) and counted with an inverted compound microscope to facilitate species determination. The community composition in the top section at this point was considered the first "generation." The process was repeated twice more (where survivors in the bottom section were used to initiate a new round of competition) to produce three experimental generations during which the species competed with one another in two soil moisture conditions. For each generation and moisture level, the proportion of the total EPN population of each species was compared by ANOVA and differences were separated by Tukey's HSD.

Relative Humidity, Hypertonicity, and EPN Survival

Glass specimen jars (150 ml volume, 5 cm diameter) containing 115 ml solution of 15, 30, and 80% (wt:wt) glycerin in water produced an estimated ambient relative humidity of 95, 90% and 50%, respectively (Foruney and Brandl, 1992). Approximately 2000 IJs of Sd or Sx in 50 µl water were pipetted onto each of three filter paper strips (2 × 0.5 cm) contained in the lids of Petrie dishes (3.8 cm diameter) that were floated on the solutions in the sealed jars at room temperature. After 24 h strips were placed into water in individual Petrie dishes where IJs rehydrated for 24 h before being counted as dead (non-motile) or alive. Non-motile specimens were touched with an eyelash probe to determine whether they responded with movement. The experiment was repeated once.

Aliquots of ca. 2000 IJs of two isolates each of Sd and Sx were also placed directly into solutions of 30% glycerol in 8-ml sample bottles (Wheaton, Corp. Millville, NJ). After 12, 18, and 24 h, three bottles containing each nematode isolate were poured onto 38-mm opening sieves and backwashed into counting dishes. After 24 h rehydration, IJ motility was evaluated as described above. In the first trial, the isolates Sd Hancock and Sx Webber were compared with one another. In a second trial Sd Bartow and Sx Arcadia were used. Data were expressed as percent survival and were transformed to arcsin-square root before analysis of variance at each time point followed by Tukey's HSD-test for mean separation. In a third trial, all four isolates were compared together in the same experiment.

Protein Expression at Different Soil Moistures

Washed sand was prepared as previously described and soil moisture adjusted to 6% or 18%. Approximately 1300–1400 IJs of either Sd or Sx in 0.5 ml of distilled water were pipetted onto the soil surface of each plate and 20 replicate plates of each treatment were sealed and maintained as previously described. After 48 h IJs were recovered by rinsing and decanting as previously described. IJs were further separated from soil debris by centrifugation in a $MgSO_4$ gradient (Kaplan and Davis, 1990). IJs were then incubated with four volumes of ice-cold acetone at −20°C overnight, after which the proteins were further cleaned with 2-D Clean-up Kit (GE) following the manufacturer's instructions. Sd and SX proteins were dissolved in rehydration buffer [7 M urea, 2 M thiourea, 60 mM dithiothreitol (DTT), 65 mM 3-(3-cholamidopropyl dimethylammonio)-1-propanesulfonic acid (CHAPS), 2% Trion X-100, 0.2% ampholytes 5–8] and briefly sonicated. After a 14,000 g centrifugation at ambient temperature for 30 min, the protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). For the first dimension of isoelectric focusing (IEF), 350 μg of solubilized proteins was dissolved in 300 μl of rehydration buffer with trace bromophenol blue dye, and loaded onto a 17-cm immobilized pH gradient (IPG) linear pH 5–8 strip (Bio-Rad). After an active rehydration step at 20°C for 13 h at 50 V, the strips were automatically focused using the following parameters: 250 V, linear, 1 h; 500 V, slow, 1 h; 1000 V, slow, 1 h; 5000 V, slow, 1 h; 10,000 V, linear, 3 h; 10,000 V, rapid, 70,000 Vh (Lu et al., 2010). The current limit for each strip was set to 50 μA. After IEF, the strips were first incubated in equilibration buffer (6 M urea, 20% glycerol, 2% SDS and 0.375 mM Tris–HCl, pH 8.8) containing 2% (w/v) DTT for 15 min with gentle shaking, then a second equilibration in equilibration buffer containing 2.5% (w/v) iodoacetamide instead of DTT for 15 min. Equilibrated IPG strips were further resolved with 9.5% SDS-PAGE gels (1.5 mm gel thickness), and the program was 5 mA/gel for 40 min, then 30 mA/gel until the bromophenol blue dye front reached the bottom of the gel. Each treatment was resolved with 2-DE at least three times to obtain reliable and statistically significant results. The gels were stained following a modified Colloidal Coomassie G-250 staining protocol (Dyballa and Metzger, 2009). The stained gels were scanned and imported into Melanie 7 software for various analyses including spot detection, matching, and quantitative intensity analysis. Three independent experiments were performed to validate the results. Only those unique or significantly different protein spots ($P < 0.05$) were selected and subjected to identification by mass spectrometry (MS).

LC-MS/MS was done in the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, Gainesville. The protein was reduced, alkalated in-gel, and digested with trypsin (Promega) at 37°C overnight. The enzymatically digested samples were injected onto a capillary trap (LC Packings PepMap) and desalted for 5 min with a flow rate 3 μl/min of 0.1% (v/v) acetic acid. The samples were loaded onto an LC Packing® C18 Pep Map nanoflow HPLC column. The elution gradient of the HPLC column started at

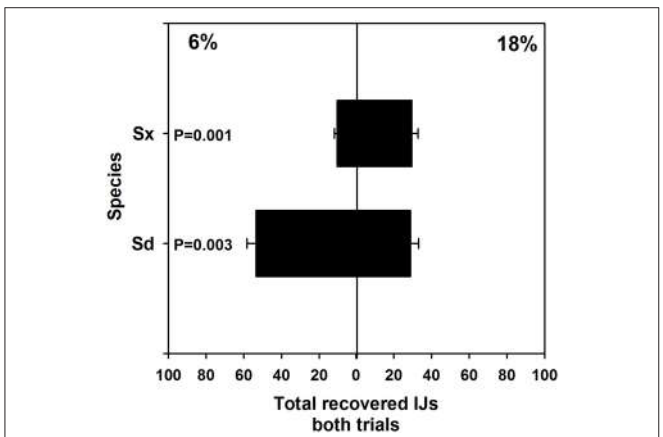

FIGURE 1 | Mean (and standard error) number of infective juvenile *Steinernema* sp. and *S. diaprepesi* recovered from the field capacity (6%) or saturated (18%) ends of soil columns with moisture gradients. Nematodes were recovered 24 h after placement in the center of the horizontal sand columns. Sd , *Steinernema diaprepesi*, Sx, *Steinernema* sp.

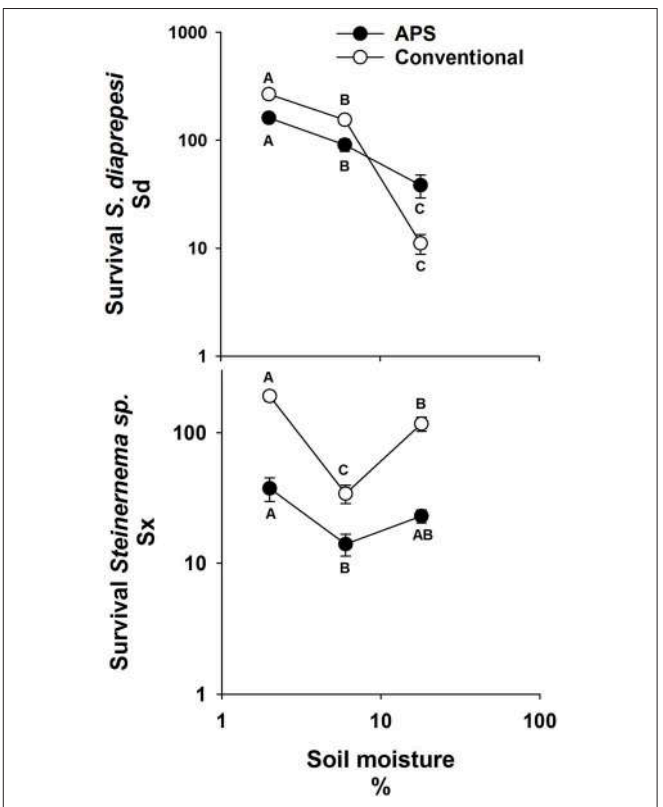

FIGURE 2 | Mean (and standard error) number of infective juvenile *Steinernema* sp. and *S. diaprepesi* recovered following 7 d of storage in field capacity (6%) or saturated (18%) sand that originated from experimental field plots managed with conventional or advanced citriculture methods. Data shown on log scaled axes. Points on the same curve with the same letters do not differ significantly ($P > 0.05$) according to Tukey's HSD-test. Sd = *Steinernema diaprepesi*, Sx = *Steinernema* sp.

3% solvent A (0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H_2O), 97% solvent B (0.1% v/v acetic acid, 96.9% v/v ACN, and 3% v/v H_2O), and finished at 60% solvent A, 40% solvent B for 60 min for protein identification. LC-MS/MS analysis was carried out on a LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, West Palm Beach, FL). The ion spray voltage was set to 2200 V. Full MS scans were acquired with a resolution of 60,000 in the orbitrap from m/z 300 to 2000. The five most intense ions were fragmented by collision induced dissociation (CID). Dynamic exclusion was set to 60 s. Tandem mass spectra were extracted. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0). Mascot was set up to search NCBI_other Metazoa databases assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 ppm. Iodoacetamide derivative

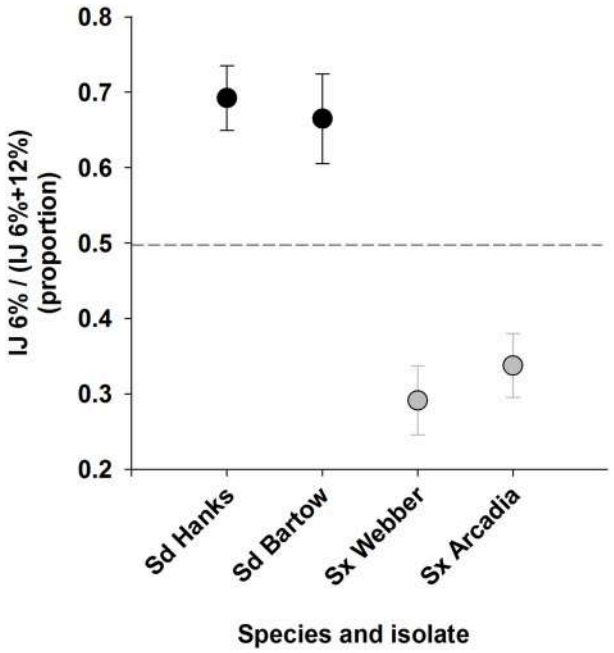

FIGURE 3 | Recovery of two isolates each of infective juvenile *Steinernema* sp. and *S. diaprepesi* recovered following 7 d of storage in field capacity (6%) or saturated (18%) sand that originated from a citrus orchard. Proportions were calculated as numbers of nematodes recovered from sand at field capacity divided by the total number of nematodes recovered from soil at both soil moistures. Sd = *Steinernema diaprepesi*, Sx = *Steinernema* sp.

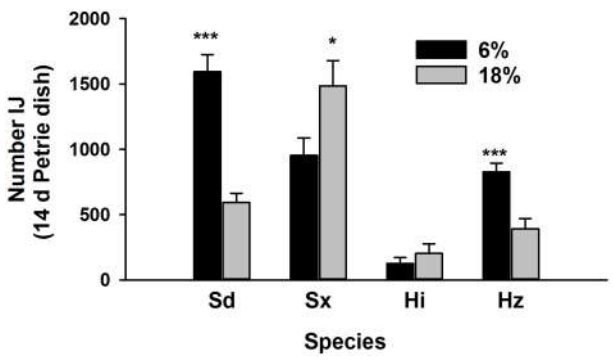

FIGURE 4 | Recovery of four species of entomopathogenic nematodes (Sd = *Steinernema diaprepesi*, Sx = *Steinernema* sp., Hi = *Heterorhabditis indica*, Hz = *H. zealandica*) after 14 days in Petrie dishes filled with sand moistened to field capacity (6%) or saturated (18%). Significant differences in recovery from each moisture for each species denoted by $^{***}P < 0.001$ and $^{*}P < 0.05$.

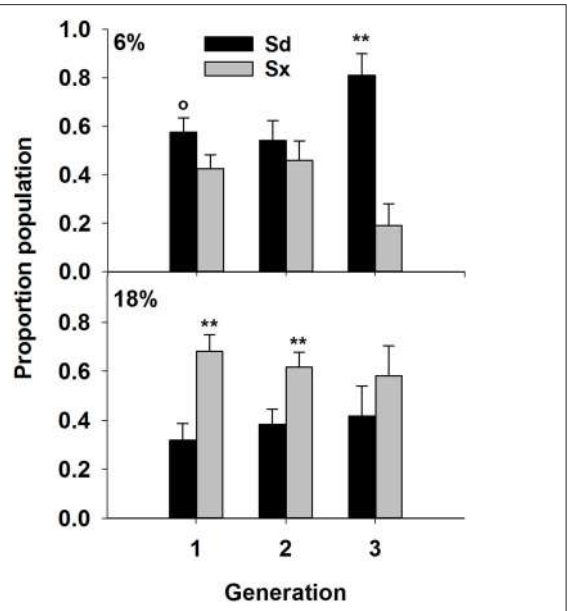

FIGURE 5 | Proportion of the total nematode population represented by Sd (*Steinernema diaprepesi*), or Sx (*Steinernema* sp.) for three "generations" of nematodes maintained with *Galleria mellonella* larve in PVC columns filled with sand at field capacity (6% moisture) or saturated (18%). Significant differences between species within a generation from each moisture for each species denoted by $^{**}P < 0.01$ and $^{\circ}P < 0.10$.

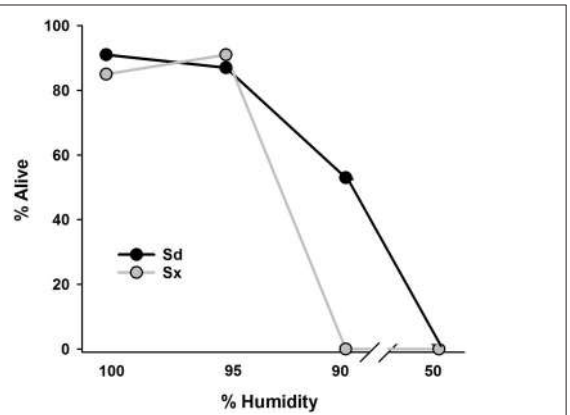

FIGURE 6 | Percentage of surviving infective juvenile Sd (*Steinernema diaprepesi*) or Sx (*Steinernema* sp.) after 24 h storage at 100, 95, 90, or 50% relative humidity. Humidity conditions were created by floating Petrie dish lids containing filter paper and EPNs on either tap water or glycerol solutions in sealed containers.

of Cys, deamidation of Asn and Gln, oxidation of Met, are specified in Mascot as variable modifications. Scaffold (version Scaffold-4.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002; Nesvizhskii et al., 2003). Protein identifications was accepted if they could be established at >95.0% probability and contain at least two identified unique peptides.

RESULTS

EPN Migration in Moisture Gradients

Pooled results from two experiments revealed that Sd and Sx migrated preferentially toward drier and wetter conditions, respectively (**Figure 1**). Sixty-five percent of the recovered Sd ($P = 0.003$) and 61% of the recovered Sx ($p = 0.001$) migrated toward their preferred moisture levels.

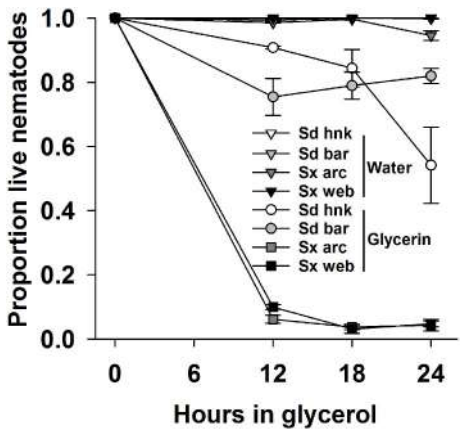

FIGURE 7 | The proportion survival of two isolates each of Sd (*Steinernema diaprepesi*) and Sx (*Steinernema* sp.) stored for 12, 18, or 24 h in water or 30% glycerin solution, then rinsed and stored for 24 h in water. Error bars are SEM.

EPN Survival and Soil Moisture in the Absence of Host Insects

Significantly more of both EPN species survived in soil managed by either cultural practice (APS or CC) when soil moisture was 2% than when it was wetter (**Figure 2**). Regardless of cultural practice, five-fold more Sd survived at 6% than at 18% moisture ($P = 0.01$), whereas just 34% as many Sx survived at 6% compared to survival at 18% ($P = 0.01$). Survival of Sd was significantly lower in APS soil than in that of CC when moisture was at 2 and 6%. Survival of Sx was also lower in APS compared to CC soil when moisture was at 2% and at 18%. The different soils did not affect survival of either species at the soil moisture least favorable for its survival.

Two isolates each of Sd and Sx responded consistently to maintenance at 6% or 18% soil moisture (**Figure 3**). Approximately two-thirds of the total Sd IJs were recovered from the dryer soil and a similar proportion of Sx IJs were recovered from the wetter soil. The proportion recovery of each species from the two soil conditions differed from 50% ($P < 0.001$) and there were no significant differences in response to moisture between the two isolates of either species.

EPN Community Structure and Soil Moisture in Presence of Host Insects

Nematode survival after 7 days in the Petrie dishes was similar to previous results (**Figure 4**). Survival of Sd and Sx was greatest at 6% ($P = 0.001$) and 18% ($P = 0.05$), respectively. Fewer Hi survived than other species and soil moisture had no effect on the species. The 6% treatment favored survival of Hz ($P = 0.001$). The three subsequent generations contained communities of just Sd and Sx (**Figure 5**). In two of the three generations, Sd was the dominant community member in soil

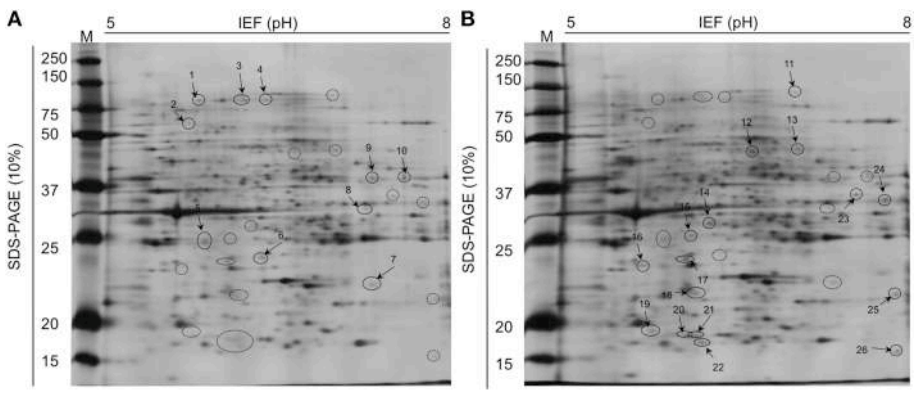

FIGURE 8 | Two-dimensional gel electrophoresis comparisons of total proteins isolated from *Steinernema diaprepesi* maintained for 48 h in sand at 6% moisture (A) and 18% moisture (B). IPG strips of linear pH 5–8 were used for isoelectric focusing and the SDS-PAGE was performed in 10% acrylaMIDE. Protein molecular weight standards are shown on the left. Spots of statistically significant differences ($P < 0.05$) between the condition were selected to be identified using mass spectrometry and listed in **Table 1**. Spots labeled 1–10 were proteins expressed at highest levels in 6% moisture sand, those from 11 to 26 were expressed at higher levels in 18% moisture sand.

TABLE 1 | Differentially-expressed proteins from *Steinernema diaprepesi* grown in sand at 6% or 18% moisture, as identified by LC-MS/MS.

	No.[a]	Protein name[b]	NCBI Accession	Fold change[c] [(18–6%)/6%]	Coverage[d] (%)	Matched unique peptides[d]	Protein identification probability[d] (%)	Molecular and biological function	Known role in nematode
Structural proteins (muscles/ movement)	1	Paramyosin	gi|127760	–4	20	20	100	Major component of thick filaments in many invertebrate	Necessary for determination of nematode thick filament length *in vivo* (Mackenzie and Epstein, 1980)
Mechanical response	2	Paramyosin	gi|127760	S6%	20	24	100	Major component of thick filaments in many invertebrate	Necessary for determination of nematode thick filament length *in vivo* (Mackenzie and Epstein, 1980)
	13	ACT-3 (Actin 3)	gi|14278	2.2	5	5	100	The actin cytoskeleton drives the cellular rearrangements underlying morphogenesis, through regulated polymerization or actomyosin contraction.	
	28	LET-99	gi|17541472	–1.8	1.3	1	99	LET-99 is a DEP (Disheveled/EGL/Pleckstrin) domain protein (nematode specific)	Required for the proper orientation of spindles after the establishment of polarity (Tsou et al., 2002). *no homolog in non-nematode
	26	CCT-2 (Chaperonin containing T-complex polypeptide 1 subunit beta)	gi|1046266	7.5	2	1	100	Known to play a role, *in vitro*, in the folding of actin and tubulin	
	10	ACT-3 (Actin 3)	gi|14278147 NP_505817	–5.2	15	2	100	The actin cytoskeleton drives the cellular rearrangements underlying morphogenesis, through regulated polymerization or actomyosin contraction.	
Gene regulation/cell division	3	HIS-44 (Histone 44)	gi|17532989	–1.7	20	3	100	One of the five main histone proteins involved in the structure of chromatin in eukaryotic cells. Featuring a main globular domain and a long N terminal tail H2B is involved with the structure of the nucleosomes of the 'beads on a string' structure	
	16	HIS-67(Histone 67)	gi|17509199	3.1	17	2	100	One of the five main histone proteins involved in the structure of chromatin in eukaryotic cells. Featuring a main globular domain and a long N terminal tail H2B is involved with the structure of the nucleosomes of the 'beads on a string' structure	

(Continued)

TABLE 1 | Continued

	No.[a]	Protein name[b]	NCBI Accession	Fold change[c] [(18–6%)/6%]	Coverage[d](%)	Matched unique peptides[d]	Protein identification probability[d](%)	Molecular and biological function	Known role in nematode
	14	HIS-67(Histone 67)	gi|17509199	S18%	26	3	100	One of the five main histone proteins involved in the structure of chromatin in eukaryotic cells. Featuring a main globular domain and a long N terminal tail H2B is involved with the structure of the nucleosomes of the 'beads on a string' structure	
	4	HIS-67(Histone 67)	gi|17509199	–4.2	29	3	100	One of the five main histone proteins involved in the structure of chromatin in eukaryotic cells. Featuring a main globular domain and a long N terminal tail H2B is involved with the structure of the nucleosomes of the 'beads on a string' structure	
	7	EEF-2, isoform a	gi|156279	–1.3	5	7	100	Main regulator of peptide chain elongation in eukaryotic cells.	
	8	EEF-2, isoform a	gi|156279	6%		8	100	Main regulator of peptide chain elongation in eukaryotic cells.	
	9	AKT-1, isoform a	gi|71983636	–7	2	1	98	Regulator of apoptosis in limiting cytokine concentrations	
	11	CYN-4 (Peptidyl-prolyl cis-trans isomerase 4)	gi|17532641	1.3	2.5	1	97	Required for body wall muscle cell development.	
Thermal response	6	GCY-6 (Guanylyl Cyclase)	gi|453232464	S6%	1	1	98	Guanylyl cyclase synthesizes cGMP from GTP in response to calcium level	Thermosensory receptor only in left neuron in terms of lateral bisymetry in *C. elegans* (Inada et al., 2006)
	27	F13E6-4	gi|71987524		2	1	97	F13E6.4 gene encodes a protein that shows sequence similarities to YAP	Involved in the thermotolerance and aging in *C. elegans*. (Iwasa et al., 2013)
	20	F13E6-4	gi|71987524	3.5	2	1	95	F13E6.4 gene encodes a protein that shows sequence similarities to YAP	Involved in the thermotolerance and aging in *C. elegans*. (Iwasa et al., 2013)
	12	F13E6-4	gi|71987524	S18%	2	1	91	F13E6.4 gene encodes a protein that shows sequence similarities to YAP	Involved in the thermotolerance and aging in *C. elegans*. (Iwasa et al., 2013)
metabolism	15	R12C12.1	gi|17535605	S18%	1.3	1	93	Glycine dehydrogenase	
	17	R12C12.1	gi|193208127	3.7	3.2	1	97	Glycine dehydrogenase	

(Continued)

TABLE 1 | Continued

	No.[a]	Protein name[b]	NCBI Accession	Fold change[c] [(18–6%)/6%]	Coverage[d] (%)	Matched unique peptides[d]	Protein identification probability[d] (%)	Molecular and biological function	Known role in nematode
	21	F46H5.3	gi|32566409	3.5	4.9	1	98	Arginine kinase	
	23	FUM-1	gi|17553882	1.4	6	2	100	Fumarase; converts fumaric acid to L-malic acid in the TCA cycle	
	22	MDH-2	gi|17554310	3.7	5	1	99	Methanol/ethanol family dehydrogenase	
Lectin detoxification	18	LEC-3	gi|17535117	2.9	4	2	100	32 kDa beta-galactoside-binding lectin lec-3	
	19	LEC-3	gi|17535117	S18%	3.8	1	99	32 kDa beta-galactoside-binding lectin lec-3	
	5	C08F11.7	gi|17538574	–2.1	4.1	1	98	Putative protein	

[a] *The spot numbers correspond to the numbers in 2DE gels in figure X.*

[b] *Proteins identification was done using C. elegans database.*

[c] *Quantitative analysis was performed with Melani 7 software.*

[d] *The three parameters of protein identification were generated using MASCOT software.*

of 6% moisture and Sx dominated communities in soil at 18% moisture.

Relative Humidity, Hypertonicity, and EPN Survival

Survival of both EPN species after 24 h on filter paper was >80% when incubated at 95% and 100% RH and none of either species survived at 50% RH (**Figure 6**). At 90% RH, about half of the Sd and none of the Sx remained motile.

When incubated in 30% glycerin solution, both Sd isolates survived at higher rates than either isolate of *Steinernema* sp. (**Figure 7**). All IJs were flattened, distorted and apparently completely desiccated upon removal from the glycerin. All appeared normally rehydrated after 24 h recovery in water; however dead IJs were internally disorganized. Survival for both isolates of Sx ranged between 2 and 10% after 12 and 24 h in glycerin with virtually no mortality of IJs in water. The survival of Sd IJs isolated from Bartow remained above 75% throughout the experiment. The Sd IJs isolated from Hancock behaved similarly until mortality increased to about half after 24 h. Both isolates of Sd survived better ($P = 0.001$) than either isolate of Sx. Results of the experiments when repeated were essentially the same.

Sd Protein Expression at Different Soil Moistures

Twenty-six proteins occurred in different amounts in IJs, depending on soil moisture conditions (**Figure 8**). Ten proteins were detected at higher concentration and 16 proteins at lower concentration in IJs stored in soil at 6% moisture for 24 h compared to IJs stored at 18% moisture. Histone 67 and Actin 3 were present in different forms at higher and lower levels in IJs from both soil moisture conditions. Differential expression occurred for proteins involved in thermo-sensation (guanylyl cyclase and F13E6-4) and mechano-sensation and movement (paramyosin, Actin 3, LET-99, CCT-2). Proteins involved in metabolism, lectin detoxification, gene regulation and cell division were also among those that differed between the two conditions.

DISCUSSION

The responses of Sd and Sx to different hydraulic and osmotic conditions were consistent with their natural geospatial patterns and with the hypothesis that they are physiologically adapted to drier and wetter soil conditions, respectively. Presumably, Sd is adapted to drier conditions in the central ridge than is Sx, in part due to a superior ability for osmoregulation and desiccation survival. Conversely, the attraction to moist soil exhibited by Sx and its ability to persist better than Sd in wetter conditions suggest the occurrence of adaptive behaviors that were not addressed in this study. These experimental results lend support to the causative nature of correlations reported between variables that modulate soil moisture and the occurrence of EPNs in Florida orchards (Campos-Herrera et al., 2013a).

Both survival and orientation by these two species were affected by soil moisture. The ability of each species to distinguish and migrate toward its favored water potential may have been detrimental for those IJs in their respective, least favorable treatment of the survival assay because those treatments were uniformly too wet (Sd at 18%) or too dry (Sx at 6%). Quiescence increases the survival rate of many nematode species by conserving energy reserves during commonly encountered, unfavorable soil conditions such as desiccation, anoxia, or host unavailability (reviewed by Evans and Perry, 2009). As for Sd and Sx in the present study, sandy soil at 2% and even lower moisture was shown previously to increase the survival rate of *Steinernema riobrave* compared to survival rates at higher moistures (Duncan et al., 1996; Duncan and McCoy, 2001). At such low water potential, the surface film of water on soil particles is unlikely to be thick enough to permit nematode motility, thereby inducing quiescence or even partial anhydrobiosis. However, where moisture is adequate for movement, as in the field capacity (6%) or saturated (18%) treatments, IJs may have responded to unsuitable water potentials with hyperactivity in search of more favorable conditions, thereby expending more energy and dying more quickly than IJs in more favorable conditions. Alternatively, if the dissolved oxygen concentration was reduced in the saturated compared to field capacity soils, differences in the capacity for anoxic quiescence may have caused the differences in survival rates of Sd and Sx (Kung et al., 1990).

The phylogenetic similarity of Sd and Sx, combined with their different phenotypic responses to water and osmotic potential, make them potentially useful to study environmental adaptation. The fundamental mechanisms of specific adaptive behaviors such as osmoregulation (Choe, 2013), anhydrobiosis (Erkut et al., 2013), and humidity sensation/orientation (Russell et al., 2014) are being increasingly resolved with respect to nematodes, primarily *Caenorhabditis elegans*. These findings are making it possible to study how suites of these behaviors are modulated by species for adaptation to specific habitats. For example, the different levels of guanylyl cyclase expressed by Sd in soil at 6 or 18% moisture may be indicative of responses to moisture variation. Russell et al. (2014) demonstrated that humidity is detected by *C. elegans* by interpreting thermos-sensory and mechano-sensory signals. Mutant worms deficient in guanylyl cyclases, required for thermosensation, were unable to perform hygrotaxis. Similarly, variation in levels of some of the proteins in this study involved in movement, metabolism and development may play roles in adaptive responses by Sd to variation in water potential. Further comparison of protein expression between populations of Sd and Sx that vary in their responses to moisture and hypertonic stress will help eventually to make the linkages between specific behaviors and habitat adaptation.

Regional geospatial patterns of EPN species are increasingly characterized, usually in conjunction with descriptive information about the sampled habitats (e.g., Mekete et al., 2005; Mwaitulo et al., 2011; Zadji et al., 2013; Valadas et al., 2014; Wang et al., 2014). Reports for a few commonly encountered species are consistent enough to speculate broadly about their biome preferences (see Hominick, 2002). More recently, surveys have been designed and analyzed to reveal associations between EPNs and specific edaphic properties that might affect EPN occurrence (Hoy et al., 2008; Kaspi et al., 2010; Kanga et al., 2011; Campos-Herrera et al., 2013a,b, 2016). This is the first report of behavioral and physiological differences between EPN species that conform to documented variation in habitat preference. Understanding the mechanisms by which EPNs adapt to a particular habitat could have practical applications by revealing how to screen existing populations, modify gene expression, and/or change habitat properties in ways that extend the geographic boundaries of otherwise promising native or introduced EPN species.

AUTHOR CONTRIBUTIONS

FE and LD conceived and conducted all bioassays, NK perfomed proteomic analysis. All authors contributed to writing the manuscript.

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The Anti-*Phytophthora* Effect of Selected Potato-Associated *Pseudomonas* Strains: From the Laboratory to the Field

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Late blight, caused by the oomycete *Phytophthora infestans*, is the most devastating disease of potato. In organic farming, late blight is controlled by repeated applications of copper-based products, which negatively impact the environment. To find alternative solutions for late blight management, we have previously isolated a large collection of bacteria from the phyllosphere and the rhizosphere of potatoes. Here we report the antagonistic potential of these strains when co-cultivated with *P. infestans* as well as with other potato pathogens. We then focused on three *Pseudomonas* strains and compared their protective impact against late blight to that of well-known biocontrol strains *in planta* using a high-throughput leaf disk assay with automated picture analysis. When sprayed on the leaves of potatoes in the greenhouse, the strains were able to survive for at least 15 days. Under field conditions, populations decreased faster but all tested strains could still be retrieved after 8 days. The most active strain *in vitro*, *P. chlororaphis* R47, was also the best protectant on leaf disks from plants grown in the greenhouse experiment, but its protection potential could not be verified in the field due to unfavorable infection conditions. However, its protective effect against *P. infestans in planta*, its survival in the phyllosphere as well as its ability to colonize the potato rhizosphere in very high population densities, suggest a potential for field application, e.g., in the form of tuber treatment or leaf spray.

Keywords: ***Phytophthora*, *Pseudomonas*, *Solanum tuberosum*, leaf disk, biocontrol**

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INTRODUCTION

Over the last decades, the need to move from intensive agriculture to a more sustainable way of food production has risen in the awareness of growers and consumers. However, crop production is threatened by a variety of abiotic and biotic factors, such as changing climate or the occurrence of disease-causing agents. In potato production, yield losses are mostly due to the oomycete *Phytophthora infestans*, which causes late blight and can lead, upon favorable infection conditions, to massive destruction of the crop within a few days (Fry, 2008). In conventional farming, late blight is typically controlled through repeated applications of various fungicides, whereas copper-based products are commonly used in organic farming (Cooke et al., 2011; Axel et al., 2012). In view of its accumulation in the soil and of its toxicity toward the soil fauna,

copper use represents an environmental hazard and alternative solutions to control late blight are needed to ensure sustainable potato production (Dorn et al., 2007). Biocontrol organisms have been suggested as a putative alternative to chemical protectants in the protection against diseases (Velivelli et al., 2014). Such antagonistic bacteria or fungi have been proven efficient under field conditions and some of them are available as commercial products and routinely used by farmers, such as *Pseudomonas chlororaphis* MA 342, which protects cereals against some seed-borne fungal diseases (Johnsson et al., 1998), or *Bacillus amyloliquefaciens* FZB42, which acts both as a plant growth promoting and as a biocontrol agent (Chen et al., 2007). However, although numerous studies have tested the effect of microbial inoculants on late blight (reviewed in Axel et al., 2012), none has so far demonstrated a protection against late blight in the field. This might be at least partly due to the fast spreading of *Phytophthora* during humid conditions, which is mediated by the production of sporangia that, depending on temperature, can either directly infect plant tissues or release motile zoospores, which in turn infect new leaves (Fry, 2008). A successful biocontrol agent would therefore need to be able to inhibit not only the pathogen's mycelial growth but also the formation and/or the germination of its sporangia, as was recently reported for a *Lysobacter* strain producing cyclic dipeptides (Puopolo et al., 2014). In an attempt to find such a biocontrol agent against potato late blight, we have previously isolated bacterial strains from the phyllosphere and rhizosphere of potato, which we hypothesized to be adapted to the host plant (Hunziker et al., 2015). In this earlier work, we reported the ability of potato-associated bacteria to inhibit growth and sporulation of *Phytophthora infestans* through the emission of volatile organic compounds (Hunziker et al., 2015). However, the gap between results obtained in controlled laboratory conditions and true protective potential in the field can be very large (Dorn et al., 2007) and further studies are needed that include testing the strains' ability to establish in sufficient densities in the targeted plant organs (roots vs. shoots) and their efficiency *in planta* as well as under field conditions. The present study investigates these questions using a subset of *Pseudomonas* strains which showed promising protective effects *in vitro*. The strains' root and leaf colonization capacity as well as their effect on plant growth and development were assessed. Their protective effect *in planta* was then analyzed using a newly developed high through-put leaf disk assay. Finally, the strains' survival and efficiency under field conditions was monitored in a microplot experiment.

MATERIALS AND METHODS

Chemicals and Culture Media

Luria-Bertani medium (LB) was used to cultivate bacteria. LB agar plates were prepared by dissolving 20 g L^{-1} of Difco LB Broth, Lennox (BD) mixed with 15 g L^{-1} agar (Agar Agar, ERNE surface AG). PIA medium was prepared by dissolving 45 g L^{-1} of *Pseudomonas* Isolation Agar (Fluka) in distilled water to which 20 ml L^{-1} of glycerol (Sigma–Aldrich) was added. Fungi and oomycetes grew on rye agar (RA), malt agar (MA), or potato dextrose agar (PDA). RA was prepared by simmering 200 g rye grains (winter rye cv. Picasso95) in tap water for ca. 1 h. The filtered liquid (1.5 mm mesh) was filled up to a volume of 1 L with tap water and 20 g L^{-1} agar was added. For the initial screen (**Table 1**), RA without glucose was used, for later experiments, RA was supplemented with 5 g L^{-1} glucose. MA was prepared with 15 g L^{-1} Difco malt extract agar (BD) and 12 g L^{-1} agar, and PDA contained 39 g L^{-1} PDA (Oxoid). One experiment was performed on water agar (WA) containing dH_2O and 6 g L^{-1} agar. When needed, rifampicin (PanReac AppliChem) was added at a concentration of 50 μg mL^{-1}.

Strains, Culture Conditions and Preparation of Inoculation Suspensions

The bacterial strains used in this study are described in a previous publication (Hunziker et al., 2015). Additionally, *Pseudomonas protegens* CHA0 and *Pseudomonas* DSMZ 13134 were included as controls in most experiments. *Dickeya dianthicola* was obtained from S. Schaerer (Agroscope). For the greenhouse and field experiments, rifampicin-resistant derivatives of selected strains were obtained by streaking a high density of pure bacterial culture onto a LB plate containing rifampicin (50 μg mL^{-1}). Spontaneous rifampicin resistant colonies were visible 2 days later; one colony for each strain was streaked on a fresh LB plate containing the same concentration of rifampicin. After 2 days, glycerol stock was prepared with each stable mutant strain. Bacterial strains were kept at −80°C in 25% glycerol for long-term storage. A polyspore isolate of *Phytophthora infestans* sampled in 2001 in Zurich Affoltern (provided by H. Krebs, Agroscope) was used for all experiments. This isolate was grown on RA in the dark at 18°C, and was regularly transferred to potato tuber slices for host passages. The fungi *Rhizoctonia solani*, *Helminthosporium solani*, obtained from P. Frei (Agroscope), were grown on PDA and MA respectively. *Fusarium oxysporum* was recovered from a contaminated *P. infestans* host passage in 2013 and was grown on RA. Long-time storage of all fungi was done in 25% glycerol at −80°C. All potato pathogens were continuously grown on agar media and agar plugs (ø 5 mm) were transferred to fresh medium plates when borders of the previous plate were reached. Plates were stored in the dark at ca 20°C. To obtain *P. infestans* sporangia suspensions, mycelium was detached from overgrown agar plates and suspended in tap water. The suspension was filtered through autoclaved gauze and the number of sporangia adjusted to the desired final concentration using a Thoma chamber (Marienfeld Superior, Germany). The suspension was stored at 5°C in the dark until use. Unless otherwise specified, bacterial suspensions were prepared by harvesting overnight LB agar cultures and resuspending the cells in 0.9% NaCl. For the microplot field application, densities were adjusted by adding tap water.

In Vitro Screening of Bacterial Strains for Activity Against Different Potato Pathogens

Thirty two bacterial strains showing antagonistic potential in a preliminary screening (Hunziker et al., 2015) were tested in

TABLE 1 | Antagonistic activity of 32 bacterial strains isolated from rhizosphere (R) or shoots (S) of field-grown potato plants.

Strain	Affiliation	*P. inf*	*H. sol*	*R. sol*	*F. oxy*	*D. dian*
R47	*P. chlororaphis*	**8**	**11**	**69**	**81**	**35**
R32	*P. vranovensis*	**10**	**14**	**51**	**88**	**41**
R84	*P. marginalis*	**22**	**36**	**81**	**92**	**38**
R82	*P. marginalis*	**15**	**12**	**91**	**82**	**73**
S49	*P. fluorescens*	**46**	**37**	**73**	**79**	**51**
R73	*Bacillus* sp.	**7**	**27**	94	**84**	88
R54	*Bacillus* sp.	**13**	**18**	98	**83**	**87**
S50	*P. moraviensis*	**10**	66	**76**	**91**	**58**
S01	*Streptomyces* sp.	**12**	**60**	104	**60**	**81**
R01	*P. moraviensis*	**30**	**66**	**95**	98	**33**
R76	*P. fluorescens*	**25**	**38**	**91**	**93**	**81**
S35	*P. marginalis*	**27**	**67**	**81**	**80**	**73**
S06	*P. frederiksbergensis*	101	**21**	**82**	99	**66**
S24	*P. frederiksbergensis*	**92**	**22**	**95**	**85**	**75**
S22	*P. syringae*	**85**	**22**	**81**	**95**	89
S19	*P. frederiksbergensis*	100	**21**	**89**	**91**	**72**
R95	*P. lini*	72	**21**	**93**	99	97
R02	*P. veronii*	**56**	**60**	**88**	**91**	92
S46	*Curtobacterium* sp.	**35**	**78**	95	**84**	97
S27	*Arthrobacter* sp.	**50**	nd	nd	**90**	94
R74	*P. frederiksbergensis*	**89**	**28**	101	**92**	**83**
R31	*Sporosarcina* sp.	**39**	nd	nd	98	99
S34	*P. jessenii*	**96**	**30**	**90**	99	**81**
R85	*Rhodococcus* sp.	**41**	84	104	**91**	**79**
R29	*Bacillus* sp.	**65**	**53**	100	**94**	**87**
S04	*P. frederiksbergensis*	**97**	**22**	97	98	91
R61	*Arthrobacter* sp.	**8**	113	**115**	99	92
S25	*Curtobacterium* sp.	77	**71**	**90**	98	93
R60	*Arthrobacter* sp.	**15**	**132**	**112**	**87**	93
R75	*P. frederiksbergensis*	82	95	98	**86**	88
R42	*Microbacterium* sp.	**14**	**153**	**107**	**92**	**89**
R96	*Flavobacterium* sp.	111	96	99	**93**	**80**

Strains were tested against the oomycete Phytophthora infestans (P. inf), the fungi Helminthosporium solani (H. sol), Rhizoctonia solani (R. sol), and Fusarium oxysporum (F. oxy) as well as against the phytopathogenic bacterium Dickeya dianthicola (D. dian). Strains are ordered according to their overall activity (most active first). Numbers indicate the average growth of 3–4 replicates in percentage of the control (inhibition in red, stimulation in green). Bold values indicate values significantly different from the control according to a Student's t-test. nd, not determined (strains that did not grow on the fungal medium used in the direct assay).

a dual culture assay for inhibitory effects toward five different potato pathogens: the oomycete *P. infestans*, the fungi *H. solani*, *R. solani*, *F. oxysporum* and the bacterium *D. dianthicola*. The antagonistic bacteria and the pathogens were grown on the same Petri dish, which was filled with either RA (*P. infestans, F. oxysporum*), PDA (*H. solani, R. solani*), or LB (*D. dianthicola*). Two strains, S27 and R31, could not be grown on PDA and were thus not tested against *H. solani* and *R. solani*. The time point of the application of bacterial strains varied, as the potato pathogens showed different growth speeds. It was on the same day for *D. dianthicola*, 1 day later for *R. solani* and *F. oxysporum*, 4 days later for *P. infestans* and 7 days later for *H. solani*. Each fungus or oomycete was inoculated as a plug and *D. dianthicola* was inoculated as 10 µl drop of liquid culture adjusted to $OD_{570} = 1$ in the center of the Petri dish. Three 10 µl drops of overnight bacteria cultures adjusted to $OD_{570} = 1$ (or LB for the control) were spotted at the border of the Petri dishes. Plates were incubated at 20°C in the dark and the pathogen growth area was assessed by picture analysis (after 3 days for *R. solani*, 7 days for *D. dianthicola* and *F. oxysporum*, 14 days for *P. infestans* and 28 days for *H. solani*) using the image processing program ImageJ (Schneider et al., 2012). This experiment was carried out in four replicates per bacterial strain (five control plates). The average growth of the pathogens in presence of the different strains was compared with that of the pathogens grown in absence of the strains (control) and a percentage of control growth was calculated.

Greenhouse Experiment

Rifampicin-resistant derivatives of selected *Pseudomonas* strains (R47, R76, S35, CHA0, *Pseudomonas* DSMZ 13134, see section above for the generation of these strains) were inoculated onto potato tuber sprouts and tested for their effect on plant development (cv. Charlotte and cv. Victoria) and for

their survival in the rhizosphere. For the sprout inoculation experiment, bacterial cells were suspended in 0.9% NaCl and adjusted to $OD_{570} = 1$. The sprouts were moistened by spraying them with a solution containing 0.9% NaCl and subsequently 1.5 mL of the bacterial cell suspension was pipetted next to the sprout. To prevent a wash out of the inoculated cells, pots were not watered during the first 24 h following inoculation. Additionally bacteria suspensions of the same strains were sprayed onto potato leaves (∼15 mL per plant), in order to assess the survival of the bacterial population for a period of 15 days. Inoculation of potato sprouts was done 1 day before potting, by the application of 1.5 mL unwashed bacterial cells suspended in 0.9% NaCl and adjusted to $OD_{570} = 1$ (0.9% NaCl was used for the control). Each treatment was replicated six times. BBCH stage and plant height (distance between the soil surface and the apical meristem) were measured once a week between the period from sprouting until flowering (4–33 days after planting). The survival of the sprout-inoculated *Pseudomonas* strains in the rhizosphere was assessed 11 weeks after potting. To this end, the washed root system of two replicate plants was cut in small pieces and suspended in 15 mL 0.9% NaCl. After a sonication step of 5 min, the suspension was tenfold serially diluted and 100 μL of each dilution was plated on a PIA plate supplemented with rifampicin (50 $\mu g\ L^{-1}$). After 3 days of incubation at 20°C in the dark, colony forming units (CFUs) were counted from the most appropriate dilution. The survival of bacteria sprayed on potato leaves ($OD_{570} = 1$) was investigated within a period of 2 weeks at the days 1, 3, 8, and 15. From each treatment three 5 mm diameter leaf disks were cut and suspended in 200 μL 0.9% NaCl. The plant tissue was homogenized using a small plastic pestle and after sonication and tenfold serial dilution (see above), 5 μL of each dilution was spotted twice on a LB plate containing rifampicin, which was then lifted to let the drop fall and spread the CFUs. After 3 days of incubation at 20°C, CFUs were counted from the most appropriate dilution.

Establishment of a High Through-put Potato Leaf Disk Assay with Automated Picture Analysis to Monitor *P. infestans* Infection

In order to determine the appropriate sporangia concentration as well as the application strategy aiming at optimum infection pressure (necrosis development and sporangiophore appearance on the leaf surface), sporangia suspensions in different concentrations ($6.25{\cdot}10^4$, $1.25{\cdot}10^5$, $2.5{\cdot}10^5$, $5{\cdot}10^5$ sporangia mL^{-1}) were applied in a 10 μL drop on the upper or the lower side of potato leaf disks (ø 17 mm), cut from potato plants cv. Victoria 39 days after planting. Leaf disks were placed on a previously watered filter paper in a standard Petri dish and inoculated with the respective sporangia suspension. The Petri dishes were placed in a lightproof plastic box and incubated at 18°C for a period of 8 days. When the leaf disks showed first infection symptoms (after 3 days), daily pictures (dimensions 5184 × 3456) were taken with a reflex camera (Canon EOS 700D) and the increase of necrotic plant tissue (days 3–7) and sporangiophore cover (day 8) was analyzed by the automated picture analysis macroinstructions developed for this purpose in the freeware program ImageJ (see Supplementary Material).

Testing the Protective Potential of Bacteria Applied on Leaf Disk Against *P. infestans*

Using this newly developed leaf disk method with automated picture analysis, the effect of selected bacterial strains (*Pseudomonas* strains R47, R76, S35, CHA0, DSMZ 13134) on disease progression was monitored. To this end, bacteria and sporangia suspensions were mixed and applied on the lower side of leaf disks (cv. Victoria, 18 replicates). The final sporangia concentration was $1.25{\cdot}10^5$ mL^{-1} and bacteria were applied at two population densities: $OD_{570} = 0.3$ and 3 (corresponding approximately to $2{\cdot}10^8$ and $2{\cdot}10^7$ cells/ml). The experimental set up was the same as described above and after the application of 10 μL of the combined suspensions, the leaf disks were incubated for 8 days at 18°C. The necrotic leaf tissue and the sporangiophores were measured with the automated picture analysis macroinstructions (see Supplementary Material) 4 days after inoculation and 8 days after inoculation respectively. A separate set of leaf disks inoculated with the mixed suspension was used to take microscope pictures of the sporangia, which were exposed to the bacteria at a concentration of $OD_{570} = 3$ (corresponding approximately to $2{\cdot}10^9$ cells/ml). Pictures were taken 4 days after inoculation, when the necrotic area of the control treatment reached 60% of the leaf disk area.

Sporangia Germination

The sporangia germination in mixed sporangia-bacteria suspension was analyzed, when sporangia were exposed to the strains in population densities of $OD_{570} = 3$, $OD_{570} = 0.3$ and $OD_{570} = 0.03$ (0.9% NaCl was used as control). Fifteen micro liter of the mixed suspension was applied on a 0.6% WA plug placed on microscope glass slides. The sporangia germination behavior was assessed after 3 days of incubation at 18°C in the dark. Treatments and controls were replicated four times and randomly placed on the glass slides. Additional control plugs ($n = 20$) were incubated separately from treated plugs to verify whether the control plugs incubated on the same glass slides as the treated ones would be influenced in any way. The number of germinating sporangia per plug was calculated as percent of germinated sporangia relative to the total number of sporangia (23–106 per plug depending on sporangia density). This percentage was then compared to the germination percentage of the control.

Microplot Experiment

In order to determine the protection potential of bacterial strains under field conditions, a microplot experiment was carried out in Zurich Affoltern, Switzerland. Each microplot consisted of one row of five potato plants. Per treatment, four replicates were allocated to four blocks in which they were randomly distributed. Each block was surrounded on all sides by a single row of border plants of the cultivar Panda (low susceptibility to late blight). The blocks with borders were separated from one another by a single

row of plants of the cultivar Bintje (high susceptibility to late blight). The plants (cv. Victoria) were planted on 15th of April 2015. Distances were 30 cm between plants and 70 cm between rows. From day 44 after planting on, the plants were regularly sprayed with bacterial suspensions of the selected strains (R47, R76, S35, CHA0 and *Pseudomonas* DSMZ 13134), according to the recommendations of the late blight decision support system Bio-PhytoPRE (Musa-Steenblock and Forrer, 2005). The treatment interval ranged between 6 days and 2 weeks. In total, six treatments were carried out. For the suspensions, overnight bacterial cultures were suspended in water and supplemented with 0.1% Nu-Film 17® (Andermatt Biocontrol), a wetting agent intended to enhance adhesion of the cells to the leaf surface. The concentration of the suspensions was adjusted to $OD_{570} = 1$. The suspensions were sprayed from above and below on the plants' leaves, each plant receiving approximately 50 mL of suspension per application. After the last application, which occurred 106 days after planting, the survival of sprayed bacteria was assessed one and 8 days after this last spraying. To this end, three leaf discs (ø 17 mm) were cut and suspended in 2 mL 0.9% NaCl. The leaf tissue was further homogenized using a Polytron PT300 homogeniser (Kinematica AG), with which the samples were shredded by 6000 rpm during approximately 30 s each. After 5 min in a sonication bath, samples were tenfold serially diluted. Five micro liter of each dilution was spotted twice on a PIA plate containing rifampicin and incubated at 20°C in the dark. CFUs were counted from the most appropriate dilution after 3 days incubation. In order to assess the protective potential of the strains, a leaf disk experiment was performed 1 day after spraying as described above. Sporangiophore cover was assessed 6 days after inoculation of the leaf disk.

Statistical Evaluation

In the initial screen (**Table 1**), a Student's *t*-test was used to compare the growth inhibition of each bacterial strain to the negative control. The evaluation of subsequent experiments was done with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA), performing one-way ANOVAs with Tukey's *post hoc* tests.

RESULTS

Growth Inhibition of Pathogens by Potato-associated Bacterial Strains in Dual Cultures

Thirty two potato-associated bacterial strains previously identified as potentially active based on their emission of volatiles (Hunziker et al., 2015) were tested for their effects in direct competition assays with five different potato pathogens: the oomycete *Phytophthora infestans*, the fungi *Helminthosporium solani*, *Rhizoctonia solani*, *Fusarium oxysporum* and the bacterium *Dickeya dianthicola*. Among those 32 strains, five *Pseudomonas* were able to significantly inhibit the growth of each target organism, although *R. solani* and *F. oxysporum* were inhibited to a much lesser extent than *P. infestans* and *H. solani* (**Table 1**). This differential reaction of the target organisms to the bacterial strains was further illustrated, e.g., by the fact that *R. solani* was more inhibited in its growth by *Pseudomonas* strains than by the other strains, while *Bacillus strains* R73 and R54 were able to drastically reduce the growth of both *P. infestans* and *H. solani* and the *Streptomyces* strain S01 was the best inhibitor of *F. oxysporum* (60% of its control growth) (**Table 1**). Within the genus *Pseudomonas*, which in general inhibited *P. infestans* more strongly than other targets, the strains affiliated *P. frederiksbergensis* (e.g., S04, S06, S19, S24, R74) all impacted *H. solani* more than *P. infestans*. In contrast, the two *Arthrobacter* strains R61 and R60 drastically reduced the growth of *P. infestans* but barely affected the other target organisms. Based on these results, we selected three promising strains, which showed significant *in vitro* growth inhibition of all pathogens, for further analysis: *P. chlororaphis* R47, the strain with the highest *in vitro* inhibition of *P. infestans,* as well as *P. fluorescens* R76 and *P. marginalis* S35, which showed comparable *in vitro* inhibition but were isolated from different plant parts (rhizosphere for R76 and phyllosphere for S35). In the following experiments, two control *Pseudomonas* strains were included for comparison purposes, *P. protegens* CHA0 (Voisard et al., 1989) and *Pseudomonas* sp. DSMZ 13134.

Effects of Sprout-inoculated Bacterial Strains on Growth and Development of Potato Plants

As a first step toward evaluating the potential of these three selected strains for practical application, it was assessed whether they showed any phytotoxic effect when applied onto the potato tubers. The bacterial strains were inoculated on the tuber sprouts of two different potato cultivars, Victoria (moderately susceptible to late blight) and Charlotte (highly susceptible to late blight), which were monitored for 33 days after planting (Supplementary Figure S1). No significant difference in overall growth between the plants developing from inoculated and non-inoculated sprouts could be observed, thus excluding a phytotoxic effect of the strains (**Figure 1**). No growth promotion was observed either, but some strains led to a more constant growth, i.e., to less variability between individual plants, for instance *P. fluorescens* R76 in the cultivar Charlotte and *P. protegens* CHA0 in both cultivars (**Figure 1**).

Survival of the Strains in the Phyllosphere and Rhizosphere of Potato Plants

The ability of the three selected strains to survive in the phyllosphere and in the rhizosphere was assessed in a greenhouse experiment, using rifampicin-resistant derivatives of the strains. The survival of bacteria in the phyllosphere was monitored at different intervals within a 15 day period after spraying the biocontrol agents onto the leaves of potato plants. After a strong decrease in population abundance within the first few days, the levels stayed almost constant during the second week and remained at ca. 100 CFUs per cm^2 (**Figure 2**). All bacterial strains survived in the two tested cultivars for the entire tested

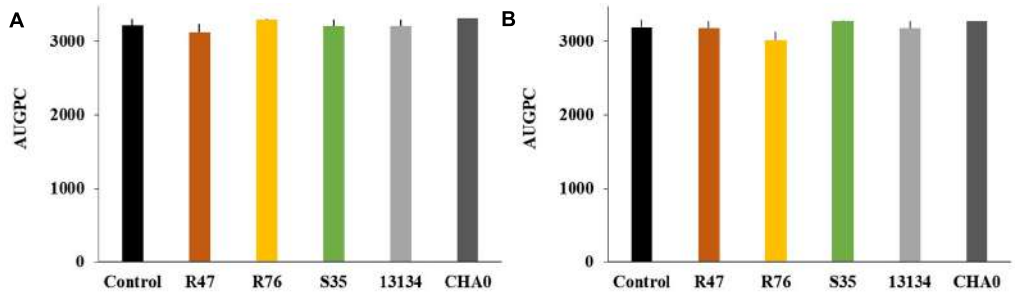

FIGURE 1 | Effects of sprout-inoculated bacteria on the growth of the potato varieties Charlotte (A) and Victoria (B). Overall growth was calculated as AUGPC (the area under growth progression curve, d(BBCH)/dt) (see Supplementary Figure S1). Averages and standard errors are shown. Sprout inoculation did not result in any significant change in the growth of cv. Charlotte [$F(5) = 1.36$, $P = 0.27$] or cv. Victoria [$F(5) = 0.74$, $P = 0.60$] as determined by one-way ANOVA.

period. For the survival in the rhizosphere, washed roots from 11 week-old sprout-inoculated potato plants were used. The abundance of the retrieved inoculated bacteria is shown in a semi-quantitative way in **Table 2**. In general, the number of bacterial CFUs from the root system of the cultivar Victoria was higher than that retrieved from the root system of Charlotte. All strains were able to establish in the rhizosphere and to compete with the natural potato microbiome, thus demonstrating their rhizosphere competence. The strain originally retrieved from the phyllosphere (S35) showed similar to higher colonization densities as the closely related *P. fluorescens* strain isolated from the rhizosphere (R76) (**Table 2**). However, highest colonization capacity was observed for *P. chlororaphis* R47: this strain was not only found in very high abundance in the pots where it had been inoculated, but it was also retrieved in significant amounts from neighboring pots (pots had been randomly placed in the greenhouse but in trays where they were connected through the bottom upon watering events), suggesting very high rhizosphere competence. It cannot be excluded that due to this invasion of *P. chlororaphis* R47, the other strains were restrained in their rhizosphere colonization and that the data presented in **Table 2** therefore underestimate their true colonization potential.

Evaluating the *In Planta* Protection Potential of the Strains Using a Leaf Disk Method

As a next step toward the evaluation of the bacterial strains' *in planta* protection potential, a high through-put leaf disk setup was developed (see Material and Methods for details). Applying the *Phytophthora* sporangia solution onto the bottom side of the leaf disk resulted in quicker disease progression than when sporangia were inoculated on the upper side (Supplementary Figure S2). Moreover, the drop containing the sporangia stayed in place until the end of the experiment, whereas when inoculated onto the upper side of the leaf disk, it often erratically spread and led to multiple infection starting points. To analyze the data in an automated and quantitative manner, a macroinstruction was developed in the freeware ImageJ to measure the necrosis development (days 3–7) and the formation of sporangiophores (day 8). Using a 10 µl drop of a $1.25 \cdot 10^5$ mL^{-1} sporangia solution led to clearly distinguishable necrosis development curves and a significant covering of the leaf disk surface by sporangiophores after 8 days (**Figure 3**).

Using the established leaf disk method, the effect of the three selected bacterial strains were investigated *in planta* in the greenhouse using Victoria as potato cultivar. Victoria, rather than Charlotte, was chosen for the greenhouse and field experiments due to its lesser susceptibility to late blight. Leaf disks from sprout-inoculated potato plants did not show greater tolerance to *P. infestans* than disks from non-inoculated control plants, suggesting that the inoculated strains did not induce resistance (data not shown). When bacteria were applied on the leaves at high concentration, all strains but *P. marginalis* S35 inhibited the formation of *Phytophthora*-induced necrotic lesions (**Figure 4A**). However, when a tenfold dilution of the inoculum was used, only the *P. protegens* CHA0 strain reduced necrotic area significantly, while the others did not. *P. fluorescens* R76, which strongly inhibited the formation of necrosis (8% of the leaf disk surface vs. 48% for the untreated control), was unable to reduce the formation of sporangiophores (**Figure 4B**). In contrast, *P. chlororaphis* R47 and *P. protegens* CHA0 significantly inhibited sporangiophore production in both inoculum densities tested. A very strong concentration-dependency was observed for *Pseudomonas* sp. DSMZ 13134, which conferred excellent protection when applied in high concentrations, but was inefficient or even favoring infection when applying a lower concentration. Autoclaved *Pseudomonas* sp. DSMZ 13134 cells did not confer any protection, suggesting that living bacteria are required for plant protection against *P. infestans*.

Inhibition of Sporangia Germination by the Bacterial Strains

The first step in *Phytophthora*'s infection process is the germination of sporangia and/or zoospores. We therefore assessed whether the bacterial strains used in the leaf disk experiments were able to inhibit this critical step. The overall germination rate in control treatments was about 35%. When applied at high densities, all strains induced a significant

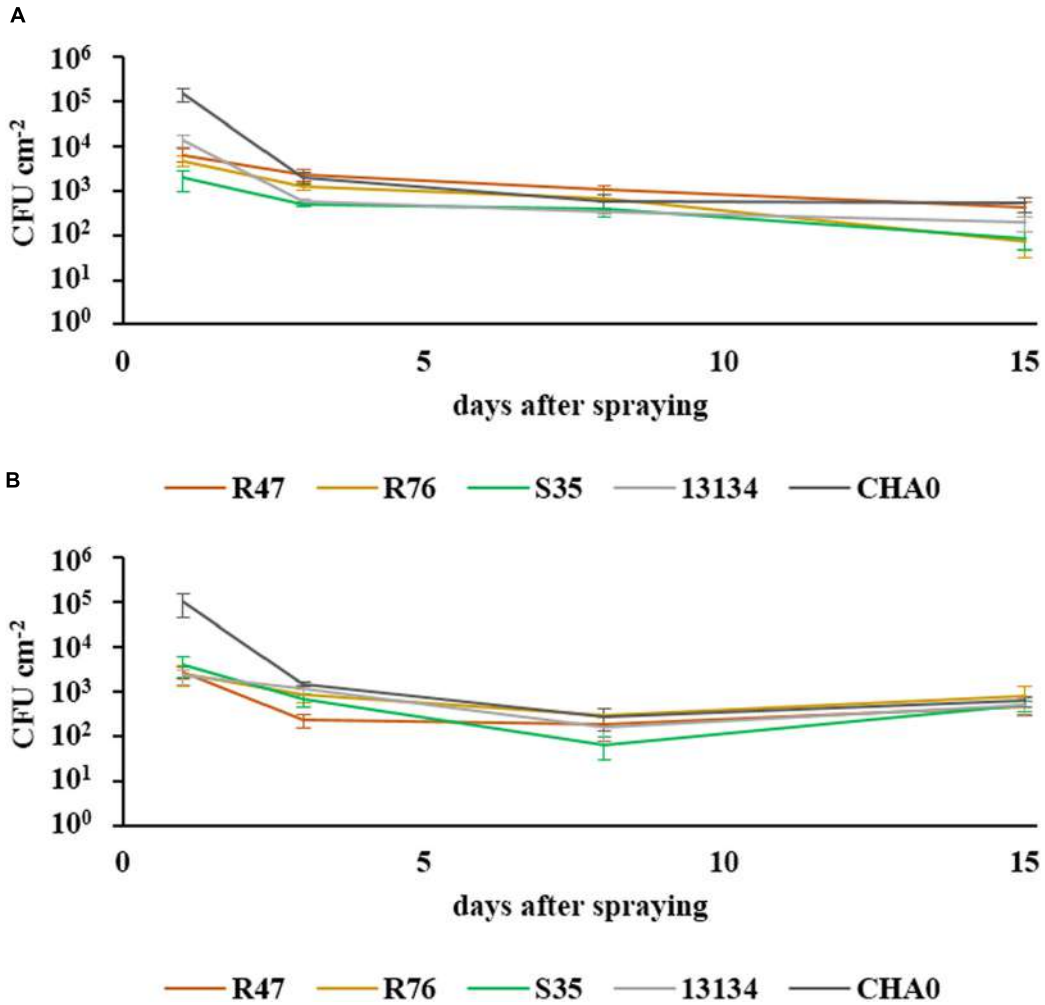

FIGURE 2 | Survival of bacteria in the phyllosphere. The survival of sprayed bacteria on the leaf surface was measured over a period of 15 days in the cultivars Charlotte **(A)** and Victoria **(B)**. Their abundance at defined intervals is shown in CFUs cm^{-2} (SEM, $n = 6$). One-way ANOVA revealed significant differences between the strains after 3 days [$F(4) = 3.92$, $P = 0.013$ for cv. Charlotte and $F(4) = 5.01$, $P = 0.004$ for cv. Victoria], but none after 8 days and only a marginal one for cv. Charlotte at 15 days [$F(4) = 2.77$, $P = 0.049$].

reduction in the percentage of germinated sporangia (**Figure 5A**). The same observation could be made with intermediate densities, except for the control strain *P. protegens* CHA0, which was not significantly different from the untreated control. Low densities of bacteria (OD = 0.03) were ineffective in reducing sporangia germination (**Figure 5A**). In addition to the reduced germination rate, morphological abnormalities such as hyphal swelling and shorter germination tubes could be observed upon treatment with the bacterial strains (**Figure 5B**). No correlation was observed between the *in vitro* effects of the strains on sporangia germination and the protection potential observed on leaf disks. However, when microscopic observations were done on the sporangia drop applied onto the leaf disks, it seemed that the strain S35, which was unable to protect leaf disks against *P. infestans*, also did not inhibit sporangia germination as drastically as the other strains (Supplementary Figure S3).

Survival and Protective Potential of the Strains in Field Conditions

To assess whether our selected strains would be able to protect potato against *P. infestans* under field conditions, we carried out a microplot experiment where potato plants were regularly sprayed with a suspension of the bacterial strains. After the last application, both survival and protection potential were assessed. One day after spraying the bacterial suspensions on the plants, all strains were still present in relatively high abundance (10^5–10^6 cm^{-2}) (**Figure 6**), but their population density dropped within the next days: after 8 days, only few cells per square centimeter of leaf could be retrieved. The microplot experiment was carried out to monitor the protective effects of the strains in field conditions, i.e., with natural *P. infestans* infection. Since this natural infection was prevented by very hot and dry weather conditions during July and August, we tested the protective effect of the strains with our leaf disk experimental setup. This revealed

TABLE 2 | Survival of bacteria in the rhizosphere.

		Inoculated strain	R47
cv. Charlotte	Control	–	+
	R47	++++	++++
	R76	++	++
	S35	+++	++
	13134	++	–
	CHA0	+++	–
cv. Victoria	Control	–	++
	R47	++++	++++
	R76	+++	++
	S35	+++	+++
	13134	+++	+++
	CHA0	+++	+++

The inoculated strains (and R47, right) were retrieved from the roots of 11-week old plants of cv. Charlotte (top) and cv. Victoria (bottom). Abundances were estimated and are expressed as follows (CFU per gram root fresh weight): –, none detected, +, 10^1–10^3, ++, 10^3–10^4, +++, 10^4–10^6, ++++, >10^6.

that the leaf disk method was, at least in our experiments, better suited for the greenhouse screen, since the infection was much less efficient in field-grown plants (**Figure 7**). The infected and the non-infected controls did not differ significantly from each other, which was mostly due to a lesser infection rate of the infected controls (see also **Figure 4**). Therefore, even though significant protective effects were observed after treatment with the strain *P. moraviensis* S35 as well as the two control strains *Pseudomonas* sp. DSMZ 13134 and *P. protegens* CHA0, these results should be interpreted with caution.

DISCUSSION

Using microorganisms as biocontrol agents seems an appealing strategy for sustainable crop protection: in the last decades, much effort has been made to isolate, characterize and use microbial strains to this end, yet the bacterial antagonists available on the market are so far only few (Velivelli et al., 2014). Indeed, many criteria have to be fulfilled for such a biocontrol agent to find its way to the farmer (Köhl et al., 2011). The first step usually taken to select for candidate biocontrol agents is an *in vitro* screening in the laboratory, such as the one we carried out against potato pathogens in the present study (**Table 1**). Compared to the first screen of the potato-associated strains reported in (Hunziker et al., 2015), which focused on volatiles, the present evaluation of the strains' potential activity against a broad range of potato pathogens yielded slightly different results: while the *Pseudomonas* strains classified as the best producers of antifungal volatiles (Hunziker et al., 2015) also were very active in the present study, other, non-*Pseudomonas* strains such as the two *Bacillus* strains R73 and R54 or the *Streptomyces* S01 were much more inhibitory to the potato pathogens when their diffusible substances, rather than only their volatiles, came into play. Moreover, the selective inhibition of *Helminthosporium solani* by strains affiliated with the species *P. frederiksbergensis*

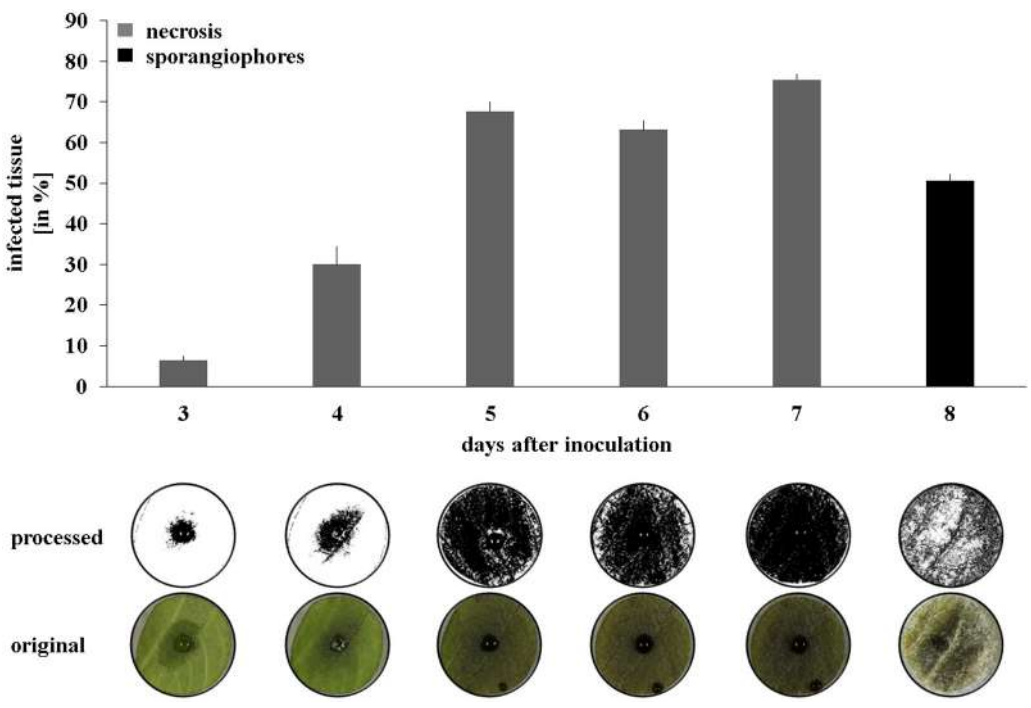

FIGURE 3 | Disease progression on potato leaf disks. Pictures were taken daily between 2 and 8 days after inoculation and processed to quantify necrosis area (days 2–7) and sporangiophore production (day 8). Averages and standard errors are shown ($n = 18$). Pictures were taken with illumination from the top for necrosis measurement and with illumination from the side for sporangiophore measurement. One representative picture and its processed counterpart are shown per day.

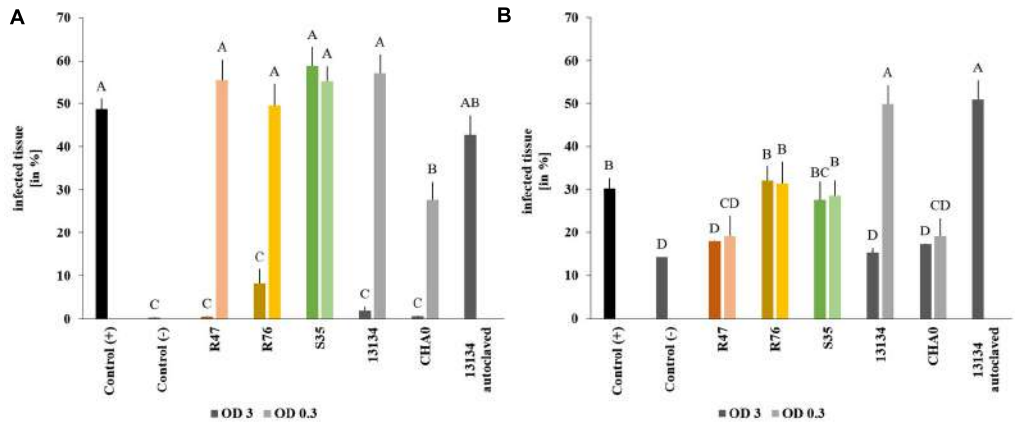

FIGURE 4 | Protective effect of the strains on leaf disks. Sporangia and bacteria were inoculated onto leaf disks. Necrosis formation was quantified after 4 days **(A)** using a macroinstruction (see Supplementary Material, macro1) and sporangiophore production after 8 days **(B)** (macro2). OD = 3 is represented by dark and OD = 0.3 by light bars. Averages and standard errors are shown (n = 18–20). Different letters indicate statistically different values (Tukey's *post hoc* test: $p < 0.05$).

seem to originate from diffusible substances, as these strains did not produce volatile compounds inhibiting *H. solani* (Hunziker et al., 2015).

However, the results obtained in such *in vitro* experimental setups only represent a metabolic potential of the strains grown on rather rich laboratory media, and no guarantee can be offered that the strains that are active *in vitro* will also be *in planta*. The reasons for this first screen in the laboratory are mainly time and space constraints. Therefore, developing a space- and time-efficient screening procedure, which would allow testing the biocontrol agents at a very early stage already on plant material rather than on artificial laboratory media would potentially yield better-suited candidate strains for further investigations. This is why we developed a high through-put leaf disk method allows the monitoring over time and the automated quantification of disease progression by picture analysis. The main difficulty in developing a reliable macroinstruction to quantify the sporangiophore production came from the white background originating from leaf veins and trichomes. Nevertheless, provided the macroinstructions are carefully adapted to each new experimental setup, the automated leaf disk quantification method represents a big step forward for the selection of promising biocontrol strains, since it provides the means to obtain in a time- and space-efficient manner quantitative data on the *in planta* disease protection potential of the strains of interest. While this assay was in our hands well-suited for greenhouse-grown plants, it was not suitable to evaluate disease progression on material from field-grown plants, since the control plants (of the same cultivar, Victoria) developed little infection (less than 20% of leaf surface infected, compared with over 30% for leaf disks from greenhouse plants, **Figures 4** and **7**). Field-grown plants are expected to show a basic level of resistance to diseases due to the multifarious biotic and abiotic stimuli they encounter in nature (Walters, 2009). Moreover, plants grown in the greenhouse show less abundant and less diverse microbial colonization than field-grown plants (data not shown), which might also explain their higher susceptibility to *P. infestans* infection, as the role of the plant microbiome in disease protection and induction of resistance is becoming increasingly clear (Bakker et al., 2013; Pieterse et al., 2014). The leaf disk method and the subsequent automated image analysis developed in the present study is therefore not meant to replace whole-plant analysis and field trials, but represents an efficient tool to replace the *in vitro* screening and to select for antagonists that are able to inhibit the pathogen when both organisms grow on the host plant rather than on rich laboratory media. Since the co-inoculation of both antagonist and pathogen would not allow to see induced effects, the setup might be adapted by either applying the antagonists 1 day before the pathogen or by spraying whole plants with the antagonists and thereafter cutting leaf disks, infecting them with *P. infestans* and monitoring disease progression.

In our case, the strain that showed the highest *in vitro* activity turned out to also be the most efficient when tested on leaf disks from greenhouse-grown plants (**Table 1**, **Figure 4**), although the very few strains selected in this study do not allow any generalization of this observation. This *Pseudomonas* strain was affiliated to the *P. chlororaphis* species according to its *rpoD* sequence (Hunziker et al., 2015), a species to which the active ingredient of the product Cerall® also belongs (Johnsson et al., 1998; Velivelli et al., 2014). This affiliation was confirmed by phylogenetic analysis based on four housekeeping genes (*16S rRNA*, *gyrB*, *rpoB*, *rpoD*), which placed this strain in a cluster comprised of *P. chlororaphis* and *P. protegens* strains (De Vrieze et al., 2015). Both species are part of the larger *P. fluorescens* group and recent phylogenetic studies indicate a close proximity between *P. chlororaphis* and *P. protegens* (Gomila et al., 2015). Strains belonging to both species include well-known biocontrol agents against pathogenic fungi (Haas and Defago, 2005) but also against insects (Kupferschmied et al., 2013; Ruffner et al., 2013). Preliminary inspection of the genomic potential of *P. chlororaphis* R47 revealed that this strain shows a similar toolset of antibiotics as other *P. chlororaphis* strains according to a recent study comparing *Pseudomonas*

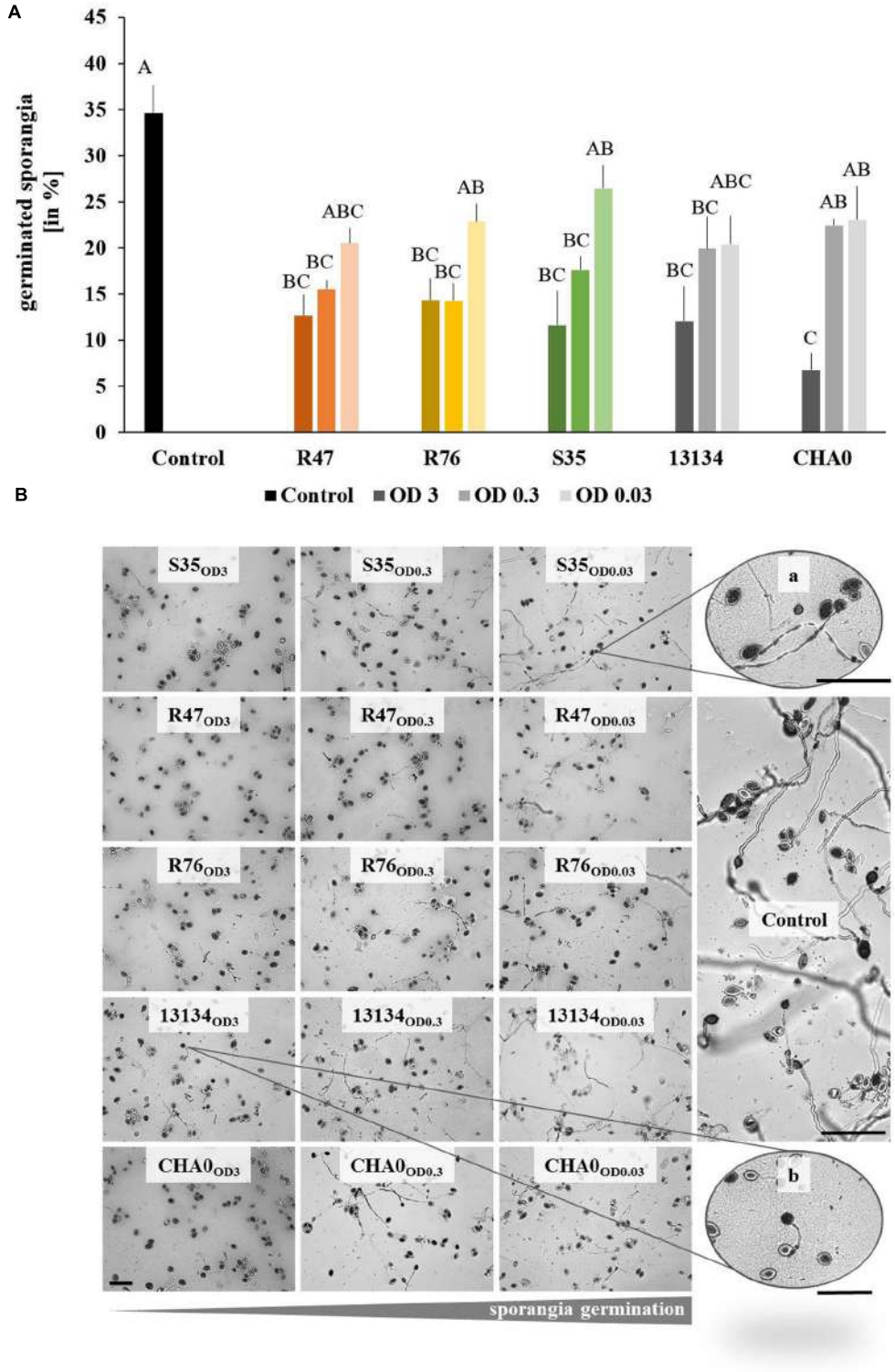

FIGURE 5 | Germination of *Phytophthora infestans* sporangia in the presence of bacteria. Sporangia and bacteria in different concentrations were co-inoculated on agar plugs and the percentage of germinated sporangia was counted after 3 days. **(A)** Percentage of germinated sporangia. Different letters indicate significant differences (Tukey's *post hoc* test: $p < 0.05$, $n = 4$). OD = 3 are represented by dark, OD = 0.3 by intermediate and OD = 0.03 by light bars. **(B)** Representative pictures of *Phytophthora infestans* sporangia germinated in the presence of different concentrations of bacteria. From left to right, decreasing bacteria concentration and increasing sporangia germination. Abnormal morphology such as hyphal swelling was observed even upon exposure to low bacteria concentrations (zoomed picture on the top right). Bars indicate 100 μm.

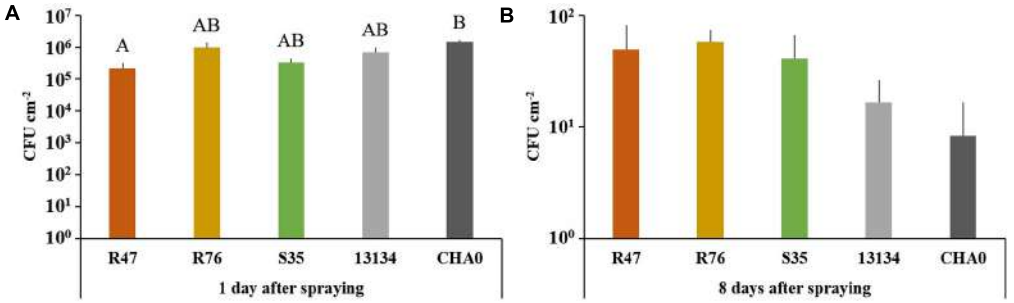

FIGURE 6 | Survival of the inoculated bacterial strains in a field microplot experiment. Abundance of bacteria were determined on leaf disks ($n = 4$) of sprayed plants collected 1 **(A)** and 8 **(B)** days after spraying. The number of colonies after 8 days did not differ significantly between the treatments according to one-way ANOVA [$F(4) = 1.13$, $P = 0.38$], even though 1 day after spraying significant differences were observed. (Tukey's *post hoc* test: $p < 0.05$, $n = 4$).

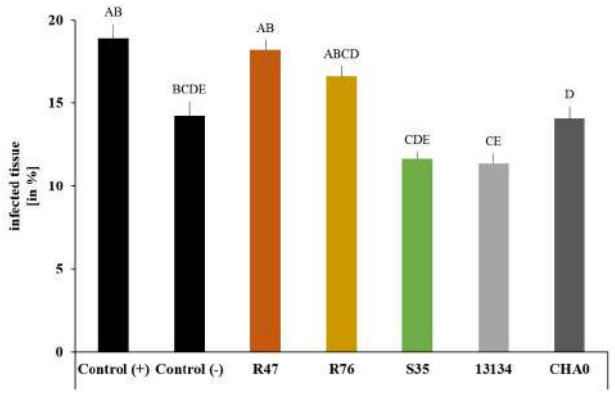

FIGURE 7 | Protective effect of the strains on leaf disks of field-grown plants. *Phytophthora infestans* sporangia and bacteria were inoculated onto leaf disks collected from field-grown plants 1 day after spraying. Control (+) are leaf disks from untreated plants infected with *P. infestans* sporangia. Control (−) are leaf disks from untreated plants not infected with sporangia. Sporangiophore production was quantified after 6 days using a macroinstruction (see Supplementary Material, macro3). Averages and standard errors are shown ($n = 52$–58, $n_{control(-)} = 14$). Different letters indicate statistically different values (Tukey's *post hoc* test: $p < 0.05$).

strains (Loper et al., 2012): the genome of *P. chlororaphis* R47 encodes the synthesis of the antibiotics hydrogen cyanide, phenazines, pyrollnitrin and 2-hexyl-5-propyl-alkylresorcinol (HPR) (data not shown), which might be involved in its anti-*Phytophthora* activity. Moreover, siderophore (pyoverdine, achromobactin) production is also encoded in the genome and might contribute to the strains' antifungal activity and, perhaps more importantly, to its remarkable rhizosphere competence (Ghirardi et al., 2012). Indeed, *P. chlororaphis* R47 strain seemed to largely surpass the other strains tested in terms of rhizosphere colonization (**Table 2**), an important feature considering the practical advantages (feasibility and cost-efficiency) of tuber treatment compared with leaf spraying. However, to successfully inhibit late blight at the shoot level, the biocontrol agent should be able to either induce resistance or to systematically colonize the upper parts of the plants. Although induction of resistance in potato has been shown for other *Pseudomonas* strains (Arseneault et al., 2014; Pieterse et al., 2014), leaf disks from *P. chlororaphis* R47- tuber inoculated plants did not show increased resistance to late blight (data not shown), suggesting that this strain was not able to induce long-lasting resistance to late blight after tuber inoculation. Preliminary data suggest that *P. chlororaphis* R47 might be able to move from the tuber to the upper parts of the plants, which would be an important feature for late blight protection. Such an endophytic colonization has been shown for other plant-growth promoting and biocontrol *Pseudomonas*, such as *P. poae* in sugar beet (Zachow et al., 2015) or *P. putida* in potato (Andreote et al., 2009).

For non-endophytic microbes, the challenge of a successful application as leaf spray is particularly high in view of the harsh conditions that prevail in the phyllosphere, such as UV irradiation, as well as extreme variations in temperature and humidity. In our greenhouse experiments, all tested bacterial strains were able to persist for 2 weeks, but in the field, their abundance underwent a more rapid decrease, although all inoculated strains could be retrieved after 8 days in our microplot experiment (**Figure 6**). In addition to the abiotic stresses prevailing in field conditions, the higher complexity of the microbiome in field-grown plants is also likely to reduce the ability of introduced bacteria to establish in leaves as well as in roots, due to the higher competition they are facing. The colonization ability of the strains might thus depend on the residing microbiome as well as on the plant variety: in our root colonization assay, consistently more bacteria could be retrieved from the rhizosphere of cv. Victoria than from that of cv. Charlotte (**Table 2**).

As in many studies preceding the current one, going from the greenhouse to the field proved a challenging step. However, we selected only few strains that had been pre-screened and characterized based on *in vitro* experiments. We hope that the leaf disk-based automated picture analysis of disease progression developed in the frame of this study will enable to skip this first time-consuming step of *in vitro* tests and to directly test the antagonists' protective potential on plant material from different potato cultivars differing in their susceptibility to late blight. In a second step, the selected strains should be tested for their ability to establish sufficient population densities on field-grown

plants that harbor their own, complex native microbiome, since colonization of these plants might be more challenging than that of greenhouse-grown plants harboring less complex microbial communities. Beneficial or antagonistic interactions between the inoculated strain and the resident microbiome, whose composition will change according to biotic and abiotic factors, might be important factors that support or prevent successful establishment of a biocontrol agent. Those strains showing *in planta* anti-oomycete activity in a broad range of cultivars, as well as the ability to establish on already colonized plants would then represent better candidates for the time-consuming field trials than those selected solely based on *in vitro* tests in Petri dishes. Finally, future research will tell whether the best use of microbial control agents to limit late blight in tomorrow's potato production will reside in application of single, highly potent strains, or of a combination of strains with different and therefore complementary abilities, or in a more global approach involving microbiome engineering and microbiome-driven selection (Mueller and Sachs, 2015).

AUTHOR CONTRIBUTIONS

AG, LW, and AB designed the research, AG, MV, DB, RG, and NB performed experiments, AG, MV, and AB analyzed the data, LW and AG wrote the MS with help from MV, AB, NB, TM, and RG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01309

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Effects Due to Rhizospheric Soil Application of an Antagonistic Bacterial Endophyte on Native Bacterial Community and Its Survival in Soil: A Case Study with *Pseudomonas aeruginosa* from Banana

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Effective translation of research findings from laboratory to agricultural fields is essential for the success of biocontrol or growth promotion trials employing beneficial microorganisms. The rhizosphere is to be viewed holistically as a dynamic ecological niche comprising of diverse microorganisms including competitors and noxious antagonists to the bio-inoculant. This study was undertaken to assess the effects due to the soil application of an endophytic bacterium with multiple pathogen antagonistic potential on native bacterial community and its sustenance in agricultural soil. *Pseudomonas aeruginosa* was employed as a model system considering its frequent isolation as an endophyte, wide antagonistic effects reported against different phytopathogens and soil pests, and that the species is a known human pathogen which makes its usage in agriculture precarious. Employing the strain 'GNS.13.2a' from banana, its survival in field soil and the effects upon soil inoculation were investigated by monitoring total culturable bacterial fraction as the representative indicator of soil microbial community. Serial dilution plating of uninoculated control versus *P. aeruginosa* inoculated soil from banana rhizosphere indicated a significant reduction in native bacterial cfu soon after inoculation compared with control soil as assessed on cetrimide- nalidixic acid selective medium against nutrient agar. Sampling on day-4 showed a significant reduction in *P. aeruginosa* cfu in inoculated soil and a continuous dip thereafter registering >99% reduction within 1 week while the native bacterial population resurged with cfu restoration on par with control. This was validated in contained trials with banana plants. Conversely, *P. aeruginosa* showed static cfu or proliferation in axenic-soil. Lateral introduction of soil microbiome in *P. aeruginosa* established soil under axenic conditions or its co-incubation with soil microbiota in suspension indicated significant adverse effects by native microbial community. Direct agar-plate challenge assays with individual environmental bacterial isolates displayed

varying interactive or antagonistic effects. In effect, the application of *P. aeruginosa* in rhizospheric soil did not serve any net benefit in terms of sustained survival. Conversely, it caused a disturbance to the native soil bacterial community. The findings highlight the need for monitoring the bio-inoculant(s) in field-soil and assessing the interactive effects with native microbial community before commercial recommendation.

Keywords: antagonistic effect, bacterial endophytes, banana, biological control, microbe–microbe interactions, *Musa* sp., *Pseudomonas aeruginosa*, soil microbial community

INTRODUCTION

With increasing awareness about the hazardous effects of agrochemicals employed in crop husbandry, there is an impetus on the usage of safe and effective microorganisms in agriculture toward protection against biotic and abiotic stresses and in crop production (Zarb et al., 2005; Thomas and Upreti, 2015). It is often observed that the growth promotion effects or the antagonistic potential shown by the bacterial strains in laboratory assays are not translated to effective biocontrol strategies in the field. The conditions in the field are different influenced by soil, water, and edaphic factors as well as the native microbial community (van Veen et al., 1997; Bakker et al., 2013; Tyc et al., 2014). The successful performance of a bio-inoculant in the agricultural field is governed by its ability to survive in field soil and the interactive effects with the native microbiome. The introduced organisms in soil are vulnerable to physical stresses and antagonistic effects by other microorganisms (Acea et al., 1988; van Veen et al., 1997). It is also important that the candidate bio-inoculant shall not cause undue biological perturbation in the native soil microbial community. Effects due to the introduced organisms on resident soil microcosm is a topic of much interest to the microbiologists (Trabelsi and Mhamdi, 2013; Tyc et al., 2014).

A gradual reduction in the population of the introduced organism in soil/field has been documented in several instances (van Veen et al., 1997; Matos et al., 2005; Kröber et al., 2014). As for the interaction effects, studies employing *Azospirillum brasilense* in maize rhizospehere (Herschkovitz et al., 2005) or *Bacillus amyloliquefaciens* in lettuce rhizosphere through molecular tools (Chowdhury et al., 2013; Kröber et al., 2014) have indicated only little or marginal changes in the rhizosphere bacterial community. The bioinoculant consortium of *Bacillus megaterium*, *Pseudomonas fluorescens,* and *Trichoderma harzianum* also did not impart any negative effects on rhizospheric microbial community (Gupta R. et al., 2015). On the other hand, significant modifying effects due to the introduced biocontrol agent *Pseudomonas jessenii* on the soil bacterial community composition of lettuce rhizosphere influenced by soil type and time span after inoculation have been documented (Schreiter et al., 2014). The microbial community varies from soil to soil and location to location. This is largely constituted by non-cultivable organisms whose community profiles can be studied deploying molecular tools (Ogram et al., 2007; Bakker et al., 2013). Cultivation based methods may not provide a full account of metabolically active cells, yet it can be a powerful tool in different spheres of microbiology and in the modern era of genomics towards exploitation of beneficial organisms or for further physiological, molecular and application studies (Steward and Rappe, 2007; Epstein, 2013; Prakash et al., 2013). Here, we consider that monitoring the cultivable bacterial community in a confined environment could serve as a representative of soil microbiota to assess the effects due the introduced organism.

Endophytic microorganisms are plant internal inhabitants and are often isolated from surface sterilized tissue or through vacuum extraction (Hallmann et al., 1997; Hardoim et al., 2015). Endophytes are known to benefit plants through growth promotion and antagonistic effects on phytopathogens and pests besides facilitating phytoremediation (Ryan et al., 2008; Gaiero et al., 2013; Kumar et al., 2014). The potential for intra-tissue colonization and their intimate association with the host (Thomas and Reddy, 2013; Thomas and Sekhar, 2014) make endophytes more valuable agents in biocontrol applications over the rhizospheric organisms (Turner et al., 2013; Upreti and Thomas, 2015). Endophytes are normally considered to be recruited by the host plant primarily from the soil community through roots (Hallmann et al., 1997; Hardoim et al., 2015). The methods for the delivery of endophytes in agriculture range from inoculation of seed, seedling or other planting propagules to soil drenching, stem injection, and foliar sprays (Hallmann et al., 1997; Puri et al., 2015). When an antagonistic microorganism is to be employed as a biocontrol agent against soil-borne pathogens or pests, it warrants that the organism be applied through soil-drenching to neutralize the pathogen/pest in the root-zone from where the endophytes should migrate to the host. In this respect, the survival of endophytic microorganisms under field conditions for a longer duration assumes significance. Endophytes invariably return to the soil at the end of the life span of the host/organ and are thus having a phase in the soil environment before re-colonizing the host (Thomas and Soly, 2009).

Recently, we cultured several bacterial endophytes from the sucker-derived shoot-tips of banana (*Musa* sp.) cv. Grand Naine (Sekhar and Thomas, 2015). The prime target was to explore by testing these isolates for potential biocontrol of banana wilt disease caused by the soil-borne vascular pathogen *Fusarium*

Abbreviations: CNA, Cetrimide- nalidixic acid- agar medium; Kan+TTC:NA medium, NA containing kanamycin and 2,3,5-triphenyl tetrazolium chloride; NA, nutrient agar; *Pau, Pseudomonas aeruginosa* banana strain 'GNS13.2a'; SATS, spotting- and- tilt- spreading; SDW, sterile distilled water; SP-SDS, single plate – serial dilution spotting.

oxysporum f. sp. *cubense* (*Foc*), a serious limiting factor in major banana growing areas world over (Stover, 1962; Ploetz et al., 2003). Evaluating the 47 endophytic banana strains against *Foc,* one isolate (GNS.13.2a) exhibited significant antagonistic activity against the pathogen in direct *in vitro* challenge assays and this isolate was identified as *Pseudomonas aeruginosa* (Sekhar and Thomas, 2015). This isolate also displayed antagonistic effects against the soil-borne pathogens, *Ralstonia solanacearum* from tomato and *Erwinia carotovora* from banana and thus a potential biocontrol agent against multiple diseases. *P. aeruginosa* is also known to be a human pathogen and there are concerns about its usage in agriculture (Matos et al., 2005; Kumar et al., 2013). Therefore, we did not consider our endophytic strain from banana as a biocontrol candidate for agricultural applications.

Pseudomonas aeruginosa has been frequently isolated as endophytes from different crop plants and most of such strains have been reported to show inhibitory activity against various phyto-pathogens. These include antagonistic activity against *Phytophthora capsici* in black pepper (Aravind et al., 2009), *Sclerotium rolfsii* in cucumber (Pandey et al., 2012), *Colletotrichum gloeosporioides* in chili (Allu et al., 2014), *Pythium myriotylum* in ginger (Jasim et al., 2014), *Fusarium oxysporum* in cotton (Yasmin et al., 2014) and wheat (Gupta G. et al., 2015), *Ralstonia solanacearum* in tomato (Maji and Chakrabartty, 2014) and *Xanthomonas* sp. infecting different crops (Spago et al., 2014). Endophytic *P. aeruginosa* is also known to be an effective biocontrol agent against nematodes (Ali et al., 2002; Kumar et al., 2013). Further, some strains of *P. aeruginosa* have been reported as plant growth promoters (Pandey et al., 2012; Gupta et al., 2013; Gupta G. et al., 2015) or useful in weed management (Lakshmi et al., 2015). Thus the endophytic strain of *P. aeruginosa* from banana (Sekhar and Thomas, 2015) appeared to form a good model system for studying the twin aspects of (i), the ability of an endophytic strain to survive in soil and (ii), effects due to the introduced microorganism with pathogen-antagonistic potential on native soil microbial community. Further, *Pseudomonas* sp. represents one of the most abundant genera of the root microbiome (Zamioudis et al., 2013). The present investigations were undertaken to assess the survivability of the endophytic *P. aeruginosa* strain from banana under non-axenic conditions in agricultural soil and rhizosphere and to gauge the effects due to the introduced organism on native microbiome by monitoring the gross cultivable bacterial community as the representative indicator of soil microbiota.

MATERIALS AND METHODS

Endophytic Bacterial Strain

Endophytic *P. aeruginosa* strain 'GNS13.2a' (NCBI 16S rRNA gene accession number KP798813) isolated from the deep-seated shoot-tip tissue of banana sucker cv. Grand Naine (*Musa* sp.; AAA genome) in a previous study (Sekhar and Thomas, 2015), referred to as *Pau* hereafter, was employed as the test organism in this study. The isolate was maintained as glycerol (30%) stock at −80°C and revived on nutrient agar (NA) followed by single colony perpetuation at each culturing. This strain was not specifically tested for human or animal pathogenicity. The experiments were conducted under containment followed by destruction of all biosamples through autoclaving or formaldehyde drenching. A Class-II vertical airflow cabinet with ULPA filter (Esco Biotech, Pvt. Ltd., Singapore) was employed during axenic works.

Identification of Bacteriological Media for *Pau* Monitoring

To select the appropriate media for capturing the culturable bacterial community and for monitoring *Pau* specifically, NA was tested compared with two known *Pau* selective media. These included (i), NA containing 60 μg ml^{-1} kanamycin and 50 μg ml^{-1} 2,3,5-triphenyl tetrazolium chloride (Kan+TTC:NA) as used by Kumar et al. (2013), and (ii), cetrimide agar base added with 15 μg ml^{-1} nalidixic acid (Goto and Enomoto, 1970) as employed by Deredjian et al. (2014). The above media were tested using pure culture of *Pau*. For this, six decimal dilutions (10^1 to 10^6) in sterile distilled water (SDW) considering 0.1 $OD_{600\ nm}$ overnight NA colony-derived culture as 10^0 'anchored stock' (Thomas et al., 2015) were applied through SATS approach (Thomas et al., 2012). The plates were observed at 30°C for 1–4 days for *Pau* cfu. The dilution level that yielded cfu in the 30–300 range was selected based on which cfu ml^{-1} of 0.1 OD stock in different media was worked out (Thomas et al., 2015).

Further, pure culture of *Pau*, irrigation grade water and rhizospheric field soil were tested on the above three media to ascertain the suitability for harnessing *Pau* cfu specifically. Here, the six serial dilutions from *Pau* anchored stock as above were tested by spotting 20 µl aliquots side by side in six sectors in a 9-cm Petri dish, a method designated as single plate-serial dilution spotting (SP-SDS) (Thomas et al., 2015). The anchored stocks (10^0) constituted 0.1 OD suspension for pure bacterial cultures, original sample for irrigation water and 1 g per 10 ml SDW for soil samples. Irrigation water was tested directly and after mixing with *Pau* stock. Soil sample suspensions were tested similarly after mixing with *Pau* culture employing six serial dilutions. The plates after surface drying were observed for bacterial cfu and specificity for *Pau* detection over 7 days.

Based on the results, NA was selected as the standard medium for growing pure cultures of *Pau* and for total culturable bacterial monitoring. Cetrimide-nalidixic acid-agar (CNA) was identified as the selective medium for the specific monitoring of *Pau* with obvious colony development within 24 h similar to NA. The GNS13.2a isolate had kanamycin and rifampicin resistance as genetic markers besides nalidixic acid. Modification of CNA with 60 μg ml^{-1} kanamycin was tried which delayed the colony growth and enumeration by 1 day with no extra benefit on cfu or selection specificity. Cetrimide agar with 50 μg ml^{-1} rifampcin (Matos et al., 2005) also did not offer any advantage over CNA while testing field soil or irrigation water for *Pau*. Further, 1–2 days-old NA plate cultures of *Pau* were tested directly and after refrigeration for cfu ml^{-1} on NA and CNA (six replications) to ensure that the inocula employed had good viability. All media formulations and supplements were sourced from M/s Hi Media Biosciences, Mumbai, India.

Pre-monitoring of Irrigating Water and Soil-Mix for *Pau*

As a prerequisite to ascertain whether the water used for irrigation, or the rhizospheric soil-mix employed in pot-culture trials harbored any *Pau,* both were checked 4 days prior to the start of the soil-monitoring experiment. Piped irrigation-grade water collected and stored in an open plastic tank in the glasshouse 1 day before was generally used for watering the soil. Aliquots of 100 μl were applied directly on CNA plates (100 nos) which altogether offered a gross detection sensitivity of 0.1 cfu ml^{-1}. The soil stock was comprised of a 1:1:1 blend of rhizospheric soil where banana was being gown, river sand and well-decayed farm yard manure (pH 7.35 ± 0.44) without any chemical or other sterilization treatments. The soil mix (50 g in 500 ml autoclaved water; 10^0 stock) was shake incubated for 1 h at 200 rpm and plated at 10^1 dilution on CNA plates (100 nos) offering a detection sensitivity of 1 cfu g^{-1} soil. The plates were monitored for microbial cfu and distinct colony types for 4 days in comparison with pure culture of *Pau*. Two colony morphotypes that emerged on CNA from water (2–4 × 10^1 cfu ml^{-1}) and soil (1–4 × 10^2 cfu g^{-1}) and distinct from *Pau* reference colonies in appearance were taken for identification through 16S rRNA gene sequence analysis as described elsewhere (Sekhar and Thomas, 2015). Mix culture of these organisms was further tested on NA and CNA along with pure *Pau* culture through SATS to ensure their distinction from *Pau* colonies.

Monitoring of *Pau* in Inoculated versus Control Soils Relative to Native Cultivable Bacterial Biome

To monitor the survival of *Pau* in inoculated versus control soils, the rhizospheric soil mix from the same lot as above in pots was employed as it was not possible to get a clear estimate under field conditions. Plastic pots (7″ height and 6.5″ diameter) were provided with 2.5 kg of dry soil-mix per pot and watered to field capacity. After 24 h under glasshouse conditions, eight replicate pots were drenched with 250 ml of 0.1 OD *Pau* suspension (about 10^8 cfu ml^{-1}) from day-2 NA plate colonies (*Pau*$^+$ soil; approximately *Pau* cfu of about 10^7 g^{-1}) and the control pots (*Pau*$^-$ soil) were applied with equal volume of SDW. First soil sampling was done on the same day (day-0) within 30–60 min. This involved inserting a 15 ml sterile Falcon tube to the soil to its full length (to a depth of approximately 10 cm) during which approximately 2 cm of compacted soil (approximately 2–2.5 g) was collected inside the tube. The sample from eight such pots was pooled and mixed thoroughly after removing the stony particles and breaking the lumps. 10 g soil was dispersed in 100 ml of SDW (10^0 stock) in a sterilized container and *Pau* and non-*Pau* cfu were assessed through SATS employing four replications per dilution. The *Pau*$^-$ soil samples were also processed similarly and monitored on NA and CNA. *Pau* colonies were located on NA based on the bluish green tinge while on CNA they appeared as shining/fluorescent cream-yellow colonies. The cumulative cfu on day-4 was used for estimating the *Pau* versus non-*Pau* cfu which was expressed as cfu g^{-1} soil. A simple assessment of the extent of bacterial variability was made on day-4 by counting the different colony types formed on NA at dilutions (10^3 or 10^4) that yielded well-delineated colonies.

The pots were left open under glasshouse conditions (day temperature range of 25–30°C; irradiance of 500–600 μE m^{-1} s^{-1} for 8–10 h) using 250 ml of irrigation-grade piped water per pot daily in the afternoon. Sampling of *Pau*$^+$ and *Pau*$^-$ soils was repeated on days-4, 7, 14, 21, and 28 with sample collection during the 30–60 min time span after watering. The irrigation water sample was routinely monitored for bacterial cfu by plating on NA and CNA. The pots thereafter were left without watering allowing the soil-mix to dry completely. The survival of *Pau* under dry soil conditions was monitored as above after another 4 weeks preceded by watering 4 h before sampling. This soil was monitored for *Pau* again after 48 h.

To assess the effect due to the moistening of dry soil in altering the total culturable bacterial cfu and the contribution of irrigating water to it, a further trial was set up. The dry soil-mix (2.5 kg in plastic pots) was applied with 500 ml of (i) sterile water or (ii) irrigation-grade water (3.2 × 10^5 cfu ml^{-1}). The initial soil bacterial cfu was monitored within 30–60 min on NA. Thereafter, the pots were kept covered with polythene sheet to avoid the lateral introduction of organisms, and were monitored for bacterial cfu again after 24 h. A third treatment involved rhizospheric soil from pots planted with tomato (6 weeks post-planting). These pots were maintained under glasshouse conditions, watered daily with irrigation-grade water (250 ml) and cfu estimations were undertaken as above.

Validation Trials Employing Rhizospheric Soil-Mix and Banana Rhizospheric Soil in Pots

A validation trial was undertaken employing a new batch of rhizospheric soil-mix watered to field capacity one day prior to *Pau* inoculation as discussed above (*Pau*$^+$ and *Pau*$^-$ soils) employing eight replicate pots. Another experiment was set up employing pots which were planted with tissue-cultured banana 'Grand Naine' saplings for the preceding 2 months (pH 7.13 ± 0.37) with daily inundation using irrigation-grade water. The *Pau*$^+$ and *Pau*$^-$ soils were monitored for *Pau* and gross cultivable bacterial flora on days-0, 4, and 7 employing CNA and NA after clearing the root tissues. Irrigation was practiced daily as above with periodic monitoring of water employing NA and CNA.

Monitoring the Survival of *Pau* in Axenic Soil Culture

For this, the soil-mix from the same lot described above was employed. Dry rhizospheric soil-mix (200 g) was wetted to field capacity employing 50 ml distilled water in 300 ml volume glass bottles with wide mouth (50 mm diameter). The screw capped bottles were subjected to autoclaving for 20 min at 121°C (1.1 kg cm^{-2}) on three consecutive days. On the third day, the bottles were kept open in the vertical airflow cabinet for 1 h followed by the addition of 10 ml of 1.0 OD *Pau* culture (about 10^9 cfu ml^{-1}) per bottle employing eight replications. The control bottles were provided with SDW. The soil was monitored

on the same day (day-0) and thereafter on days 1, 4, 7, 14, 21, and 28 on CNA for the extent of *Pau* cfu and on NA to check for any lateral introduction of non-*Pau* cfu during samplings. The soil from eight replicate Pau^+ and Pau^- bottles was collected separately in 50 ml tubes, weighed aseptically and dispersed in sterile water (1 g per 10 ml) followed by SATS of decimal dilutions on NA and CNA.

Monitoring of *Pau* in Established Axenic Soil Culture Following Lateral Introduction of Soil Microbiome

With a view to assess the response of established *Pau* flora in axenic soil to the exposure to native soil microbiota, *Pau* population was initiated in 3× autoclaved soil-mix in glass bottles as above followed by the introduction of soil microbiome. For this, the baseline *Pau* cfu was assessed by pooling the soil from two sets of *Pau* inoculated bottles 2 weeks post-inoculation (Pau^+ axenic soil sets-I and II) employing four replications. The set-I was applied with 5 ml of SDW while the set-II was added with 5 ml of the supernatant from the soil-mix suspended in SDW (1 g per 10 ml). *Pau* and non-*Pau* cfu were assessed on NA after 48 h. Thereafter, the set-I was applied with 5 ml SDW while the set-II was applied with 5 ml of 1.0 OD suspension of pooled bacterial inoculum prepared from NA colonies of soil bacteria derived from the plating of soil-mix 2 days before. The bottles were kept open in the vertical airflow cabinet for 4 h to evaporate away the excess moisture, and the *Pau* versus non-*Pau* cfu was assessed on NA and CNA soon after.

Testing the Interactive Effects of *Pau* with Soil Microbiota in Suspension

This trial was undertaken to ascertain whether the low cfu of non-*Pau* isolates on NA during the day-0 monitoring of Pau^+ soil arose from the antagonistic effects by *Pau* on the agar plate or due to the interactive effects in the soil itself. The soil suspensions of Pau^+ soil samples from glasshouse pots prepared on days-0, 4, 7, or 14 in SDW and left under sealed conditions in 50 ml falcon tubes (to avoid the lateral introduction of microorganisms) were monitored on CNA and NA after 7 days of stationary incubation. The cfu on the date of original sampling (days-0, 4, 7, or 14) was adopted as the base reference point. As a control to test the ability of the organism to survive under static non-aerated conditions, the axenic culture of *Pau* in SDW under identical conditions was employed.

Testing the Interactive Effects of *Pau* with Cultivable Soil Bacterial Isolates in Agar Plates

With a view to assess that the cfu reduction of *Pau* was arising from the interactive effects between *Pau* and soil bacteria, 10 random representative colony types that developed on NA from Pau^+ soil during the day-0 sampling of soil in the first trial were selected. A bacterial lawn of individual soil isolates was prepared on NA by applying 100 μl of 0.1 OD inoculum in peptone-salt (Thomas et al., 2012) in 9-cm diameter plates. After allowing 1 h for the organism to establish, 25 μl of 0.1 OD *Pau* inoculum prepared in SDW was applied at the center of the agar plate (6–7 mm diameter well) followed by air-drying in the vertical airflow cabinet for 25–30 min. The reaction of *Pau* to the test isolate and *vice versa* was assessed based on (i), the diameter of spreading *Pau* colony growth from the center of the well, (ii), the extent of clear zone, if any, between *Pau* and the isolate in the lawn, and (iii), the extent to which the lawn of the test isolate was pushed to the outer edge of NA plate by *Pau*. The test plates were scored on a – to ++++ scale for the above characteristics representing none, low, medium, high or significant. Based on the pooled information, four categories of interactive effects were documented: (i), no mutual antagonism (with or without dominance by *Pau*), (ii), aggressive antagonistic effect by *Pau* on the soil isolate, (iii), significant anti-*Pau* effect by the soil isolate, and (iv), mutual antagonism. The experiment was repeated with additional 20 distinct colony types selected from NA plates that were employed for the monitoring of Pau^+ pot-soil on day-7 (4th day after SATS) and 20 colony types from the control soil.

A direct confrontation assay was set up where the lawn of *Pau* on NA prepared using 100 μl of 0.1 OD culture 4 h after plating was applied with 5 μl of 0.1 OD inoculums of the test organisms. Based on the outcome, preparation of *Pau* lawn on NA using 0.001 OD inoculum (based on pre-trials employing 100 μl of 0.01, 0.001, or 0.0001 OD inoculum) followed by spotting with the challenge isolate as above (5 μl of 0.1 or 1.0 OD inocula) was tried. The ability of different isolates to grow or establish on *Pau* lawn at different strengths or any clear-zone development between *Pau* and the test isolate was recorded 1–4 days from the start of the experiment.

Observations and Statistical Analysis

The ability of *Pau* strain to survive in soil *vis-à-vis* the interactive effects on soil bacterial community were assessed primarily based on the cumulative cfu data recorded as on day-4 from the date of plating on CNA and NA. The survival of *Pau* was assessed based on the percent difference in cfu keeping the day-0 cfu in soil as the reference point. The extent of non-*Pau* cfu in Pau^+ soils was assessed with reference to the native bacterial cfu in Pau^- control soil samples on NA. During soil samplings, eight replications were employed for Pau^+ and Pau^- treatments. For all cfu comparisons, unless mentioned differently, two independent serial dilutions were prepared from pooled soil/water samples and applied as per SATS on two plates per dilution, thus constituting four replications. For statistical analysis, the cfu data were subjected to logarithmic transformation and Single Factor ANOVA or *t*-test (assuming equal variance) using the Data Analysis package of Microsoft Excel 2010. The mean (logarithmic scale) ± standard deviation values are presented.

RESULTS

Validation of Selective Medium for *Pau* Monitoring

Testing the *Pau* serial dilutions on three media, NA and CNA showed colonies in the 30–300 range at 10^5 dilution of the original 0.1 OD stock within 18–24 h while it took

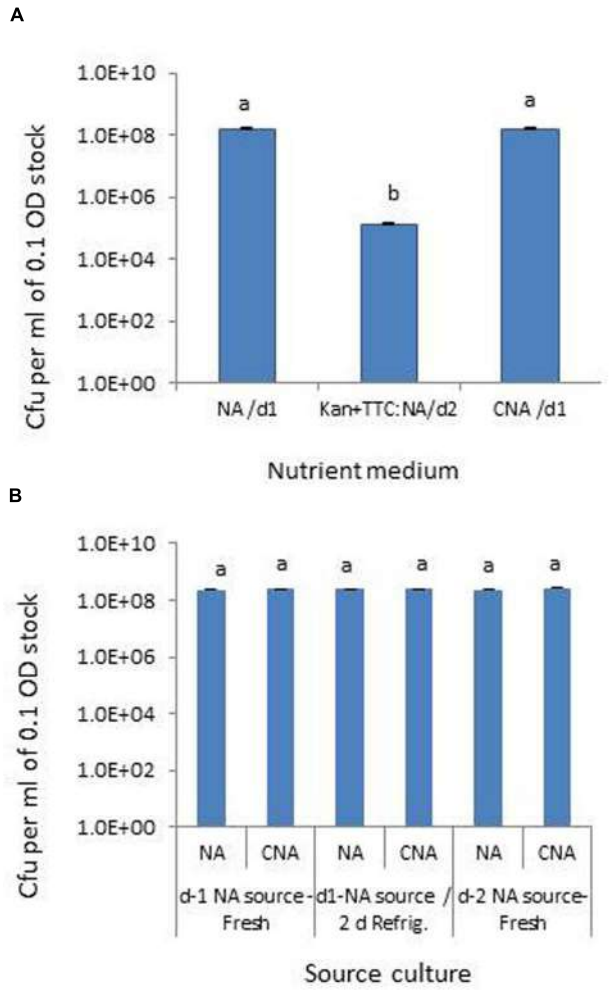

FIGURE 1 | Assessing different media for the selective monitoring of *Pseudomonas aeruginosa* (*Pau*) based on cfu ml^{-1} of 0.1 OD anchored stock (A) and testing of source *Pau* cultures of different ages for cell viability based on cfu (B). NA/d1 and CNA/d1 in **(A)** indicate cfu recorded on respective media on day-1 and Kan+TTC:NA/d2 cfu observed on day-2. Vertical bars indicate standard deviation. Bars with same letters are not significantly different at $P = 0.05$.

an extra day for colony emergence on Kan+TTC:NA with obvious cfu at 10^1 to 10^3 dilutions of the stock culture only. CNA gave similar cfu estimates as for NA within 24 h while Kan+TTC:NA showed significantly low estimate ($P < 0.0001$; **Figure 1A**). *Pau* colonies on NA showed a blue-green tinge and they tended to fade away after 4–5 days whereas on CNA they remained delineated with a fluorescing yellow shade for several days. A comparison of *Pau* day-1, day-2 NA source cultures versus day-1 NA plate culture refrigerated for two nights showed similar cfu estimates for different age groups on both NA and CAN ($P = 0.104$; **Figure 1B**). This endorsed the usage of day-1 or day-2 NA cultures directly or after 1–2 days of refrigeration as start culture without loss of viability.

Now, testing the different dilutions of *Pau* pure culture, irrigation-grade water and soil samples (with or without *Pau* addition) in SP-SDS on the above mentioned three media (**Supplementary Figures S1A–E**) indicated (i), similar cfu at 10^5 dilution in NA and CNA on day-1 itself, (ii), Kan+TTC:NA required an extra day for colony growth displaying countable cfu at 10^2 dilution, (iii), Kan+TTC:NA supported the growth of several non-*Pau* organisms from irrigation water and soil, (iv), NA displayed considerable bacterial variability considering the diverse colony types, (v), CNA did not support any water or soil isolates, (vi), *Pau*-mixed irrigation water and soil showed characteristic *Pau* type colonies on NA distinguishable from the native bacterial flora based on colony features, and (vii), CNA showed pure *Pau* colonies with cfu on par with *Pau* colony counts on NA. It was quite striking that Kan+TTC:NA supported the growth of diverse microorganisms including fungi from soil while NA normally did not support fungal growth. This was validated in a repeat trial where the irrigating water and soil suspensions were mixed with 10^2 serial dilution of *Pau* 0.1 OD stock (data not shown). *Pau* colonies from *Pau*-added soil and water were clearly distinguishable on NA when they were not entirely masked by the native bacterial colonies at higher cfu.

Pre-monitoring of Irrigating Water and Rhizospheric Soil-Mix for *Pau*

Testing up to 10 ml water sample directly for the presence of *Pau* (100 μl in 100 CNA plates) indicated some *Pau*-unlike cfu in the range of 1–8 per 100 μl (1.0–8.0 × 10^1 cfu ml^{-1}) which was too low considering the cfu ml^{-1} of about 10^8 for 0.1 OD *Pau* stock. The soil sample used at 10^1 dilution showed a meager 1–4 cfu per plate (1.0–4.0 × 10^2 cfu g^{-1} soil) which did not include any *Pau* as per the colony characteristics. There were two colony types other than *Pau* that were supported on CNA. These were identified as *Pseudomonas plecoglossicida* (predominant) and *Pantoea ananatis* (occasional). Testing the mix culture of *Pau* with the above two organisms indicated that *Pau* colonies were clearly distinguishable on NA and CNA based on the bluish green/yellow tinge and colony size by day-2 (**Figure 2**).

Monitoring of *Pau* Inoculated versus Control Soils with Reference to Soil Bacterial-Biome

The salient observations from the monitoring of *Pau* in potted *Pau*$^+$ soils on the date of inoculum application (**Figures 3** and **4**) included: (i) the *Pau*$^+$ soil yielded only *Pau* colonies on CNA to the tune of 7.1 × 10^6 g^{-1}, (ii), both *Pau* and non-*Pau* colonies were easily identified on NA registering a slightly higher *Pau* cfu (9.7 × 10^6 g^{-1}) than on CNA, (iii), no fresh *Pau* colonies emerged after day-1 on NA from *Pau*$^+$ soil while non-*Pau* colonies continued to emerge for 2–4 days, and (iv), the non-*Pau* cfu in *Pau*$^+$ soil was significantly lower (4.6 × 10^6 g^{-1}) than the corresponding value for un-inoculated control (1.8 × 10^7 g^{-1}; $P = 0.0071$). *Pau*$^-$ soil did not exhibit any colony growth on CNA nor yielded any *Pau*-like colonies on NA (**Figures 3** and **4**). Beyond 4–5 days, *Pau* colonies tended to fade away or vanish from NA plates, also causing the waning of a major

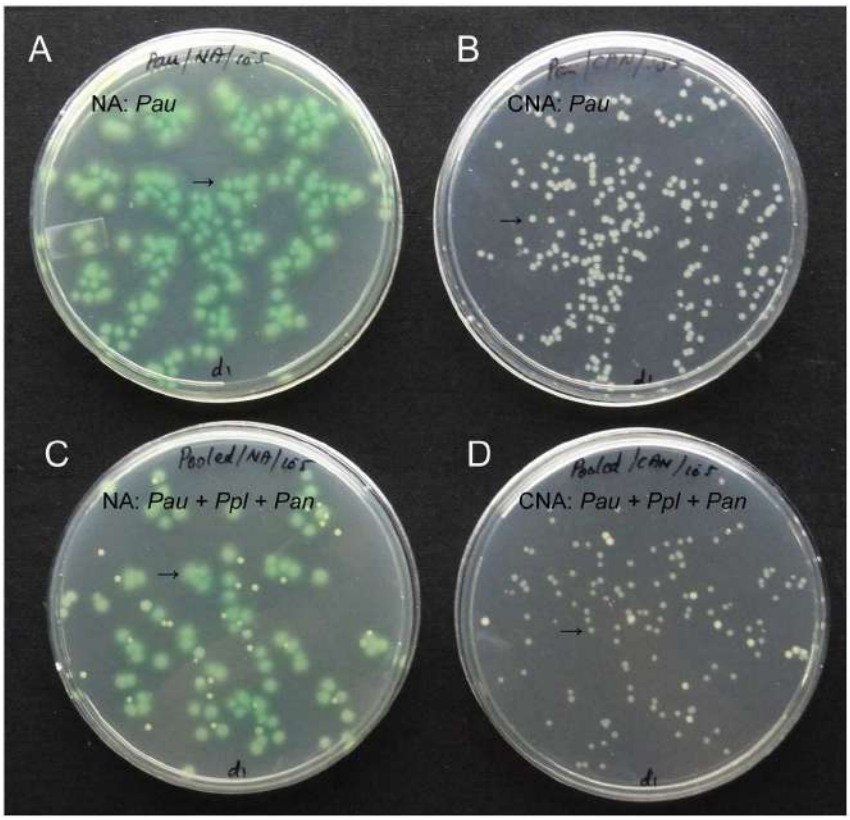

FIGURE 2 | Pure culture of *P. aeruginosa* (*Pau*) showing cfu at 10^5 of the 0.1 OD anchored stock as bluish-green tinged larger colonies on nutrient agar (NA, A) or as white distinct colonies on CNA (B), or a mix culture with *P. plecoglossicida* (*Ppl*) and *Pantoea ananatis* (*Pan*) showing clearly identifiable *Pau* colonies on NA (C) and CNA (D; *Pau* indicated by arrow).

share of non-*Pau* colonies. Thus, the gross cfu estimate on day-4 with colony enumerations on days-1, 2, and 4 was adopted for monitoring *Pau* versus non-*Pau* cfu on NA. Marking the initially formed colonies on the reverse of the plate facilitated clearer cfu enumeration.

Monitoring the Pau^+ and Pau^- soils in the pots kept under glasshouse conditions on day-4 indicated a significant reduction in *Pau* cfu in Pau^+ soil compared with the day-0 cfu registering 2.2×10^5 (−97%; $P < 0.0001$) and 2.5×10^5 cfu g^{-1} (−97.4%; $P < 0.0001$) on CNA and NA, respectively (**Figure 5**). On the other hand, the non-*Pau* cfu in Pau^+ soil showed a rise to 1.3×10^7 g^{-1} (from day-0 cfu of 4.6×10^6 g^{-1}; 182%; $P < 0.0001$). This indicated a gradual build-up of native bacterial community following the initial counter effect by *Pau*. The cfu of non-*Pau* organisms on NA from control soil (3.4×10^7 g^{-1}) also showed a significant increase from day-0 (1.8×10^7 g^{-1}; 93.3%; $P = 0.0016$) to day-4 indicating a surge in native bacterial community following the watering of dry soil which was commenced 1 day prior to the start of the trial.

The day-7 sampling indicated a further reduction in *Pau* cfu from day-4 cfu in Pau^+ soil (7.9×10^4; $P < 0.001$, and 9.8×10^4 g^{-1}; $P = 0.002$, respectively, on CNA and NA). The non-*Pau* cfu here showed a significant increase (4.5×10^7 g^{-1}) over the day-0 (4.7×10^6 g^{-1}; $P < 0.001$) and day-4 cfu (1.3×10^7 g^{-1}; $P < 0.001$) indicating native bacterial community build up after the initial attack by *Pau*. The level attained here by day-7 (4.5×10^7 g^{-1}) reached nearly close to that recorded for the un-inoculated soil (5.8×10^7 g^{-1}; $P = 0.05$). The sampling done on day-14 indicated a further drop in *Pau* population which continued through day-21 and almost stabilized by day-28 (**Figure 5**). It was not feasible to document *Pau* cfu of Pau^+ soil on NA at this stage due to the too few colonies which were masked by high non-*Pau* cfu. During this phase both the Pau^+ and Pau^- sets also displayed a gradual reduction in non-*Pau* cfu indicating stabilization of native bacterial community to similar cfu levels in both cases.

Monitoring the Pau^+ soil after another 4 weeks with an intervening 4-weeks dry spell showed barely any *Pau* (1.0×10^2 to 2.0×10^2 cfu g^{-1}) indicating poor survival of *Pau*. The native non-*Pau* population in both Pau^+ and Pau^- soils appeared comparable which was similar to the levels documented before the onset of 1 month dry spell (7.4×10^6 and 7.9×10^6, respectively; $P = 0.66$). The sampling 48 h after the rehydration of above soils showed a slightly more but no significant increase in *Pau* colonies in Pau^+ soil (1.4×10^3 cfu g^{-1}). It was striking to note that the cfu of non-*Pau* organisms capable of growing on CNA showed an increase in Pau^+ soil (4.0×10^3 cfu g^{-1}) while the Pau^- soil did not show such colonies. In other words, the

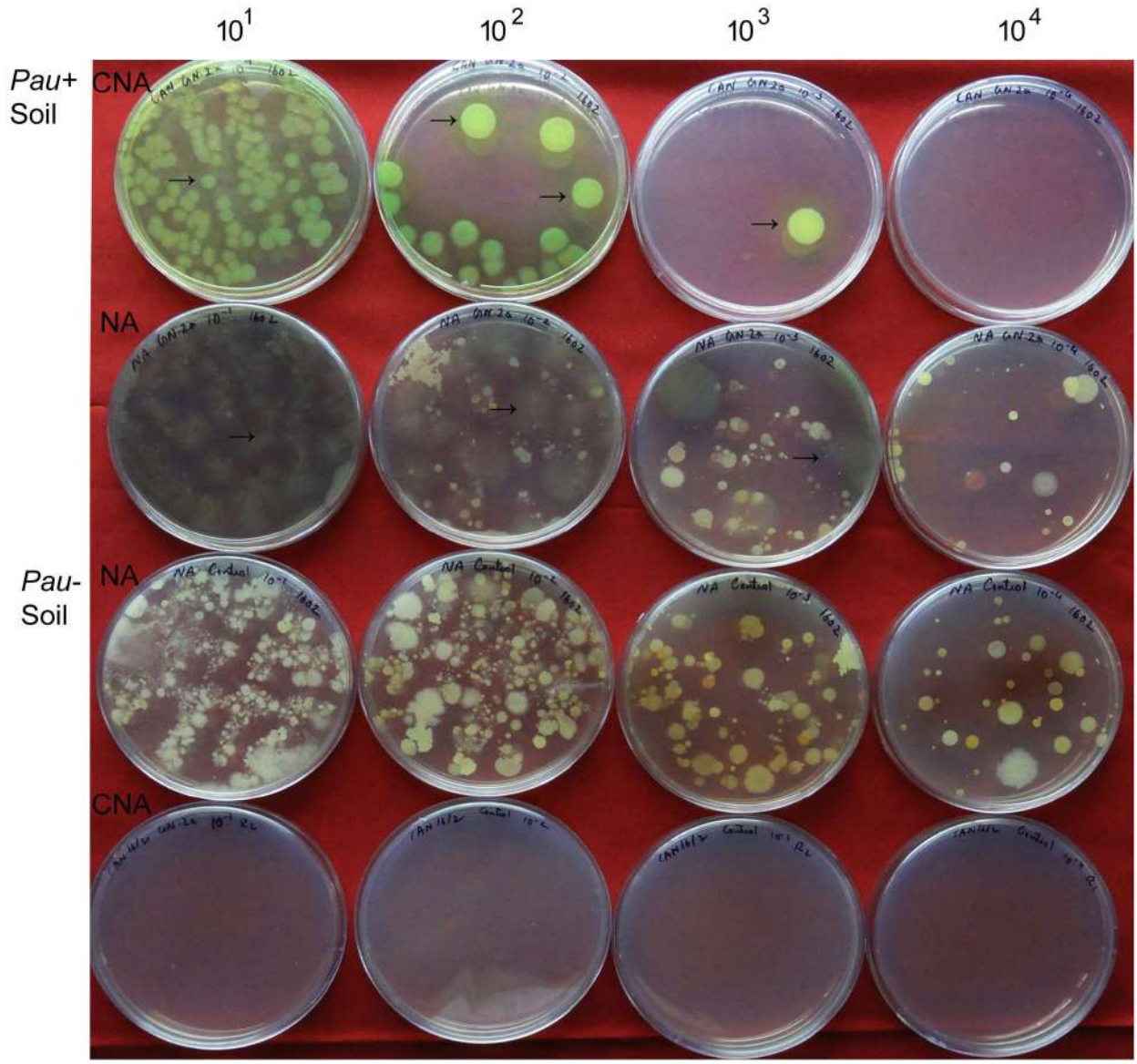

FIGURE 3 | Plating of serial dilutions (10^1 to 10^4) of *Pau*$^+$ and *Pau*$^-$ rhizospheric soil mix on CNA and nutrient agar (NA) at the start of the experiment (day-0) and documentation of *Pau* (⟶) and non-*Pau* cfu as on day-4 after plating (row 1: *Pau* colonies from *Pau*$^+$ soil on CNA; row 2: *Pau* cfu on NA appearing brownish and vanishing gradually with evident non-*Pau* bacterial cfu; row 3: *Pau*- soil showing non-*Pau* colonies on NA; row 4: no colony growth on CNA from *Pau*- soil; Colony counts were taken on days 1, 2, and 4 with the marking of initially formed ones on the reverse of the plate).

non-*Pau* organisms supported on CNA appeared more rampant in *Pau*$^+$ soil than in un-inoculated soil.

The irrigating water did not show any *Pau* colonies on CNA during the periodic monitoring. The control soil sample also did not yield any *Pau*-like colonies on CNA but the *Pau*$^+$ soil showed a few *Pau*-unlike colonies capable of growing on CNA during the 8 weeks monitoring. Identification of these distinct colony morphotypes confirmed them to be not *Pau*; these included *P. plecoglossicida*, *P. monteilii, P. taiwanensis,* and *Achromobacter* sp. detected in very few counts on CNA (0–4 cfu) applied with the 10^1 dilution of soil suspension. These were distinguishable from *Pau* colonies when grown together based on color and size on CNA and NA. Randomly selected supposedly *Pau*-colonies were confirmed to be so with 16S rRNA typing.

The trial assessing the contribution of soil wetting to the build-up of native bacterial cfu in dry soil and the relative contribution from the irrigation water indicated that the cfu hike in dry soil 24 h after the application of sterile water was mainly contributed by the activation of bacterial cells to cultivation and/or their multiplication than the direct contribution from irrigating water. This was evident from the cfu levels in the treatment inundated with irrigation-grade water (3×10^5 cfu ml^{-1}) which showed a relatively lower amount of cfu increase (**Figure 6**). The rhizospheric soil of tomato showed identical

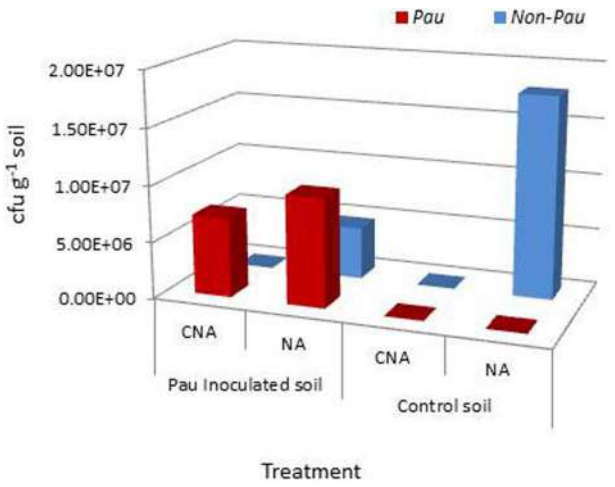

FIGURE 4 | Extent of *P. aeruginosa* (*Pau*) and non-*Pau* bacterial cfu in potted rhizospheric soil mix within 30–60 min of *Pau* application as per the cfu estimations on the selective CNA medium and NA; (cumulative of cfu recorded on day-1 to day-4).

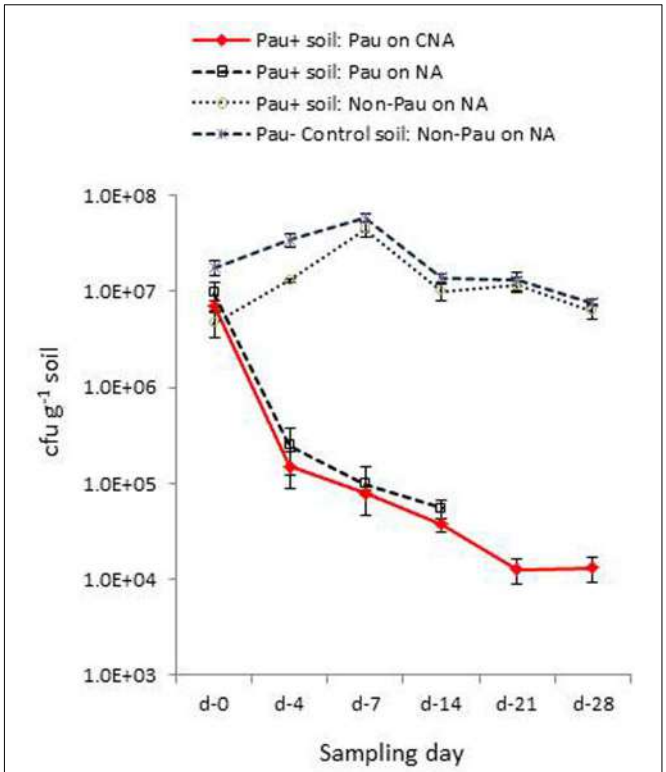

FIGURE 5 | Extent of *P. aeruginosa* (*Pau*) and non-*Pau* bacterial cfu in potted *Pau*-inoculated (*Pau*$^+$) and control (*Pau*$^-$) rhizospheric soil mix on different sampling dates commencing from the date of *Pau* application (day-0) as per the cfu estimations on selective CNA and NA (cumulative of cfu recorded on day-1 to day-4; vertical bars indicate standard deviation).

cfu during day-0 and day-1 samplings indicating a microbially buffered condition therein.

Validation Trials Employing Rhizospheric Soil-Mix and Pots with Banana

The low survival of *Pau* in field soil and the adverse effect on soil bacterial community were verified in this repeat trial where the dry soil was watered to field capacity 1 day prior and applied with the *Pau* inoculum/sterile water as discussed above (**Figure 7A**). Monitoring of soil on day-0, day-4, and day-7 for *Pau* and non-*Pau* bacterial cfu on CNA and NA endorsed that (i), *Pau* was less fit to survive in soil, (ii), *Pau* application disturbed the native bacterial community instantly and (iii), the soil bacterial community showed a revival following the reduction in *Pau* population.

The parallel trial employing pots which were planted with banana 'Grand Naine' plants showed a similar pattern of significant reduction in *Pau* cfu within a weeks' time as in the trial employing dry soils (**Figure 7B**). The native bacterial cfu in *Pau*$^+$ soil also appeared significantly low compared with the respective figure for *Pau*$^-$ soil initially and then showed a gradual cfu build up as observed earlier. One striking observation in this trial was a stable gross bacterial cfu for *Pau*$^-$ rhizospheric soil from day-0 to day-7 in line with the observations on tomato rhizosphere soil indicating a stabilized condition when watering was practiced on a regular basis.

Monitoring of irrigation water confirmed that there was no lateral introduction of *Pau*. On the other hand, a significant share of non-*Pau* cfu and considerable diversity (25–30 distinct colony types) were observed in irrigation water. This explained the inoculum and diversity build-up in *Pau*$^+$ soil after the initial adverse effect on native bacterial community by *Pau*. While the direct piped water from the irrigation source tank showed 4.3×10^4 to 7.6×10^4 cfu ml^{-1} with ≥15–20 diverse colony types, the stored water in the glasshouse in the open tank showed 1.3×10^5 to 3.2×10^5 cfu ml^{-1} with ≥25–30 colony types. This explained to some extent the variations in the non-*Pau* cfu and diversity detected in the *Pau*$^+$ and *Pau*$^-$ soils on some sampling dates.

Monitoring of *Pau* under Axenic Soil Culture

On the date of inoculation of axenic soil with *Pau*, CNA and NA registered 1.30×10^8 and 1.33×10^8 *Pau*-cfu g^{-1}, respectively, ($P > 0.05$). During the monitoring over the next 1 week, a significant increase in *Pau* cfu was observed under the axenic conditions as monitored on CNA and NA (**Figure 8**). This amounted to 2.5×, 4.0×, 5.5× increase over the day-0 base cfu as on day-1, day-4, and day-7, respectively, on CNA ($P < 0.001$ in all instances on both CNA and NA). Thereafter, *Pau* cfu showed a gradual dip but the population was still higher than that of the base level (2.8×, 2.6×, and 2.4× on day-14, day-21, and day-28, respectively). The corresponding figures while monitoring the samples on NA were 2.3×, 4.5×, 6.0×, 3.4×, 3.3×, and 2.7×, respectively, over the day-4 cfu. The un-inoculated control soil did not show any colony growth ensuring the aseptic conditions following 3× autoclaving.

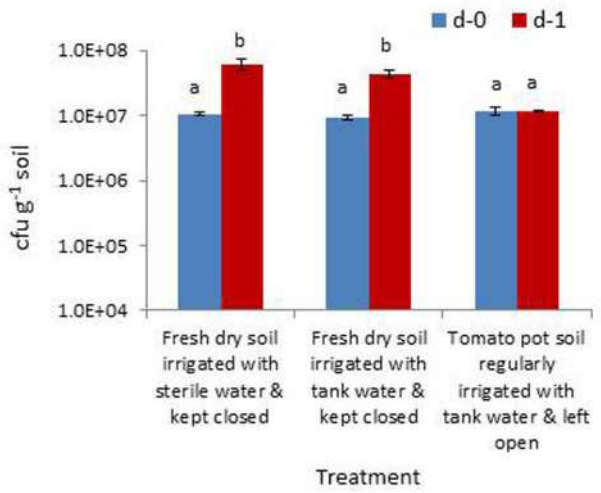

FIGURE 6 | Assessing the extent of increase in gross bacterial cfu due to the moistening of dry rhizospheric soil mix with sterile water, the contribution from irrigating water and the monitoring of rhizospheric soil of tomato (Bars with same letters are not significantly different at $P = 0.05$).

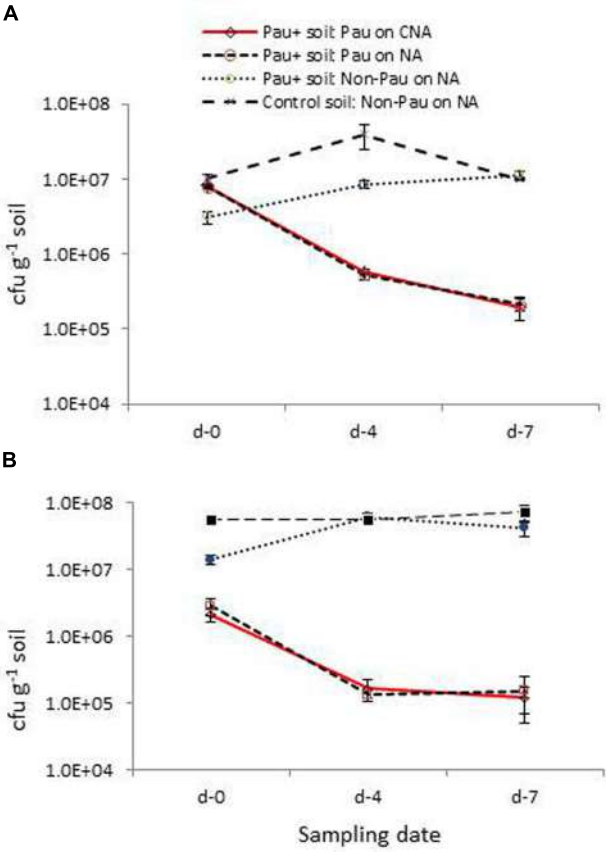

FIGURE 7 | Validation trials employing fresh field soil (A) and micro-flora stabilized banana rhizospheric soil (B): extent of *P. aeruginosa* (*Pau*) and non-*Pau* organisms in potted *Pau*-inoculated (*Pau*$^+$) and control (*Pau*$^-$) field soils on different sampling dates commencing from the date of *Pau* application (day-0) as per the cfu estimations on CNA and NA (cumulative of cfu recorded on day-1 to day-4; vertical bars indicate standard deviation).

Monitoring of *Pau* in Established Axenic Soil with Lateral Introduction of Soil Microbiome

Both the sets of bottles (axenic-I and II) displayed similar *Pau* cfu at the start of the experiment (**Table 1**). The monitoring 2 days after the addition of SDW to set-1 showed an increase in *Pau* cfu by 4.6× over the cfu at the start of the experiment while set-II applied with soil suspension showed only 3.2× hike over control which was significantly low compared with the *Pau*$^+$ soil. The non-*Pau* cfu was not assessable as the incorporation of soil suspension added only an estimated 2×10^4 cfu g^{-1} which was masked by *Pau* during the monitoring on NA plates. The monitoring of soil 4 h after the addition of NA-grown soil inoculum showed a 50% reduction in *Pau* cfu over the pre-application cfu (with the detection of a high non-*Pau* cfu). On the other hand, SDW-applied control set under axenic conditions showed a 43% increase in *Pau* cfu over the weighted average. The results overall indicated an adverse effect on established *Pau* cfu with the laterally introduced soil microbiome.

Testing the Interactive Effect with Soil Microbiota in Suspension

The monitoring of the soil suspensions prepared on days-0, 4, 7 or 14, a week later indicated that *Pau* was counteracted by other organisms in the suspension significantly affecting its population levels (**Figure 9**). Soil suspension also offered the entire soil microbiome including bacterial, fungal, and protozoan mircocosms in cultivable and non-cultivable forms. The monitoring of pure *Pau* suspension under identical axenic conditions indicated that the organism was capable of surviving stationary incubation with no change in cfu. The non-*Pau* community showed a revival after the initial attack by *Pau* in the sample prepared on day-0. The suspensions from days-4, 7, and 14 sampling showed a less fitness for the survival of both *Pau* and non-*Pau* sets.

Testing the Interactive Effects of *Pau* with Soil Isolates in Agar-Plate Assay

The control NA applied with *Pau* in the agar-well at the center of the plate showed growth covering almost 30–35 mm with a plate radius of 45 mm over 3–4 days. In dual culture plates, the growth of *Pau* varied from nil or negligible around the pit to quite active growth to the extent of 15–20 mm radius as documented after 3–4 days of application. A clear-zone was evident between *Pau* and the soil isolates in most instances. The extent of lawn formed by the soil strains in dual culture plates also varied significantly depending on the isolate ranging from full growth reaching the pit domineering over *Pau* to the extent of being pushed to the outer periphery. In extreme cases, there was no obvious lawn development at all. Based on the

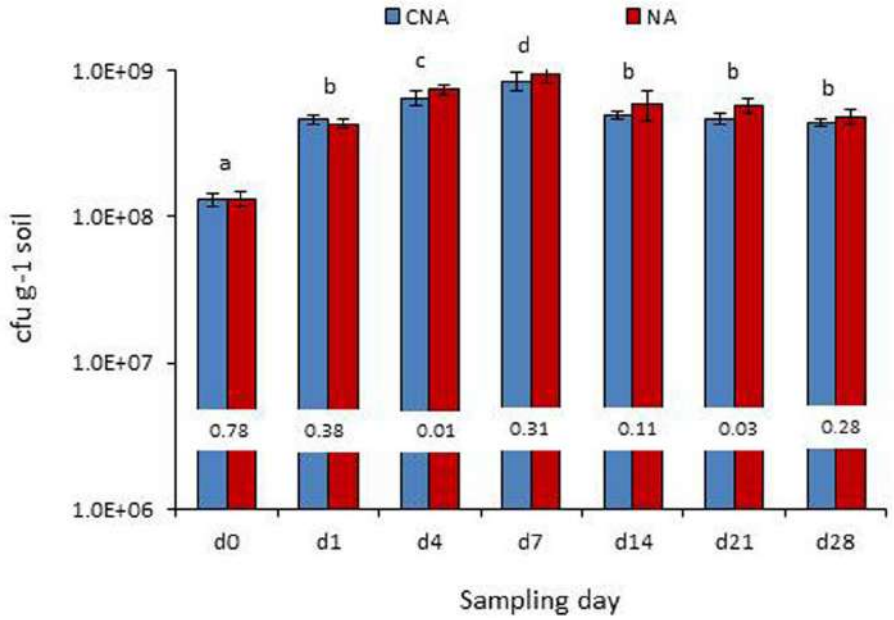

FIGURE 8 | Monitoring of the *P. aeruginosa* (*Pau*) inoculated soil under axenic conditions (bottles which were maintained under axenic conditions) employing the selective CNA and NA on different dates from the initial inoculation (Bars with same letters are not significantly different at $P = 0.05$; Values on the bar indicate *P*-value at 0.05 between the *Pau* cfu on CNA and NA).

TABLE 1 | Effect due to the lateral introduction of soil microbial suspension or soil derived bacterial inoculum on the population of *Pseudomonas aeruginosa* established in axenic soil for 2 weeks[a].

Description of treatments/observations	Axenic set-I[a]	Axenic set-II[a]	Significance[b]
Base *Pau* population on NA after 2 weeks under axenic conditions (cfu g^{-1})	*Pau* cfu: 4.0×10^7	*Pau* cfu: 4.5×10^7	NS ($P = 0.1619$)
First treatment imposed	Applied with 5 ml sterile water, closed, and kept under ambient	Applied with 5 ml supernatant of soil suspension @ 1 g 10 ml^{-1} (4×10^6 cfu /5 ml; final 2×10^4 cfu g^{-1} bottled soil)	–
Cfu g^{-1} soil 48 h post-application of water or soil suspension	*Pau* cfu: 18.6×10^7	*Pau* cfu: 14.6×10^7 Non-*Pau* cfu: Too low for detection at the dilutions for *Pau* cfu enumeration	** ($P = 0.004$)
Second treatment imposed	Applied with 5 ml sterile water to 200 g soil	Applied with 5 ml of 1.0 OD suspension of pooled inoculum from plate grown colonies 2 days after plating the soil suspension (estimated non-*Pau* cfu: 10^9 ml^{-1}; 2.5×10^7 g^{-1} soil)	
Cfu g^{-1} soil 4 h post-second treatment (bottles kept open for 4 h in the vertical air-flow cabinet before sampling)	*Pau* cfu: 28.9×10^7	*Pau* cfu: 6.9×10^7 Non-*Pau* cfu: 1.7×10^7	** ($P = 1.7 \times 10^{-6}$)

[a]*Bottles with 200 g wet soil were autoclaved thrice followed by the addition of 10 ml of 1.0 OD P. aeruginosa suspension in SDW and incubated under ambient conditions for 2 weeks with screw cap closure. Cfu assessments were made on NA and expressed as cfu g^{-1} soil.*
[b]*Significance with reference to difference in Pau cfu between axenic sets-I and II; ** significant at P = 0.05; NS, not significant.*

above observations, four different categories of responses were documented: (i), null effect, (ii), severe antagonistic effect by *Pau* on the soil isolate, (iii), severe anti-*Pau* effect displayed by the soil isolate, and (iv), mutual antagonistic effects (**Supplementary Table S1**).

The spot application of soil isolates on *Pau* lawn formed on NA after plating the 0.1 OD culture and allowing 1 h for *Pau* to establish wholly inhibited the growth of test isolates. No obvious colony growth was observed from the applied spots even after 3–4 days in all the 50 test isolates. Reducing the *Pau* cell population employing 0.1, 0.01, or 0.001 OD inoculums also showed proper lawn development. Use of 0.001 OD *Pau*-inoculum for lawn plating followed by immediate spotting of soil isolate using 0.1 or 1.0 OD inoculum showed the growth of the test organism at the applied spots at 1.0 OD in a few instances but not at 0.1 OD (**Supplementary Table S1**). Thus, the presence of *Pau* even at lower cell densities before the addition of challenge isolate appeared to inhibit their establishment under the nutrient rich conditions in the plates.

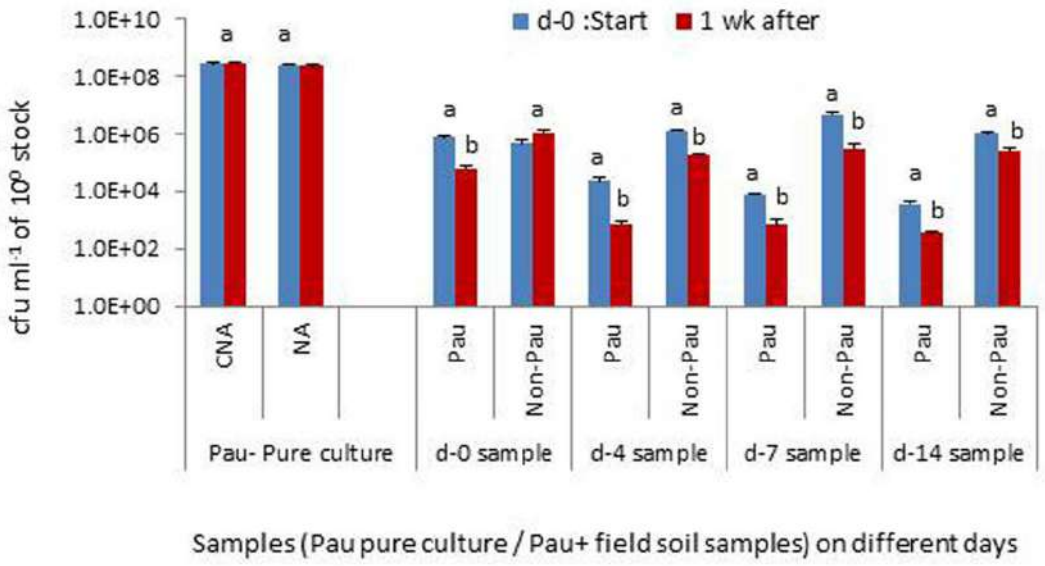

FIGURE 9 | Testing the interactive effect of *P. aeruginosa* (*Pau*) with soil microbiota in suspension employing the soil suspensions prepared on days 0, 4, 7 or 14, with static incubation for 1 week after the preparation, in comparison with the pure culture employing CNA for *Pau* and NA for non-*Pau* estimations (10^0 stock refers to 0.1 OD anchored stock for the pure culture and 1 g per 10 ml for the soil suspension; Bars with different letters in a treatment set are significantly different at $P = 0.05$).

DISCUSSION

The present investigations have brought out valuable information on the effects due to the introduction of an agriculturally significant bacterium, *P. aeruginosa*, possessing multiple pathogen-antagonism potential on the native soil microbial community and the response of the soil microbiome to the introduced microorganism. Besides, it has added the much desired information on the survivability of an endophytic strain artificially inoculated into the field soil which is generally lacking. For an organism to be effective as a biocontrol or growth promoting agent in agriculture, it should be able to sustain under field soil-conditions in sufficient populations for a minimum duration (van Veen et al., 1997). It should not disturb the native microbial community drastically which otherwise be playing diverse significant roles in the rhizosphere (Hartmann et al., 2009; Trabelsi and Mhamdi, 2013). Contrary to this, *P. aeruginosa* proved to be not a competitive inoculant for soil and its application in agricultural soil instantly affected the non-target organisms, thereby disturbing the soil microbial community dynamics. The effect, however, was transient as the non- *P. aeruginosa* cfu showed restoration on par with control soil within 1 week. This perhaps was contributed by the multiplication of survivors or the addition of new organisms through water/air, with the reduction in the population of the inoculant. The present trials were carried out in pot cultures considering the feasibility of monitoring the inoculant and the soil microbiome unlike the open field but employing the rhizospheric soil with the natural soil associates.

Studies exploring the effects due to the introduced organism on soil bacterial or microbial community depict varying conclusions depending on the organism or the soil type (Herschkovitz et al., 2005; Chowdhury et al., 2013; Kröber et al., 2014; Schreiter et al., 2014; Gupta R. et al., 2015). Most of the above studies have relied on molecular approaches/metagenomics rather than cultivation which on one hand gave a wider community impact effect but did not involve verification with direct challenge assays. Both cultivation-based and cultivation-independent approaches have inherent advantages and disadvantages. In this report, the cultivable fraction was analyzed as a whole representative of the soil microcosm and it facilitated the documentation of four categories of interaction effects varying from severe antagonistic effect by *P. aeruginosa* on the soil isolate, severe anti- *P. aeruginosa* effect displayed by the soil isolate or mutual antagonistic effects to nil effects. We did not target the identification of different isolates as the soil and irrigation water contained multitude of organisms, the cultivable fraction constitutes a minor fraction of the microbial community and that the soil microbial communities vary with location and time.

Pseudomonas aeruginosa was employed in this study as a model system considering the wide range of antagonistic activity reported against various pathogens including bacteria (Maji and Chakrabartty, 2014; Spago et al., 2014) and fungi (Aravind et al., 2009; Allu et al., 2014) and pests like nematodes (Ali et al., 2002; Kumar et al., 2013). The bacterium is known to produce different antimicrobial compounds which include mainly phenazines (Anjaiah et al., 1998; Mavrodi et al., 2006; Jasim et al., 2014). The putative biocontrol agent exerted significant adverse effects on non-target native microbiome at variance from the conventional wisdom of nurturing the soil microbiome. It is likely that such responses vary with the aggressiveness of the inoculants or the

extent of antimicrobial activity. Considering the spectrum of antagonistic effects by *P. aeruginosa* cited above, it could be viewed as an aggressive species to which the soil community perhaps needed to respond likewise. This proposal is hypothetical at this stage but forms an aspect for future research. The observations in this study emphasize the need for pre-testing the bio-inoculant for possible adverse effects on native microbial community before recommending it as a potential biocontrol agent, particularly for organisms with broad anti-microbial activity.

Pseudomonas aeruginosa, in this study, proved to be a poor survivor in agricultural field soil. The sustenance and proliferation of the strain under gnotobiotic conditions but poor survival in field soil suggested that this was not a mere fitness issue but mostly due to the antagonistic effects by the native microbial community. This was endorsed by the observations from the lateral introduction of soil-microbiome in *Pau*-established axenic soil and the interactive effects of *Pau* in soil suspension both of which involved the gross microbial community including cultivable and non-cultivable microorganisms. The direct confrontation assays in agar plates also endorsed the significant role of microbe–microbe interactions though it involved a representative group of cultivable environmental bacteria. The soil microbial community includes bacteria, fungi, and viruses. The introduced organism faces challenge due to the antagonistic effects or competition from native microorganisms and is vulnerable to predation by protozoa which could affect its survivability (England et al., 1993; van Veen et al., 1997; Trabelsi and Mhamdi, 2013; Tyc et al., 2014, 2015).

Besides the above biotic factors, the environmental fitness of the introduced organisms in soil is also influenced by several abiotic factors such as soil type, pH, temperature, water content, periodicity of wetting and drying, and soil constituents including organic carbon and mineral nutrition (Acea et al., 1988; van Veen et al., 1997; Bakker et al., 2013; Trabelsi and Mhamdi, 2013; Tyc et al., 2014, 2015). The inoculant is also vulnerable to desiccation. Considering that the same soil-mix was employed for axenic studies and that *P. aeruginosa* monitoring in field soil was carried out in pot trials under glasshouse conditions where there was a better control over watering and edaphic factors, the observations suggest that the poor survival of *P. aeruginosa* under field conditions was governed by interactions with the soil microbiome rather than due to environmental factors. The situation in the field may be still harsher with alternating spells of wetting and drying or prolonged dry/wet spells compared to the controlled conditions in the glasshouse.

The low fitness of *P. aeruginosa* strain employed in this study did not appear to arise from its endophytic origin as the same has been reported with environmental strains too. Weir et al. (1996) observed a 100-fold reduction in the cfu of *P. aeruginosa* in dry soil inoculated with rifampicin-resistant environmental strain UG2Lr over 3 weeks in comparison with cells encapsulated in dry alginate beads before application. A significant interaction effect between *P. aeruginosa* and the soil community was documented in wheat rhizosphere that the invasibility was inversely related to the level of native microbial diversity (Matos et al., 2005). Adopting cultivation-based as well as molecular monitoring, *P. aeruginosa* was not detected in most native agricultural soils but sparsely observed in manure-amended soils although the bacterium showed good survival in organic-manures (Deredjian et al., 2014). Further, employing clinical and environmental strains of *P. aeruginosa*, the introduced organism showed a decline to below detectable levels after 3–5 weeks under non-sterile microcosms while the population was maintained high under sterilized microcosms (Deredjian et al., 2014). It may be argued that an endophytic strain is more suitable for plant colonization. The 'GNS.13.2a' strain showed colonization of banana roots. However, it is imperative that endophytic microorganisms go through a phase in the soil or environment at the end of the life of the plant or the organ. Besides, soil drenching of inoculum constitutes the best form of application toward biocontrol of soil-borne pathogens. Decline in the populations of introduced bacteria in soils have been documented for a host of organisms irrespective of the source of their original isolation (van Veen et al., 1997). The form in which endophytes survive intra-plant or in soil may be different from the nutrient rich monoculture during *in vitro* growing.

There is a general criticism that the significant pathogen and pest antagonistic effects displayed by the test organisms in the laboratory trials are not always translated to successful biocontrol strategies in the field (Acea et al., 1988; van Veen et al., 1997; Upreti and Thomas, 2015). In effect, the application of *Pau* in field soil did not serve any net beneficial effect in terms of its sustained survival. It rather caused an unwarranted transient disturbance in the soil community dynamics which otherwise was at harmony as observed with the undisturbed banana and tomato control rhizospheric soils. The population of surviving *Pau* in field soil applied with nearly 10^7 cfu g^{-1} reached barely 0.01–0.025% of the initial population within 1 week in the instance of immediately wetted soil, and about 0.05% level for the microbially buffered banana rhizospheric soil. Such low population level in soil would perhaps be insufficient to offer a formidable and sustained protection against pathogens which survive in the field even under harsh conditions.

It is pertinent to mention that a vast majority of trials recommending the use of pest/pathogen antagonistic organisms are based on mere laboratory assays with no systematic monitoring of the survival of the organism or the effect due to the introduced organism on the native microorganisms. The observations here suggest the need for investigations on soil survival and microbe–microbe interaction effects before recommending the commercial adoption of bio-inoculants as the preservation and nurturing of native flora is also important in sustainable agriculture. The significance of microbe–microbe interactions under field conditions has not received much attention which is being gradually recognized now (Trabelsi and Mhamdi, 2013; Tyc et al., 2014, 2015). This also calls for the detailed analysis of microbe–microbe interactions while formulating microbial consortia rather than mere compatibility testing in nutrient plates and also testing the combined effect of the microbial consortium on soil microbiome.

Pseudomonas aeruginosa strains are known to be a human pathogen (Alonso et al., 1999; Morita et al., 2014). Therefore, it does not form a choice candidate toward biocontrol or plant growth promotion applications unless it is vividly established that the isolates infecting humans or animals and the plant/soil associated isolates are different and the possibility of horizontal gene transfers are low. The study by Kumar et al. (2013) showed the uniqueness of the endophytic isolate from pepper from other clinical isolates but the former also possessed the virulent genes as the clinical strains. A high degree of genomic conservation between *P. aeruginosa* isolates from diverse environments including clinical strains has been documented which raises concerns about the usage of this organism in agriculture (Grosso-Becerra et al., 2014). Therefore, authentic information on the relatedness between the plant isolates and human opportunistic pathogenic isolates, effect on native endophytes and the possible transmission to different plant parts all need to be considered before recommending *Pau* in agricultural applications. *P. aeruginosa* is also reported as a plant pathogen (Walker et al., 2004).

Reports highlighting the benefits of *P. aeruginosa* in terms of plant growth promotion and antagonistic effects on phytopathogens and pests and recommending the organism in agriculture are continuing to emerge as per the recent research publications cited. These studies do not mention about the monitoring of the survival and the effects on native microflora nor do they consider the potential hazards to human or animal systems. Whether the beneficial effects arise from the endophytic colonization by *P. aeruginosa* and any consequential effects on the endophytic microbiome need further investigations. The potential of transmission of the inoculant to banana fruits which is consumed in fresh form is another aspect for future research. Such investigations would be strengthened with the adoption of metagenome based analyses (Knief, 2014) facilitating interaction studies between the introduced strain and native microbiome covering cultivable and non-cultivable communities.

CONCLUSION

Pseudomonas aeruginosa proved to be a poor survivor in agricultural soil with a quick decline in the cfu of the inoculant within a week while it showed survival and proliferation under axenic conditions. Its application in the rhizospheric soil caused an unwarranted disturbance to the native soil bacterial community which in turn fought back and showed restoration of population on par with the control soil within this time span. The observations explain the cause of poor translation of some of the laboratory results ascribable to the poor survival of the inoculant in agricultural soil and highlight the need for monitoring the sustenance and performance of the introduced organism under field soil conditions which would be different from the nutrient-rich conditions during *in vitro* assays. It also pinpoints the essentiality to assess the interactive effects of the bio-inoculant with native microbial community and the plausible adverse effects on resident soil flora before commercial recommendation.

AUTHOR CONTRIBUTIONS

The experiments were planned and executed together by PT and AS. PT undertook the data analysis, interpretation, and manuscript preparation. This publication bears the Institute contribution No. 38/2015.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00493

FIGURE S1 | Testing of media formulations for the selective monitoring of *Pseudomonas aeruginosa* (*Pau*) by spotting six serial dilutions of the socks in a SP-SDS format employing pure culture of *Pau* (A), irrigation water (B), field soil (C), irrigation water mixed with *Pau* (D) and field soil sample mixed with *Pau* (E). NA, Kan+TTC NA and CNA represent nutrient agar, formulation as per Kumar et al. (2013) and CNA as per Goto and Enomoto (1970), respectively; f > indicates fungal colony growth.

TABLE S1 | Testing the interactive effects between banana endophytic strain of *Pseudomonas aeruginosa* with soil bacterial isolates in agar plate assays.

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REVIEW
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Controlling weeds with fungi, bacteria and viruses: a review

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Weeds are a nuisance in a variety of land uses. The increasing prevalence of both herbicide resistant weeds and bans on cosmetic pesticide use has created a strong impetus to develop novel strategies for controlling weeds. The application of bacteria, fungi and viruses to achieving this goal has received increasingly great attention over the last three decades. Proposed benefits to this strategy include reduced environmental impact, increased target specificity, reduced development costs compared to conventional herbicides and the identification of novel herbicidal mechanisms. This review focuses on examples from North America. Among fungi, the prominent genera to receive attention as bioherbicide candidates include *Colletotrichum*, *Phoma*, and *Sclerotinia*. Among bacteria, *Xanthomonas* and *Pseudomonas* share this distinction. The available reports on the application of viruses to controlling weeds are also reviewed. Focus is given to the phytotoxic mechanisms associated with bioherbicide candidates. Achieving consistent suppression of weeds in field conditions is a common challenge to this control strategy, as the efficacy of a bioherbicide candidate is generally more sensitive to environmental variation than a conventional herbicide. Common themes and lessons emerging from the available literature in regard to this challenge are presented. Additionally, future directions for this crop protection strategy are suggested.

Keywords: bioherbicide, herbicide resistance, turf, *Colletotrichum*, *Phoma*, *Sclerotinia*, *Xanthomonas*, *Pseudomonas*

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Recent History of Weed Control

Weeds are a problem in both crop production and turfgrass systems, associated with declines in crop yields and quality, as an esthetic nuisance and as a source of allergenic pollen (Stewart-Wade et al., 2002; Oerke, 2006; Gadermaier et al., 2014). Since the post-World War II introduction of the first selective herbicides, 2,4-D and MCPA, such products have significantly changed the management techniques that are employed by farmers and other managers of anthropogenic ecosystems (Mithila et al., 2011). The primary benefit offered by selective herbicides is the ability to control certain weed species without harming crops, based on physiological differences between species. This ability has enabled significant yield increases in many crops, and continues to be an important aspect of agroecosystem management (Mithila et al., 2011). Currently, there are 25 known herbicide target sites at the molecular level (Heap, 2015), e.g., disruption of EPSP synthase required for branched amino acid synthesis by glyphosate (Sammons and Gaines, 2014) or interference of auxin pathways by 2,4-D (Grossmann, 2010). Despite this variety, in many cases a limited number of herbicide mechanisms have been continuously employed by operators based on the low cost or ease of use associated with those products (Beckie, 2011; Mithila et al., 2011). This practice has in many cases created artificial selection pressure on weed populations, causing the widespread emergence of herbicide-resistant weeds (Beckie et al., 1999; Green and Owen, 2011; Mithila et al., 2011;

Darmency, 2013). As of June, 2015, resistant-weed populations have been reported in association with 22 of the 25 known herbicide targets (Heap, 2015).

The introduction of glyphosate-resistant transgenic crops brought a novel strategy for controlling weeds to the array of options available to operators (Green and Owen, 2011). This approach was notably different from previous selective weed control options in that it enabled the application of a broad spectrum herbicide that controlled almost all plants that were not engineered to tolerate glyphosate (Green and Owen, 2011). The unprecedented efficacy and ease of use associated with this weed control system led to its rapid adoption throughout much of the world, in many cases completely replacing former weed control practices (Beckie, 2011; Green and Owen, 2011). However, as with the generation of selective herbicides before it, the common practice of continuous glyphosate use led to the emergence of resistant weed populations, beginning in 1996 with the observation of glyphosate-resistant rigid ryegrass (*Lolium rigidum*) in Australia (Green and Owen, 2011). As of the writing of this report, there are 32 glyphosate-resistant weed species throughout the world (Heap, 2015).

It is apparent that as new herbicides are developed, weeds will continue to evolve in response to whatever selective pressure that is applied. For this reason, the continuous development of novel weed control methods is essential to the ongoing maintenance of agricultural yields. These developments are needed both to control weed populations that are resistant to currently available modes of action, as well as to diversify weed control platforms in order to delay the emergence of new resistance traits. Additionally, increasing public concern with the negative effects of pesticide residues, particularly in residential areas (e.g., turfgrass), has led to increasing demand for alternative methods of controlling weeds and other pests (Knopper and Lean, 2004; Bailey et al., 2010; Belair et al., 2010).

Biological Control of Weeds: Introduction and Scope of Review

Biological control as a general term refers to the introduction of organisms into an ecosystem with the intention of controlling one or more undesirable species (Charudattan, 2001; Bailey et al., 2010). Within the context of controlling weeds and otherwise invasive plant species, this field of study has increasingly focused on bacteria and fungi in the past five decades (Li et al., 2003), although viruses have also been considered for this purpose in select cases (Ferrell et al., 2008; Elliott et al., 2009; Diaz et al., 2014).

There are two primary fields of application within the study of biological weed control. Classical biological control refers to the release of a natural predator or pathogen of a pest species with the anticipation that it will be able to persist in the environment and provide ongoing reduction of the pest species population throughout an entire ecosystem (Dane and Shaw, 1996; TeBeest, 1996; Shaw et al., 2009). On the other hand, inundative biological control (also referred to as the bioherbicide strategy) refers to the application of propagation materials such as fungal spores or bacterial suspensions in concentrations that would not normally occur in nature, with the intention of destroying a pest species within a managed area (Johnson et al., 1996; TeBeest, 1996). The inundative biological control strategy is more relevant to the needs of agriculture and turf management, as it can generally be implemented through the application of inoculum as liquid sprays or solid granules in a similar manner to conventional herbicides (Auld et al., 2003; Caldwell et al., 2012).

In Canada, a significant number of biological agents for control of insects, plant pathogens and weeds has been approved by the Pest Management Regulatory Agency (PMRA), with 24 such products registered between 1972 and 2008, and the majority of these registrations occurring between 2000 and 2008 (Bailey et al., 2010). An even greater number of microbes and microbe-derived chemicals has been registered with the United States Environmental Protection Agency (EPA) for crop, forest, or ecological management, with 53 such products registered between 1996 and 2010 (EPA, 2014). As of 2014, 47 different microbial strains were approved in the EU for the purpose of controlling fungi or insects (European Parliament, 2014). Surprisingly, there are no microbes approved for the control of weed species in the European Union (European Parliament, 2014).

This review will focus on the use of biological agents to control weeds, including fungi, bacteria and viruses, with examples provided from North America. The review will discuss incentives to adopt these technologies, factors in the real world that affect their efficacy, and challenges to their commercialization. The review will conclude by examining future directions to accelerate progress in this promising field.

Incentives to Adopt Biological Agents to Control Weeds

The use of bioherbicides in lieu of traditional chemical inputs has the potential to offer a number of benefits to managers of ecological systems, pesticide producers and the general public. Most proponents of biological control strategies cite reduced environmental impact as the primary benefit associated with such management techniques (Auld and Morin, 1995; Johnson et al., 1996; Li et al., 2003; Ghosheh, 2005). This argument has been put forth on the basis of increased target specificity (Auld and Morin, 1995), the rapid degradation of residual biological weed control agent metabolites (Li et al., 2003), and the inability of bioherbicide species to propagate without human assistance (Johnson et al., 1996; Hoagland et al., 2007). It has also been argued that the unintended dispersal of introduced biological weed control species can be limited through the employment of agents that cannot survive without their particular host, such as certain strains of *Xanthomonas* (Schaad et al., 2001). The development cost associated with bioherbicides has also been reported to be generally lower than the cost of developing a comparable chemical agent (Auld and Morin, 1995; Li et al., 2003). Finally, as the public perception of pesticides is generally negative, the development and implementation of lower-risk pest control strategies has the potential to capture the increased willingness of consumers to pay premium prices for foods produced through these methods (Anderson et al., 1996; Bazoche et al., 2014). This has been specifically investigated by McNeil et al. (2010) through a telephone survey of Canadian consumers, in which 70% of participants indicated preference for foods produced using

biological control agents rather than synthetic insecticides. It is likely that a similar trend would emerge in regard to consumer preference for biological herbicides over conventional herbicides.

The pressure to develop novel weed control strategies has been additionally increased by the removal of several effective but environmentally problematic pesticides from various markets (Charudattan, 2001). Biological weed control strategies can potentially address this need and provide novel modes of action that will inhibit the growth of weeds that are resistant to more commonly used herbicides. Additionally, it is also possible that in some cases biological control agents could be applied in combination with herbicides to attack weed species through multiple modes of action (Auld and Morin, 1995; TeBeest, 1996).

Biological Control of Weeds Using Fungi

A list of the biological weed control candidates described in this article is summarized (**Table 1**). Most commercial biological weed control products researched in North America have been based on formulations of fungal species, however, few have been successful in the long term. Examples include BioMal, a formulation of *Colletotrichum gloeosporioides* f.sp. *malvae*, introduced for the control of round leaf mallow (*Malva pusilla*) (Mortensen, 1988; PMRA, 2006), and *C. gloeosporioides* f.sp. *aeschynomene*, which was released for control of northern jointvetch (*Aeschynomene virginica*) in the United States in 1982 as Collego (Daniel et al., 1973; Menaria, 2007), and again in 2006 as LockDown (EPA Registration Number 82681-1) (Bailey, 2014). Additionally, Sarritor, a formulation of *Sclerotinia minor* was introduced for the control of dandelion (*Taraxacum officinale*), white clover (*Trifolium repens*) and broadleaf plantain (*Plantago major*) in turf (PMRA, 2010).

Within the scientific literature, three genera of fungi have received the majority of attention as bioherbicide candidates (**Table 1**). In addition to the aforementioned BioMal and Collego, several other species within the genus *Colletotrichum* have been investigated. Additional examples include *C. truncatum*, which has been investigated to control hemp sesbania (*Sesbania exaltata*) (Schisler et al., 1991), and *C. orbiculare*, which was investigated for its potential to control spiny cocklebur (*Xanthium spinosum*) (Auld et al., 1988, 1990). An investigation of the genomes of *C. gloeosporioides* and *C. orbiculare*, found that both species contained a number of candidate genes predicted to be associated with pathogenesis, including plant cell wall degrading enzymes and secreted disease effectors including small secreted proteins (SSPs), the latter of which were shown to be differentially expressed *in planta* according to stage of infection, suggesting that some of these proteins may have specific roles in the infection process (Gan et al., 2013). There is also evidence that both of these *Colletotrichum* species have the ability to produce indole acetic acid (Gan et al., 2013), a plant hormone, derivatives of which are well established herbicide templates (Grossmann, 2010).

Three species within the genus *Phoma* have also received attention as potential agents for biological weed control (**Table 1**). *P. herbarum*, a fungal pathogen originally isolated from dandelion leaf lesions in Southern Ontario, has been investigated for control of dandelions in turf (Neumann and Boland, 1999; Stewart-Wade and Boland, 2005). *P. macrostoma* has also been investigated for similar purposes as it has been observed to specifically inhibit the growth of dicot plants (Bailey et al., 2011, 2013; Smith et al., 2015). The 94-44B strain of this species has been registered for control of broadleaf weeds in turf systems in Canada and the US (Evans et al., 2013). An investigation of 64 strains of *P. macrostoma*, including 94-44B, found that the bioherbicidal activity of these species was limited to a genetically-homogeneous group of strains, all of which were isolated from Canada thistle (Pitt et al., 2012). Through mass spectrometry, *P. macrostoma* has been recognized to produce photobleaching macrocidins (Graupner et al., 2003) that do not affect monocots (Bailey et al., 2011). As the activity of *P. macrostoma* is most apparent on new growth, it has been suggested that these compounds are transported in the phloem of the host plant (Graupner et al., 2003). Unfortunately, the specific phytotoxic mechanism of macrocidins remains unknown (Schobert and Schlenk, 2008; Zhao et al., 2011; Mo et al., 2014). Despite this, macrocidins and other molecules within the tetramic acid family have received significant attention as templates for the development of novel synthetic herbicides (Barnickel and Schobert, 2010; Yoshinari et al., 2010; Zhao et al., 2011). Additionally, an anthraquinone pigment has been isolated from a *P. macrostoma* strain and shown to have herbicidal effects on several prominent weeds of Central India (Quereshi et al., 2011). Anthraquinone pigments produced by other fungi have also been demonstrated to cause necrosis on wheat leaf blades (Bouras and Strelkov, 2008) and a variety of cultivated legumes (Andolfi et al., 2013). Although the phytotoxic mechanism underlying the effects of these compounds has not been fully characterized, the development of necrosis after exposure to the anthraquinone lentisone was found to be light dependent, a potential clue for the eventual determination of the mechanism associated with this class of molecules (Andolfi et al., 2013). Also of note within this genus is *Phoma chenopodicola*, which has been investigated as a potential control agent for lamb's quarters (*Chenopodium album*) (Cimmino et al., 2013). A phytotoxic diterpene, chenopodolin, has been isolated from this species, which was found to cause necrotic lesions on lamb's quarters (*Chenopodium album*), creeping thistle (*Cirsium arvense*), green foxtail (*Setaria viridis*) and annual mercury (*Mercurialis annua*) (Cimmino et al., 2013). Two additional fungal isolates of the genus *Phoma* have also been found to cause a modest degree of stem rot on *C. arvense*, however, these isolates were not identified at the species level (Skipp et al., 2013).

Two species within the aforementioned *Sclerotinia* genus have been investigated for their potential to control weeds. Abu-Dieyeh and Watson (2007a) found that *Sclerotinia minor* effectively controlled dandelions with and without the presence of turf species in greenhouse conditions. A follow up trial including application of *S. minor* in field conditions confirmed these results (Abu-Dieyeh and Watson, 2007b). As noted earlier, *S. minor* strain IMI 344141 was introduced to the Canadian lawn care industry under the product name Sarritor in 2010, however, it is no longer commercially available (Watson and Bailey, 2013; see Challenges in Commercialization). A relative of *S. minor*, *S. sclerotiorum*, has been observed to have phytotoxic activity against creeping

TABLE 1 | Summary of North American biocontrol agents discussed.

Bioherbicide agent	Target weed	Specificity of control agent	Intended system	Product formulation	Stage of development	Initial scientific report
Fungal agents						
Colletotrichum gloeosporioides f.sp. *aeschynomene*	Northern jointvetch (*Aeschynomene virginica*)	Also affects *Sesbania exaltata* Boyette et al. (2011)	Field crops: Rice, soybean	Aqueous spore suspensions Sandrin et al. (2003)	Registered with EPA in 1982, no longer available	Daniel et al. (1973)
Colletotrichum gloeosporioides f.sp. *malvae*	Round leaf mallow (*Malva pusilla*)	Lethal effect limited to *Malvaceae* family Mortensen (1988)	Field crops: Wheat, rye, flax, lentil, barley, canola, sunflower, soybean, oats, mustard, sugar beet and buckwheat	Aqueous spore suspension Mortensen (1988)	Registered with PMRA from 1992 to 1994 (marketed by Philom Bios Inc.)	Mortensen (1988)
Colletotrichum orbiculare	Spiny cocklebur (*Xanthium spinosum*)	Known pathogen of the *Cucurbitaceae* Harata and Kubo (2014)	Pasture and field crops	Aqueous spore suspension Auld et al. (1988)	Research phase	Auld et al. (1988)
Colletotrichum truncatum	Hemp sesbania (*Sesbania exaltata*)	Pathogenicity reported as limited to *Leguminosae* Boyette (1991) Minor pathogenicity on *Matricaria perforata* Hynes et al. (2010)	Field crops	Aqueous spore suspension Boyette et al. (2007)	Research phase	Boyette (1991)
Phoma chenopodicola	Lamb's quarters (*Chenopodium album*), Creeping thistle (*Cirsium arvense*), Green foxtail (*Setaria viridis*), Annual mercury (*Mercurialis annua*)	Not tested on other species	Field crops such as sugar beet and corn	Active ingredient isolated from live culture of *P. chenopodicola* using organic solvents [MeOH-H_2O (1:3) solution] Cimmino et al. (2013)	Research phase	Cimmino et al. (2013)
Phoma herbarum	Dandelion (*Taraxacum officinale*)	Reported as a potential control agent for *Trianthema portulacastrum* Ray and Vijayachandran (2013)	Turf	Suspension of mycelia in potato dextrose broth Neumann and Boland (1999)	Research phase	Neumann Brebaum (1998); Neumann and Boland (1999)
Phoma macrostoma	Dicot plants	Affects most monocots but not dicots Bailey et al. (2011)	Turf	Granules composed of mycelial fragments and flour (final iteration of inoculum formula) Bailey et al. (2011)	94-44B strain registered with EPA and PMRA in 2011	Graupner et al. (2003); Bailey et al. (2011)
Sclerotinia minor	Dandelion (*Taraxacum officinale*), White clover (*Trifolium repens*), and Broadleaf plantain (*Plantago minor*)	Wide host range, predominantly dicot species Melzer et al. (1997)	Turf	Barley grits containing actively growing mycelia Abu-Dieyeh and Watson (2006)	Registered with PMRA as Sarritor in 2010, no longer available	Riddle et al. (1991)
Chondrostereum purpureum strain HQ1	Re-growth of deciduous trees and shrubs	Wide host range Setliff (2002)	Forestry	Paste containing mycelia PMRA (2002)	Registered as Mycotech Paste with PMRA in 2002, and with EPA in 2005	Setliff (2002)

(continued)

TABLE 1 | *continued*

Chondrostereum purpureum strain PFC 2139	Re-growth of deciduous trees and shrubs	Wide host range Setliff (2002)	Forestry	Paste containing mycelia EPA (2004)	Registered as Chontrol Paste with EPA in 2004, and with PMRA in 2007, commercially available in both regions	Setliff (2002)
Puccinia thlaspeos	Dyer's woad (*Isatis tinctoria*)	*Isatis tinctoria* only EPA (2002)	Ecological management	Spray containing leaf fragments of infected *Isatis tinctoria*	Registered as Woad Warrior with EPA in 2002, no longer commercially available	Lovic et al. (1988)
Alternaria destruens	Dodder species (*Cuscata* spp.)	Observed to affect several unspecified crop species EPA (2005)	Alfalfa, cranberries, carrots, peppers, tomatoes, eggplant, blueberries, and woody ornamentals	Wettable power and granules containing spores	Registered as Smolder G and Smolder WP with EPA in 2005, no longer commercially available	Simmons (1998)
Phytophthora palmivora	Strangler vine (*Morrenia odorata*)	Weak pathogen of some crop species Ridings et al. (1973)	Citrus orchards	Aqueous spore suspension	Registered as DeVine with EPA in 1981, re-registered in 2006. No longer commercially available	Burnett et al. (1973)
Bacterial agents						
Pseudomonas fluorescens strain D7	Downy brome (*Bromus tectorum*)	Effect is limited to *Bromus tectorum* Kennedy et al. (2001)	Field crops	Cell-free filtrate prepared from nutrient broth culture Gealy et al. (1996)	Research phase	Kennedy et al. (1991)
Pseudomonas fluorescens strain BRG100	Green foxtail (*Setaria viridis*)	Not identified	Not specified	Granules prepared from live culture in nutrient broth, oat flour, maltose and molasses Caldwell et al. (2012)	Research phase	Quail et al. (2002)
Pseudomonas fluorescens strain WH6	Inhibits most of the species tested	Non-specific	Not specified	Cell-free filtrate Banowetz et al. (2008)	Research phase	Banowetz et al. (2008)
Viral agents						
Tobacco Mild Green Mosaic Tobamovirus	Tropical soda apple (*Solanum viarum*)	Also affects *Capsicum* spp. and *Nicotiana* spp. Font et al. (2009); EPA (2015)	Pastures	Extract from virus-infected tobacco leaf in sodium phosphate buffer used in experimental phase	Registered with EPA in 2015	Ferrell et al. (2008)
Araujia Mosaic Virus	Moth plant (*Araujia hortorum*)	Also affects *Morrenia odorata, Oxypetalum caeruleum* and *Gomphocarpus* spp.	Ecosystem management	Virus-infected leaves of *Morrenia odorata* in sodium phosphate buffer	Research discontinued because of pathogenicity on *Gomphocarpus fruticosus* Elliott et al. (2009)	Elliott et al. (2009)
Unspecified virus resembling Tobacco Rattle Virus	*Impatiens glandulifera*	Also affects species within *Chenopodium* and *Nicotiana* Kollmann et al. (2007)	Ecosystem management	Virus-infected leaves of *Impatiens glandulifera* in phosphor extraction buffer	Research phase	Kollmann et al. (2007)
Óbuda Pepper Virus	*Solanum nigrum*	Wide host range Tobias et al. (1982)	Ecosystem management	Mechanical inoculation	Research phase	Kazinczi et al. (2006)
Pepino Mosaic Virus	*Solanum nigrum*	Wide host range including *Amaranthaceae, Chenopodiacee, Compositae, Convolvulaceae, Malvaceae, Plantaginaceae* and *Solanaceae* Papayiannis et al. (2012)	Ecosystem management	Mechanical inoculation	Research phase	Kazinczi et al. (2006)

thistle (*Cirsium arvense*) (Skipp et al., 2013). Production of oxalic acid by both *S. minor* (Briere et al., 2000) and *S. sclerotiorum* (Magro et al., 1984) has been observed to play a role in the virulence of these fungi on their host plant. Oxalic acid production can be encouraged through addition of sodium succinate to *S. minor* growth media, and cultures grown on sodium succinate-enriched media caused greater development of necrotic tissue when applied to dandelion than cultures grown on non-enriched media (Briere et al., 2000). Oxalic acid acidifies the host tissue, enabling cell wall degradation, and also interferes with polyphenol oxidase (PPO), which normally assists in plant defense (Magro et al., 1984). Low concentrations of oxalic acid have also been shown to suppress the release of hydrogen peroxide, another plant defense molecule, in cell cultures of soy and tobacco (Cessna et al., 2000).

In addition to the other examples described earlier in the text, several other fungi have been registered as bioherbicides for use in forestry or ecosystem management in Canada and the US (Bailey, 2014), though in general, there appears to be limited research about these strains with respect to their mode of action. Two separate strains of *Chondrostereum purpureum* have been registered in Canada and the US for controlling regrowth of deciduous tree species in coniferous plantations (Bailey, 2014). This fungal species is a naturally occurring pathogen of deciduous trees in North America (Setliff, 2002). Although the potential host range of this species is fairly wide, wound infection is a key element of successful infection in most cases (Setliff, 2002). *C. purpureum* strain HQ1 was registered under the product name Mycotech Paste with the PMRA in 2002 (PMRA Reg. No. 27019) and the EPA in 2005 (EPA Reg. No. 74128-2). Registration of this strain with the PMRA ended in 2008. Another strain of this species, PFC 2139, was registered under the product name Chontrol Paste with the EPA in 2004 (EPA Reg. No. 74200-E/R) and with the PMRA in 2007 (PMRA Reg. No. 27823 and 29293). Both registrations are currently active and this product remains commercially available.

Another fungus, *Puccinia thlaspeos*, was registered with the EPA in 2002 under the product name Woad Warrior for control of Dyer's woad (*Isatis tinctoria*) (EPA Registration Number 73417-1). This fungus is an obligate parasite and requires a living host to reproduce, however, inoculum can be produced from dried and ground plant material of its target weed (Thomson and Kropp, 2004). This product is no longer commercially available (Bailey, 2014).

Alternaria destruens strain 059 was registered with the EPA in 2005 under the product names Smolder WP and Smolder G (EPA Reg. Nos. 34704-825 and 34704-824, respectively). This product, originally isolated from *Cuscuta gronovii* growing in unmanaged conditions in Wisconsin, is intended for control of dodder species (*Cuscuta* spp.) (Cook et al., 2009), however, it is not commercially available (Bailey, 2014).

A final bioherbicide that bears mentioning is DeVine, a formulation of the fungus *Phytophthora palmivora* (Kenney, 1986). This product was registered with the EPA in 1981 and again in 2006 (Bailey, 2014; EPA Reg No. 73049-9). *P. palmivora* was originally isolated from strangler vine (*Morrenia odorata*) in Florida and was used to control the same species in citrus orchards (Ridings, 1986). Although this product was re-registered in 2006, it is no longer commercially available (Bailey, 2014).

Biological Control of Weeds Using Bacteria

A number of bacteria have also been investigated as potential biological weed control agents (**Table 1**). Of these, *Pseudomonas fluorescens* and *Xanthomonas campestris* have attracted the most attention. Biological weed control using bacteria has been suggested to have several advantages over the use of fungi, including more rapid growth of the bioherbicide agents (Johnson et al., 1996; Li et al., 2003), relatively simple propagation requirements (Li et al., 2003), and high suitability for genetic modification through either mutagenesis or gene transfer (Johnson et al., 1996).

As mentioned above, *P. fluorescens* has received much of the attention as a biological weed control agent (**Table 1**). There are many strains of this species, some of which are beneficial to plants (Gamalero et al., 2005), whereas others are inhibitory (Banowetz et al., 2008). Among studies into the suppressive effects of *P. fluorescens*, three strains have been investigated in especially great detail, all of which have been observed to inhibit plant growth and/or germination through the production of extracellular metabolites (Kennedy et al., 1991; Quail et al., 2002; Banowetz et al., 2008).

Pseudomonas fluorescens strain D7, originally isolated from the rhizospheres of winter wheat (*Triticum aestivum*) and downy brome (*Bromus tectorum*) in Western Canada, has been observed to selectively inhibit growth and germination of a number of grassy weeds, most notably downy brome (Kennedy et al., 1991, 2001; Gealy et al., 1996). By selective removal of compounds from cell-free filtrates, the growth-inhibiting activity associated with this strain was attributed to a combination of extracellular peptides and a lipopolysaccharide, which were suggested to work in conjunction to express herbicidal activity (Gurusiddaiah et al., 1994). No subsequent reports regarding mechanism were found in the available literature.

Conversely, *P. fluorescens* strain WH6 has been observed to affect the germination of a much broader range of plant species, significantly inhibiting germination of all species tested (21 monocot species and 8 dicot species) with the exception of a modern corn (*Zea mays*) hybrid (Banowetz et al., 2008). The germination-inhibiting activity of the WH6 strain has been attributed to the production of a compound originally referred to as Germination Arrest Factor (GAF; Banowetz et al., 2008). The active component of GAF has been identified through nuclear magnetic resonance spectroscopy and mass spectrometry as 4-formylaminooxy-L-vinylglycine (McPhail et al., 2010), and its biosynthesis has been proposed to begin with the amino acid homoserine (Halgren et al., 2013). This class of compounds, the oxyvinylglycines, has been shown to interfere with enzymes that utilize pyridoxal phosphate as a cofactor, including enzymes involved in nitrogen metabolism and biosynthesis of the plant hormone ethylene (Berkowitz et al., 2006; Halgren et al., 2013). Interestingly, GAF has also been recognized to express specific bactericidal activity against *Erwinia amylovora*, the bacterium that causes fire blight in orchards (Halgren et al., 2011). The

genome sequence of *P. fluorescens* strain WH6 has been published (Kimbrel et al., 2010), and gene knockouts were used to identify several biosynthetic and regulatory genes involved in GAF/ 4-formylaminooxy-L-vinylglycine production (Halgren et al., 2013; Okrent et al., 2014). Strain D7 was also included in the original investigation of strain WH6, however, as culture filtrates of strain D7 prepared in the same manner as WH6 did not possess germination-inhibiting activity the authors suggested that GAF was not responsible for the activity associated with strain D7 (Banowetz et al., 2008).

The production of extracellular metabolites with phytotoxic effects has also been observed in an additional *P. fluorescens* strain, referred to as BRG100, which has been recognized to have suppressive activity on the grassy weed green foxtail (*Setaria viridis*) (Quail et al., 2002; Caldwell et al., 2012). The herbicidal compounds produced by this species, referred to as pseudophomin A and B, have been characterized through a combination of serial chromatography, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), chemical degradation, and X-ray crystallography (Quail et al., 2002; Pedras et al., 2003). Unfortunately, neither the biosynthetic pathway involved in the production of these compounds nor the specific biochemical effects of these molecules on green foxtail have been characterized at this time. However, the full genome sequence of this strain has been published (Dumonceaux et al., 2014) and a detailed projection of the costs and technical requirements for the mass production of this biocontrol agent has been reported (Mupondwa et al., 2015).

The other bacterial species that has received much of the attention as a candidate biological weed control agent is *Xanthomonas campestris* (**Table 1**). Most notably within this species, the strain *X. campestris* pv. *poae* (JT-P482) was registered in Japan in 1997 for control of annual bluegrass (*Poa annua*) under the product name Camperico (Imaizumi et al., 1997; Tateno, 2000). The activity of this species is specific to *Poa annua* and *Poa attenuata*, and was not reported to affect other turf species tested (Imaizumi et al., 1997). A separate strain of *X. campestris* (isolate LVA-987) has also received attention as a potential control agent against horseweed (*Conyza canadensis*) (Boyette and Hoagland, 2015). No discovery of phytotoxic compounds was reported in any of the aforementioned investigations into application of *X. campestris* as a bioherbicide, however, compounds with phytotoxic activity have been previously isolated from the *vitians* pathovar of this species (Scala et al., 1996), and it is possible that phytotoxic metabolites play a role in the suppression of *Poa annua* and *Conyza canadensis*. Although the cause of host-plant suppression was not indicated in the above studies, the infection process of *X. campestris* pv. *campestris* (*Xcc*) in brassica crops has been well characterized. Briefly, *Xcc* can colonize the xylem of the host plant and use this pathway to spread throughout the organism (Duge de Bernonville et al., 2014). The success of *Xcc* in reaching the host xylem is contingent on its interaction with receptor proteins of the host plant that can recognize pathogen associated molecular patterns (PAMPs), potentially resulting in elicitation of plant defense responses such as programmed cell death and increased production of reactive oxygen (Guy et al., 2013).

Biological Control of Weeds Using Viruses

In select cases, viruses that affect weed species have also been considered as bioherbicide candidates. This strategy is more commonly considered for management of invasive species in broader ecosystems rather than specifically managed areas. Viruses have been suggested to be inappropriate candidates for inundative biological control due to their genetic variability and lack of host specificity (Kazinczi et al., 2006). Examples of viruses that have been investigated for the potential to control invasive or undesirable species include Tobacco Mild Green Mosaic Tobamovirus for control of tropical soda apple (*Solanum viarum*) in Florida (Ferrell et al., 2008; Diaz et al., 2014), and *Araujia* Mosaic Virus for control of moth plant (*Araujia hortorum*) in New Zealand (Elliott et al., 2009). A patent on the former biological control agent has been filed (Charudattan et al., 2009) and EPA approval for use on fenced-in pasture areas was granted in 2015 (EPA, 2015). A virus resembling Tobacco Rattle Virus has also been proposed as a control agent for *Impatiens glandulifera*, an invasive weed of concern in central and western Europe (Kollmann et al., 2007). Similarly, Óbuda Pepper Virus (ObPV) and Pepino Mosaic Virus (PepMV) have been proposed as agents to reduce overall populations of the weed *Solanum nigrum* (Kazinczi et al., 2006). The biological activities of viruses are very distinct from pathogenesis caused by bacteria or fungi, and may present additional opportunities for biological weed control in some situations.

Real World Factors that Affect the Efficacy of Bioherbicides

The research pipeline from the screening stage to field conditions faces a number of unique challenges (**Figure 1**). One commonly reported challenge is the need for continuous moisture availability during the period in which the biocontrol agent infects the plant (Auld et al., 1990; Schisler et al., 1991; Auld and Morin, 1995; Stewart-Wade and Boland, 2005; Boyette and Hoagland, 2015). In a review of bioherbicide technology published by Auld and Morin (1995), it was reported that dew periods of more than 12 hours are commonly necessary for bioherbicide candidates to successfully infect their hosts. A variety of techniques to provide this moisture have been tested, with varying degrees of success. In order to prolong the period of leaf wetness necessary for successful infection of dandelion by *Phoma herbarum*, several vegetable oil emulsions were included in aqueous inoculants, however, these additives were found to be phytotoxic, thus obscuring the benefit to infection that may have been caused by their addition (Stewart-Wade and Boland, 2005). Timing inoculant application to prolong the leaf wetness period (e.g., application at dawn or dusk) has also been suggested as a simple method of maximizing infection, although the success of this technique can be highly sensitive to environmental fluctuations (Auld and Morin, 1995). In some cases, solid inoculant media have also been investigated. The most common method for developing solid inoculant media is to propagate the candidate biological weed control species on grains which will subsequently be applied directly to the field or incorporated with other moisture-retaining materials

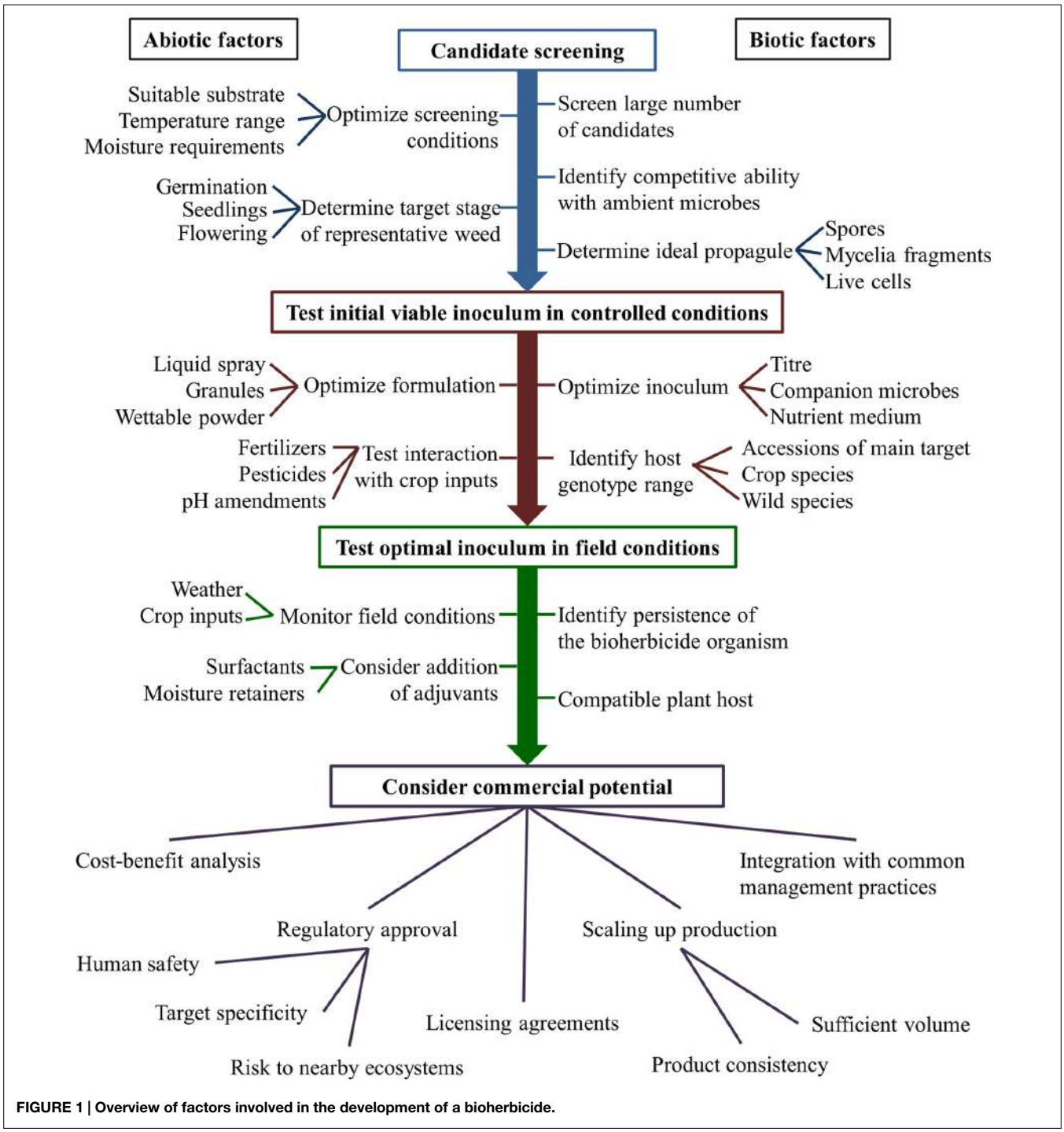

FIGURE 1 | Overview of factors involved in the development of a bioherbicide.

such as calcium alginate, oils or vermiculite (Auld et al., 2003). Granular applications have the advantage of prolonging the in-field survival of introduced biological weed control agents through the provision of moisture and nutrients, however, they are also generally associated with a more gradual rate of infection (Auld et al., 2003).

The interplay of temperature and humidity also has a significant effect on the success or failure of infection by many pathogens (Ghosheh, 2005) and may alter the efficacy of biological control agents (Casella et al., 2010; **Figure 1**). Cold air can retain less total moisture than warm air, and thus the relative humidity is more commonly elevated at lower temperatures. Elevated humidity is generally beneficial to successful bioherbicide colonization because it decreases evaporation rates, thus increasing the duration of leaf wetness following inoculant application (Casella et al., 2010). In investigating the efficacy of the biological weed

control product Sarritor, it was found that infection rates were highest when the temperature remained below 20°C and the relative humidity was high (Siva, 2014). Similar requirements have been suggested by Auld et al. (1990), who observed that successful infection of spiny cocklebur (*Xanthium spinosum*) by biological weed control agent *Colletotrichum orbiculare* may have been contingent on elevated humidity. As with any species, most biological control species will have a fairly finite temperature range in which they can survive, as well as a narrower range in which their activity will be maximized. For example, Imaizumi et al. (1997) found that ambient temperatures below 25°C (day) and 20°C (night) caused decreased efficacy of *Xanthomonas campestris* pv. *poae* in the suppression of annual bluegrass (*Poa annua*). Similarly, the efficacy of *X. campestris* isolate LVA-987 in controlling horseweed (*Conyza canadensis*) was found to require ambient temperatures between 20°C and 35°C, with peak efficacy between 25°C and 35°C (Boyette and Hoagland, 2015). This parameter will be different for any given biological control candidate species and thus temperature and humidity should be tracked throughout any efficacy trials involving biological weed control agents.

Quorum sensing refers to the ability of a bacterium to differentially express genes based on its population density (Rutherford and Bassler, 2012). The effect of bacterial and fungal population densities can in some cases inform the behavior of these species, and in some cases affect whether a pathogen is virulent or latent (Bowden et al., 2013; Lu et al., 2014). This is an important factor in the characterization of potential bioherbicides, however, testing inoculant media with varying population densities (**Figure 1**) is not a common practice within the investigation of biological weed control strategies, nor is the phenomenon of quorum sensing commonly discussed in the related literature. However, apparent latent periods in the life cycle of biological weed control candidate species have been occasionally reported (Romero et al., 2001; Paynter et al., 2006), and it is possible that quorum sensing effects could explain these cases of asymptomatic infection.

It is possible that interactions with fertilizers and pesticides could affect the infectiousness of a candidate biological weed control agent (Boyetchko, 1997; **Figure 1**). For example, an investigation of the ability of *P. macrostoma* to control dandelions in turf found that co-application with a high rate of nitrogen fertilizer improved its efficacy, whereas co-application with phosphorus had no effect, and potassium sulfate decreased efficacy (Bailey et al., 2013).

Challenges in Commercialization

Despite the promise shown by many bioherbicides, few have achieved long-term commercial success, in part due to the challenges to achieving consistent efficacy in field conditions noted above. For example, amongst the fungal bioherbicides described in this review, only LockDown (*C. gloeosporioides* f.sp. *aeschynomene*) remains commercially available (Bailey, 2014). In the case of BioMal (*C. gloeosporioides* f.sp. *malvae*), the narrow target specificity (only round leaf mallow) of the product made for a market niche that was too small to cover production costs (Cross and Polonenko, 1996). Additionally, significant challenges were encountered in maintaining product consistency while scaling up production volumes (Boyetchko et al., 2007). For these reasons, Philom Bios, the original commercial producer of this bioherbicide, discontinued its production in 1994, only 2 years after registration (Boyetchko et al., 2007). The strain was later licensed to Encore Technologies in 1998, however, challenges with maintaining product consistency under mass production led to the abandonment of the project (Boyetchko et al., 2007). In the case of Sarritor (*S. minor* strain IMI 344141), the commercial failure has been attributed to challenges with increasing production volume and product consistency, as well as inconsistent efficacy of the product due to the narrow range of environmental conditions in which successful infection will occur (Watson and Bailey, 2013). Unfortunately, the current commercial status of Camperico (*X. campestris* pv. *poae* JT-P482), described above, is unclear (Bailey, 2014).

Future Directions

Mechanism of Action

As noted above, the mechanism(s) behind the suppressive activity of a given biocontrol agent is in many cases only partially understood. Future research into the mechanisms underlying these effects will be important to achieve consistent efficacy with biocontrol agents, as well as to evaluate potential impacts on human and ecosystem health. This in turn will be of value to gaining regulatory approval. Additionally, understanding bioherbicidal mechanisms may generate novel chemical herbicides to overcome current resistance traits (Boyetchko et al., 2009), and will likely also be of peripheral value to the field of plant pathology.

Transition to the Field

Translating effects observed in a controlled environment to field conditions is a significant challenge to the development of successful biocontrol agents (**Figure 1**), and it is common for projects to conclude at this juncture. Thus, the development of new delivery formulations intended to improve the in-field stability of biocontrol agents is as important as the discovery of the agents themselves. Widespread testing of a given biocontrol agent in a variety of locations, similar to plant variety testing, is essential to understanding the feasibility of introducing that agent on a broad scale. Finally, the production of commercially relevant quantities of viable inoculum or culture extract must also be considered, as techniques employed in the laboratory are frequently impractical for industrial-scale production. Lessons from other industries such as pharmaceuticals and probiotic foods will likely be valuable in addressing this challenge.

Extraction of Herbicidal Compounds

As discussed earlier, in some cases a particular herbicidal compound can be extracted from a live culture (Kennedy et al., 1991; Quail et al., 2002; Banowetz et al., 2008). This strategy can yield a more stable control agent, the efficacy of which will not be contingent on the continued survival of a given organism in an

uncontrolled environment. Although the differentiation between naturally and synthetically sourced pesticides may be arbitrary in terms of their potential effect on human and environmental health, such compounds will likely be more acceptable in the public eye than those produced through traditional chemistry.

New Sources of Bioherbicide Candidates

This review has focused on a limited number of genera which have received an especially great degree of attention as bioherbicide candidates for turf and field crop situations. Considering the degree of taxonomic diversity among microbes, there are opportunities to employ other genera as bioherbicides in the future. Most of the studies discussed in this review employed microbes that were originally isolated from diseased individuals within the population of a weed species (Kennedy et al., 1991; Neumann and Boland, 1999; Ghosheh, 2005). However, there are additional ecological niches from which potential biological weed control candidates can be discovered. For example, most plant species form relationships with a variety of microbes, referred to as endophytes, which colonize the internal environment of the plant without causing disease (Duan et al., 2013; Ali et al., 2014). There is evidence that endophytes can play a role in nutrient accumulation, drought tolerance and disease resistance (Compant et al., 2010; Johnston-Monje and Raizada, 2011; Mousa and Raizada, 2013). Growth-promoting endophytes have been shown to reduce weed populations in pastures by inoculating the desired grass species, enabling them to compete with weeds more effectively (Saikkonen et al., 2013; Vazquez-de-Aldana et al., 2013). It has been reported that some plant-inhabiting microbes will express host-specific behavior, acting as an endophyte in some plant species but as a pathogen in another (Gomes et al., 2013). Additionally, some endophytes have also been reported to produce compounds that are phytotoxic to non-host species (Waqas et al., 2013; Zhang et al., 2013; Li et al., 2014). These phenomena could potentially be applied to controlling undesirable weed species. Endophyte-based weed control may have unique advantages over the application of pathogens such as improved ability of candidate microbes to persist in field conditions through having a more consistent ecological niche within their plant host, or the provision of other benefits to their host such as nutrient acquisition or disease resistance.

Conclusion

Although there are many challenges and constraints inherent in the development of biological herbicides, the increasing prevalence of both herbicide-resistant weeds (Green and Owen, 2011) and public concern with pesticide use (McNeil et al., 2010) creates a strong impetus for continued investigation in this field. These strategies will be of especially great value to organic production systems and to regions where cosmetic pesticide bans are in place. With continued investigation in this field, there is significant potential for the development of new weed control strategies that can be employed to delay herbicide resistance, produce food in accordance with consumer concerns, and reduce the environmental impact of modern agriculture and ecosystem management. Although there is a considerable number of candidate species that have been considered for this purpose, the major challenge to successful implementation of this strategy is the development of techniques to maintain consistent efficacy in field conditions.

Author Contributions

DPH conceived and wrote the manuscript, and MNR edited the manuscript. Both authors approved the final manuscript.

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REVIEW
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Novel Routes for Improving Biocontrol Activity of *Bacillus* Based Bioinoculants

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Biocontrol (BC) formulations prepared from plant-growth-promoting bacteria are increasingly applied in sustainable agriculture. Especially inoculants prepared from endospore-forming *Bacillus* strains have been proven as efficient and environmental-friendly alternative to chemical pesticides due to their long shelf life, which is comparable with that of agrochemicals. However, these formulations of the first generation are sometimes hampered in their action and do not fulfill in each case the expectations of the appliers. In this review we use the well-known plant-associated *Bacillus amyloliquefaciens* type strain FZB42 as example for the successful application of different techniques offered today by comparative, evolutionary and functional genomics, site-directed mutagenesis and strain construction including marker removal, for paving the way for preparing a novel generation of BC agents.

Keywords: plant growth-promotion, biocontrol, *Bacillus amyloliquefaciens* subsp. *plantarum*, mersacidin, bacillomycin D, surfactin, bacilysin, harpin genes

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INTRODUCTION

As stated by Compant et al. (2005) in their excellent review, pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. Approximately 25% of the world's crop yield is lost every year due to plant pathogens (Lugtenberg, 2015). As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations (Schäfer and Adams, 2015).

However, due to the negative impact on environment caused by agrochemicals, disease control by beneficial bacteria as an alternative to chemical pesticides in plant protection is steadily increasing and begins to replace in part chemical pesticides (Qiao et al., 2014). It has been shown that applying spore formulations of the plant-beneficial bacterium *Bacillus amyloliquefaciens* does not affect the composition of rhizosphere microbial community (Chowdhury et al., 2015a). An increasing number of farmers are recognizing the need for other avenues for pest control that are not as damaging to the environment and the land. According to a comprehensive study of BCC Research, global markets for biopesticides will grow from USD54.8 billion in 2013 to USD83.7 billion to 2019[1].

[1] www.bccresearch.com/market-research/chemicals/biopesticides-chm029e.html

Thereby, biological preparations from spore-forming *Bacillus* sp. are preferred, because their long-term viability facilitates the development of commercial products (Borriss, 2015a). These plant-associated bacteria are characterized by their simultaneous plant-growth promoting (PGP) and biocontrol (BC) activity. It should be mentioned here, that both features are linked with each other and should not artificially separated by regulatory authorities: Plant growth promoting effects strengthen plants and made them more resistant against pathogens and *vice versa* suppression of pathogens enhances plant health and reduces harvest losses (Kamilova et al., 2015). Unfortunately, the success of such biologicals in agriculture is still hampered by sometimes inconsistent field performance due to insufficient knowledge about basic mechanisms of interactions between bacilli and plants, although some progress has been made in last decade (Ravensberg, 2015).

Plant-associated *B. amyloliquefaciens* strains belonging to subsp. *plantarum* (*methylotrophicus*) (Borriss et al., 2011; Dunlap et al., 2015) are distinguished from other representatives of endospore-forming *B. amyloliquefaciens* by their ability to colonize plant rhizosphere, to stimulate plant growth and to suppress competing phytopathogenic bacteria and fungi. Due to their biofertilizer and BC properties they are becoming increasingly important as a natural alternative to chemical pesticides and other agrochemicals (Borriss, 2011). We have directed our research on *B. amyloliquefaciens* FZB42^T, the type strain for *B. amyloliquefaciens* subsp. *plantarum*. Since its first description (Krebs et al., 1998) more than 70 articles dealing with FZB42 have been published[2]. In order to reveal the specific genomic features linked with the properties beneficial for plant growth and BC, we have sequenced the whole genome of FZB42 as the first example of Gram-positive plant beneficial bacteria (Chen et al., 2007).

Comparative genome analysis, transposon mutagenesis, transcriptome and proteome analysis of this model organism have been proven as valuable means to analyze its plant growth promoting and BC activities (Chowdhury et al., 2015a). Ten giant gene clusters covering nearly 10% of the whole genome and responsible for non-ribosomal and ribosomal synthesis of secondary metabolites with antimicrobial and nematocidal action were identified (Borriss, 2013). In addition, an incomplete gene cluster directing immunity against the type B lantibiotic mersacidin was detected (**Table 1**). In this review we will describe several possibilities offered today by *in vitro* techniques for enhancing the beneficial action of bioformulations based on *B. amyloliquefaciens* FZB42, and its close relatives SQR9 and NJN6, isolated by the laboratory of Qirong Shen, Nanjing Agriculture University.

PHYLOGENOMICS OF *Bacillus amyloliquefaciens*

The genus *B. amyloliquefaciens* harbors members of different ecotypes (plant-associated and non-plant associated, Reva et al., 2004). Our analysis based on the use of all core genes of a set of 42 genomes to maximize the sequence support for the phylogenetic tree (Zdobnov and Bork, 2007) and used the pipeline provided by the EDGAR software (Blom et al., 2009). According to phylogenomic analysis *B. amyloliquefaciens* is clustered into three taxonomic units which could be considered as 'subspecies' (**Figure 1**):

(1) *B. amyloliquefaciens* subsp. *plantarum* (*B. methylotrophicus*)
(2) *B. amyloliquefaciens* subsp. *siamensis (B. siamensis)*
(3) *B. amyloliquefaciens* subsp. *amyloliquefaciens*

Interestingly, the two available genomes of *B. siamensis* formed a separate taxonomic unit within the *B. amyloliquefaciens* subspecies complex suggesting that the taxonomic classification of *B. siamensis* has to be reconsidered. As reported recently (Dunlap et al., 2015), *B. methylotrophicus* and *B. amyloliquefaciens* subsp. *plantarum* are not distinguishable by their core genome sequences and form together a robust taxonomic unit comprising all plant-associated representatives of the genus *B. amyloliquefaciens* (group 1). Overall, the *B. amyloliquefaciens* pan genome consists of 8652 CDS, whilst the core genome consists of 2104 CDS with *Bacillus amyloliquefaciens* FZB42 (NC_009725) used as reference.

The pan genome derived only from representatives of *B. amyloliquefaciens* subsp. *plantarum* and *B. methylotrophicus* (plant-associated group 1) comprises 7936 CDS, which is reflecting the high flexibility in adapting to plant-associated lifestyle. The core genome formed by the 35 *B. amyloliquefaciens* subsp. *plantarum* and 3 *B. methylotrophicus* genomes consists of 2295 CDS suggesting that 54 genes of the core genome are unique for the subsp. *plantarum* (*B. methylotrophicus*) and do not occur in the non-plant-associated subsp. *amyloliquefaciens* and in *B. siamensis* (Qiao et al., 2014). Within these singletons are the genes involved in non-ribosomal synthesis of the polyketides difficidin (Chen et al., 2006) and macrolactin (Schneider et al., 2007), an iturin-like compound (e.g., bacillomycin or iturinA, Borriss et al., 2011), and several genes involved in carbohydrate degradation and transport, such as glucuronate isomerase (*uxaC*), 2-keto-3-deoxygluconokinase (*kdgK*), 2-keto-3-deoxygluconate -6-isomerase-6-phosphate aldolase (*kdgA*), endo-1,4-beta-glucanase (*eglA*), and saccharifying amylase (*amyE*). Many of these genes, unique for plant-associated *B. amyloliquefaciens* seem to be acquired by horizontal gene transfer. FZB42 contains 17 genomic islands (Chen et al., 2007). Certain DNA islands appear to be linked with the plant-associated lifestyle. Island 7 (28,754 bp) for instance, contains genes with striking similarity to genes involved in extracellular arabinogalactane hydrolysis, galactose uptake by a sugar-specific phosphotransferase system IIABC and galactose catabolism in enterococci, lactobacilli and *Erwinia carotovora* (Chen et al., 2007). It can be assumed that acquisition of this molecular toolbox, comprising several elements derived from other soil- and plant-associated bacteria has enhanced the ability of FZB42 to exploit plant-derived polysaccharides.

A recent comparative analysis of core genomes from 28 *B. amyloliquefaciens* subsp. *plantarum* and 32

[2]http://www.nordreet.de/bacillus-consulting/literatur/

TABLE 1 | Genes and gene cluster encoding for secondary metabolites in *Bacillus amyloliquefaciens plantarum* FZB42.

Metabolite	Genes and gene cluster	Size (bp)	Genome position (bp)	MIBiG	Effect against	Reference
Sfp-dependent non-ribosomal synthesis of lipopeptides (NRP)						
Surfactin	*srfABCD*	28,544	341,664-370,208	BGC0000433	virus, *Mycoplasma*	Koumoutsi et al., 2004
Bacillomycin D	*bmyCBAD*	39,113	c1,908,427-c1,869,309	BGC0001090	fungi	Koumoutsi et al., 2004
Fengycin	*fenABCDE*	37,669	c1,968,997-c1,931,328	BGC0001095	fungi	Koumoutsi et al., 2004
Bacillibactin	*dhbABCDEF*	11,954	c3,032,970-c3,021,016	BGC0001185	microbial competitors	Chen et al., 2007
Sfp-dependent non-ribosomal synthesis of polyketides (PKS)						
Macrolactin	*mlnABCDEFGHI*	53,253	1,391,841-1,445,094	BGC0000181	bacteria	Schneider et al., 2007
Bacillaene	*baeBCDE, acpK, baeGHIJLMNRS*	72,437	1,700,345-c1,772,782	BGC0001089	bacteria	Chen et al., 2006
Difficidin	*dfnAYXBCDEFGHIJKLM*	69,523	c2,276,743-c2,346,266	BGC0000176	bacteria	Chen et al., 2006
Sfp-independent non-ribosomal synthesis (NRP)						
Bacilysin	*bacABCDE, ywfG*	5,907	c3,593,877-c3,599,784	BGC0001184	bacteria	Chen et al., 2009b
Ribosomal synthesis of processed and modified peptides (bacteriocins, lantibiotics, RiPPs)						
Plantazolicin	*pznFKGHIAJC DBEL*	9,891	726,457-736,348	BGC0000569	*B. anthrax*, nematodes	Scholz et al., 2011
Amylocyclicin	*acnBACDEF*	4,112	c3,048,678-c3,044,568	BGC0000616	related bacteria	Scholz et al., 2014
Mersacidin (partial)	*mrsK2R2FGE*	4,828	c3,774,552-c3,769,734	BGC0000527	Gram-+ bacteria	Borriss, 2013

The available MIBiG entries (Medema et al., 2015) are indicated.

B. amyloliquefaciens species identified 193,952 bp of sequences that are present within the subsp. *plantarum* core genome but absent in the *B. amyloliquefaciens* core genome (Hossain et al., 2015). Among these genetic loci there were 73 genes shared by all 28 *plantarum* strains but were not present in any strains of subsp. *amyloliquefaciens*. The putative functions of these genes included transportation (7 genes), regulation (7 genes), signaling (1 gene), carbon degradation (10 genes), synthesis of secondary metabolites (19 genes), and hypothetical proteins (12 genes). Hossain et al. (2015) hypothesized that some of these gene products may be involved in interactions with plants.

Genes involved in ribosomal synthesis of several bacteriocins, such as mersacidin (Borriss, 2013), plantazolicin (Scholz et al., 2011), and amylocyclicin (Scholz et al., 2014), were detected in several representatives of *B. amyloliquefaciens* subsp. *plantarum,* but are not part of the *plantarum* core genome. We hypothesize that most of the genes, unique in subsp. *plantarum* are involved in plant-bacteria interactions and in suppressing plant pathogens.

Bacillus amyloliquefaciens subsp. *plantarum (methylotrophicus)* FZB42^T

We have proposed to choose FZB42^T as model strain for plant-associated PGP and BC Bacilli for the following reasons (Borriss, 2011):

(1) The strain is available for scientific research from public strain collections (BGSC 10A6 and DSM23117), despite that the strain is commercialized by ABiTEP GmbH Berlin and successfully applied in agri- and horticulture[3].
(2) The whole genome sequence of FZB42^T has been determined in 2007, as the first representative of gram-positive BC bacteria. Its 3,918-kb genome, lacks extended phage insertions, which occur ubiquitously in the related *Bacillus subtilis* 168 genome (Chen et al., 2007). Nearly 10% of the genome is devoted to synthesizing antibiotics, siderophores and bacteriocins (Chen et al., 2009a; Borriss, 2013).
(3) In contrast to most environmental *Bacillus* strains, FZB42 is naturally competent and amenable to genetic transformation using a modified one-step protocol (Idris et al., 2007). In order to assign unknown gene functions, we generated more than 200 mutant strains targeted in 74 different genes involved in synthesis of secondary metabolites, volatiles, biofilm formation, alternative sigma factors and global transcription regulators (**Figure 2**). Moreover, strain derivatives of FZB42 were labeled by stable chromosomal integration of the green fluorescent protein (GFP+). Those strains were found extremely useful for studying root colonization after bacterial inoculation (Fan et al., 2011; Chowdhury et al., 2015b). The engineered mutant strains can be ordered from the Nord Reet UG Greifswald, Germany[4].
(4) FZB42 is closely related to other BC *Bacilli* with industrial importance (FZB24, QST713, GB03, D747, MB1600, GA1, SQR9, NAUB3, YAU B9601), which are often wrongly assigned as being *B. subtilis,* but are also belonging to the same subspecies *plantarum* (*methylotrophicus*) as FZB42 (Borriss et al., 2011). Studies performed with FZB42 and its derivatives are therefore of general interest and valuable for understanding the mechanisms of action in this important group of endospore-forming plant-associated bacteria.

PGPR BACILLI ENGINEERED FOR ENHANCED EFFICIENCY

A first step in improving efficiency of BC bacilli is identification of target genes involved in BC and root colonization. As stated above, the FZB42 genome harbors a rich arsenal of genes probably involved in synthesis of antimicrobial compounds.

[3]http://www.abitep.de/de/produkte.html

[4]http://www.nordreet.de/bacillus-consulting/research/

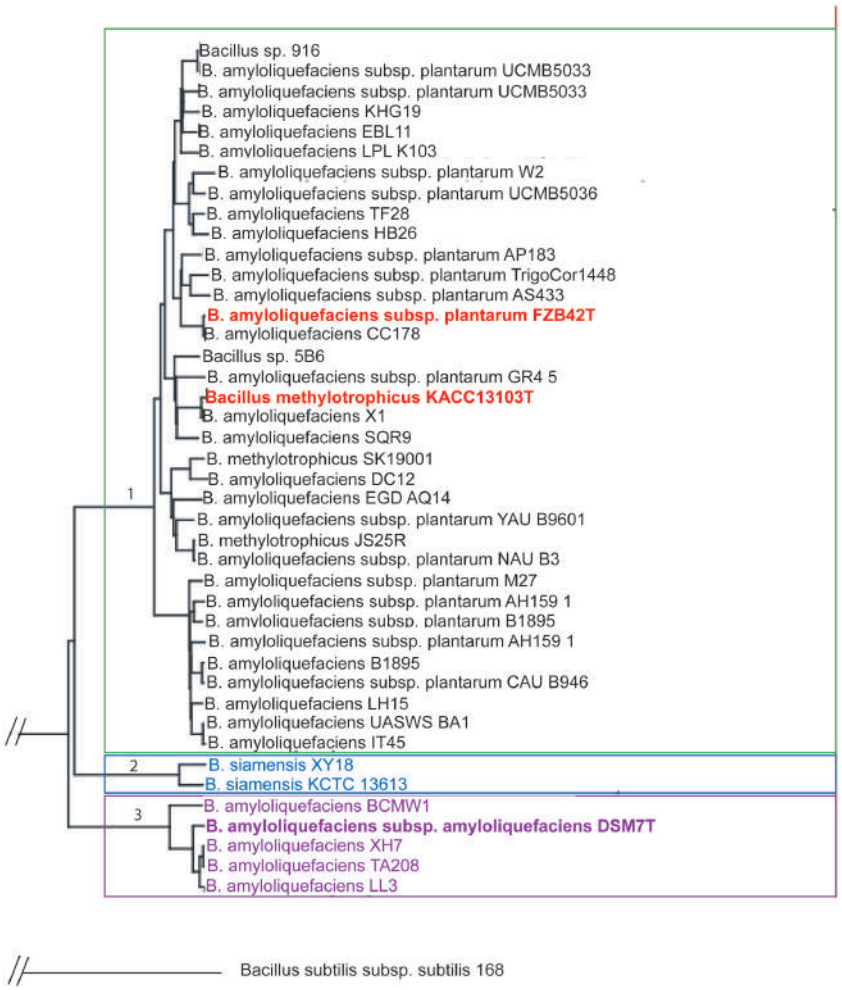

FIGURE 1 | Phylogenetic tree of *Bacillus amyloliquefaciens* chromosomes currently available in public databases. Based on the core genome of 2104 CDS the divergence of the plant-associated bacteria (*B. amyloliquefaciens* subsp. *plantarum*) and *B. siamensis* and *B. amyloliquefaciens* subsp. *amyloliquefaciens* was quantified with FZB42T employed as reference to construct the tree according to Blom et al. (2009). Every set of orthologous genes found in all genomes was separately aligned using the multiple alignment tool MUSCLE (Edgar, 2004). The alignments were concatenated to one huge multiple alignment. A distance matrix was calculated from this alignment and finally a phylogenetic tree was constructed based on this distance matrix using the Neighbor-Joining method. The two latter methods are used in the PHYLIP implementations by Felsenstein (http://evolution.genetics.washington.edu/phylip.html). The Neighbor-Joining method was chosen as it is a heuristic approach with a very good computational efficiency, making it well suited for large datasets resulting from the core genome based tree construction.

By applying a combined approach using gene knock-out mutants and chemical mass spectroscopy as analytic tools, we identified during last decade a total of 10 gene clusters involved in Sfp-dependent non-ribosomal synthesis of defined cyclic lipopeptides, c-LPs (4) and polyketides (3), Sfp-independent non-ribosomal synthesis of bacilysin, and ribosomal synthesis of the highly modified bacteriocins plantazolicin and amylocyclicin (Chowdhury et al., 2015a).

Identification of Target Genes to Improve the Efficiency of PGPR Bacilli

Biocontrol

Several case studies using site-directed mutants were performed in order to demonstrate the antibacterial effect exerted by the polyketide difficidin and the dipeptide bacilysin. Difficidin was characterized as a highly unsaturated 22-membered macrocyclic polyene lactone phosphate ester (Wilson et al., 1987), and bacilysin, consisting of non-proteinogenic L-anticapsin and N-terminal L-alanine, was first isolated from *B. subtilis* (Kenig and Abraham, 1976). FZB42 was found efficient against the gram-negative pathogen *E. amylovora*, the causative agent of fire blight, a serious disease of orchard trees. Surprisingly, a mutant strain blocked in the production of difficidin (CH8 Δ*dfn*) suppressed fire blight disease nearly in the same range as wild type FZB42. Moreover, a *sfp* mutant strain (CH3 Δ*sfp*) unable to synthesize non-ribosomally lipopeptides and polyketides did still suppress growth of *E. amylovora*, suggesting that besides action of polyketides another antagonistic principle exist. In contrast, a double mutant impaired in non-ribosomal synthesis and bacilysin (RS06 Δ*sfp* Δ*bac*) was

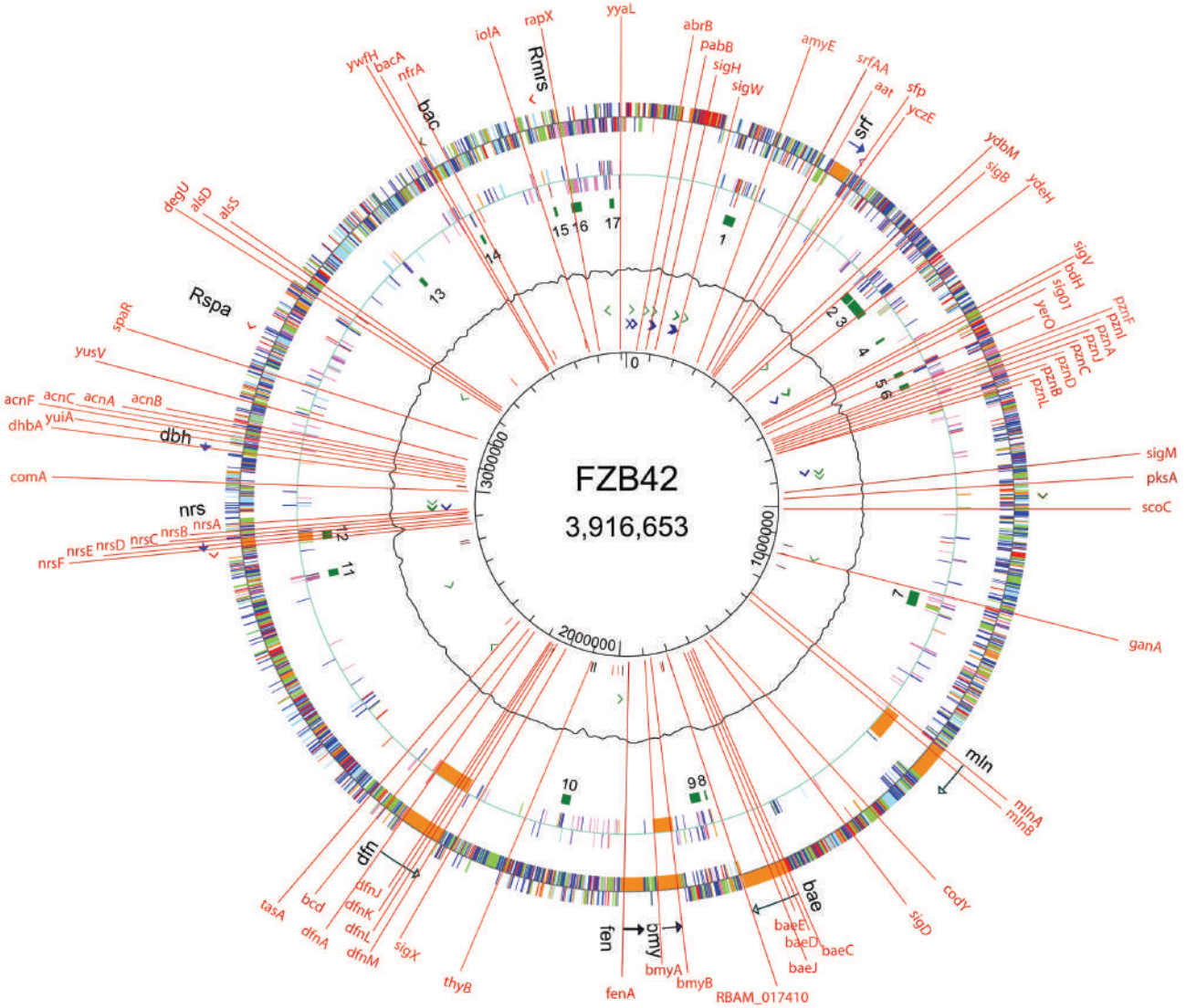

FIGURE 2 | Site directed mutations introduced into the genome of FZB42. Mutants impaired in synthesis of secondary metabolites (non-ribosamal sythesis, *sfp*, surfactin, *srf*, plantazolizin, *pzn*, macrolactin, *mln*, bacillaen, *bae*, bacillomycin D, *bmy*, fengycin, *fen*, difficidin, *dfn*, unidentified peptide, *nrs*, siderophore, *dhb*, amylocyclicin, *acn*, bacilysin), volatiles (*alsS*, *alsD*, *bdh*), sugars (*ganA, iolA*), amylase (*amy*E), global regulators (*abrB*, *scoC*, *degU, codY*), alternative sigma factors (*sigH, sigW, sigB, sigV, sig01, sigM, sigD, sigX*), competence (*comA*), biofilm formation (*srfA, tasA*), oxidative stress response (*nfrA*), plant growth promotion (*RBAM-017410*), auxotrophy (*pabB*), and others (*yczE, ydbM, ydeH, yerO, yuiA, yusV, spaR, ywfH, rapX, yyaL*).

unable to suppress *E. amylovora* indicating that the additional inhibitory effect is due to production of bacilysin (Chen et al., 2009b).

A similar study using appropriate mutant strains of FZB42 was performed recently, demonstrating that difficidin and bacilysin are also efficient against two different *Xanthomonas oryzae* pathovars, causative agents of damaging rice diseases (bacterial blight and bacterial leaf streak). Agar diffusion tests performed with several FZB42 mutant strains (**Figure 3**) revealed that the inhibitory effect of mutant CH8 (Δ*dfn*) deficient in production of difficidin was clearly reduced compared to wild type FZB42. The double mutant RS06 (Δ*sfp* Δ*bac*) was completely unable to suppress *X. oryzae* pv *oryzae* and *X. oryzae* pv *oryzicola* suggesting that difficidin and bacilysin act as antagonists of the pathogenic *Xanthomonas* strains (Wu et al., 2015a).

Among 24 strains, *B. amyloliquefaciens* FZB42 showed the strongest bactericidal activity against the cyanobacterium *Microcystis aeruginosa*, the causative agent of harmful algal blooms in freshwater lakes and rivers. Surprisingly, the site-directed *sfp* mutant CH03, impaired in Sfp-dependent non-ribosomal synthesis of lipopeptides and polyketides including difficidin, was able to inhibit growth of *M. aeruginosa* in the same magnitude as the wild type. Random transposon mutagenesis using the mariner transposon TnYLB-1 revealed that mutant strains bearing transposon insertions within the *aroA* and *aroE* gene failed completely to inhibit *M. aeruginosa*. Products of the

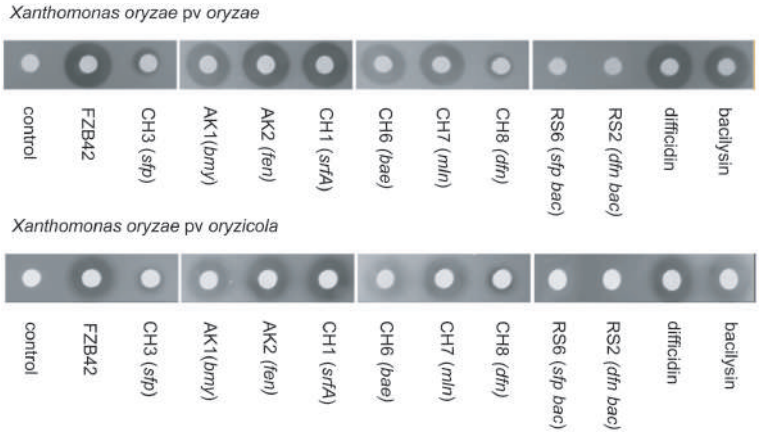

FIGURE 3 | Suppression of rice pathogens by FZB42 and its mutant strains. (Top) agar diffusion assay using *Xanthomonas oryzae* pv.*oryzae* as indicator strain. **(Bottom)** agar diffusion assay using *Xanthomonas oryzae* pv. *oryzicola* as indicator strain. Bacilysin and difficidin, FZB42 and several mutant strains not impaired in bacilysin and difficidin synthesis have a clear antaogonistic effect against both pathogens whilst mutant strains RS02 and RS06, deficient in synthesis of bacilysin and difficidin, were unable to suppress both pathovars of *X. oryzae*. Mutant strains unable to synthesize difficidin but able to synthesize bacilysin (CH3, CH8) were found clearly repressed in their antagonistic action.

aro genes are involved in synthesis of aromatic amino acids and it is known that all *aro* mutants are impaired in bacilysin synthesis. Therefore, a knock-out mutation within the *bacB* gene was constructed and as expected the mutant was unable to inhibit growth of *M. aeruginosa* suggesting that bacilysin is responsible for inhibition (Wu et al., 2014).

Induced Systemic Resistance (ISR)

Plant defense triggered by surfactin, microbial volatile organic compounds (mVOCs) and other hitherto unidentified compounds is a main factor in suppressing plant pathogens by plant-associated bacteria (Pieterse et al., 2014). Selected plant-associated Bacillus *strains* emit mVOCs consisting of 2,3 butandiol and acetoin that can elicit plant defense (Ryu et al., 2004). Synthesis of 2,3 butandiol from pyruvate via 2-acetolactate and acetoin is governed by the products of the *alsS*, *alsD*, and *bdhA* genes in *B. subtilis* (Renna et al., 1993). *B. amyloliquefaciens* NJN-6 produces volatile compounds (VOCs) that inhibit the growth and spore germination of *Fusarium oxysporum* f. sp. *cubense*. Among the total of 36 volatile compounds detected, 11 compounds completely inhibited fungal growth. The antifungal activity of these compounds suggested that VOCs can play important roles over short and long distances in the suppression of *Fusarium oxysporum* (Yuan et al., 2012). However, except acetoin and 2,3 butandiol, the genes responsible for synthesis of the volatiles are unknown.

Root Colonization

A necessary precondition for the PGP and BC action of plant beneficial bacteria is their root colonization activity (Lugtenberg et al., 2001). In contrast to *Pseudomonas fluorescens* and some other gram-negative bacteria, bacilli are known as comparable "weak" colonizers of plant roots, and PGP bacilli are hardly to detect later than 3 months after their application (Bais et al., 2004; Chowdhury et al., 2013).

After identifying genes involved in root colonization and other plant-bacteria interactions, gene targeting techniques are useful techniques in order to generate strains with enhanced rhizosphere competence, given that no additional resistance genes are introduced. Enhanced root colonization and BC activity was gained in *B. amyloliquefaciens* SQR9 by disruption of the *abrB* gene encoding a global regulator of gene expression in *Bacillus* (Weng et al., 2013). Other genes, involved in expression of antimicrobial compounds can also be targeted. In *B. subtilis*, the response regulator DegU and its cognate kinase, DegS, constitute a two-component system that regulates many cellular processes, including exoprotease production and genetic competence. Phosphorylated DegU (DegU-P) activates its own promoter and is degraded by the ClpCP protease (Ishii et al., 2013). In plant associated FZB42 the global transcriptional regulator gene *degU* was shown to control non-ribosomal synthesis of secondary metabolites, such as the antifungal lipopeptide bacillomycin D (Koumoutsi et al., 2007), and the antibacterial bacilysin (Mariappan et al., 2012), in FZB42. In an interesting study Xu et al. (2014) demonstrated that stepwise phosphorylation of DegU in *B. amyloliquefaciens* SQR9 can influence BC activity by coordinating multicellular behavior and regulating the synthesis of lipopeptide and polyketide antibiotics in a different manner. Results from *in vitro* and *in situ* experiments and quantitative PCR (qPCR) studies demonstrate that unphosphorylated DegU achieved by a knock out mutation of the *degQ* kinase gene impairs complex colony architecture, biofilm formation, colonization activities, and BC efficiency of *Fusarium* wilt disease but increases the production of the polyketides macrolactin and bacillaene. By contrast, enhanced DegU_P production achieved by *degQ* and *degSU* overexpression does significantly improve complex colony architecture, biofilm formation, colonization activities, production of the antibiotics bacillomycin D and difficidin, and efficiency of BC of *Fusarium* wilt disease.

The transcriptional levels of genes involved in biofilm formation, *yqxM* and *epsD*, were evaluated in response to organic acids via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Results suggested that root exudates containing the OAs both induced the chemotaxis and biofilm formation in *B. amyloliquefaciens* NJN-6 (Yuan et al., 2015).

In summary, research with knock-out mutants deepens our knowledge about molecular reasons for the strong antimicrobial activity observed in FZB42 and might contribute to a more efficient use, however, our final goal, developing of biopesticides with constant and enhanced efficiency against plant pathogens needs further, more direct, efforts.

PGPR BACILLI ENGINEERED FOR ENHANCED EFFICIENCY IN BIOCONTROL

It is generally assumed that suppression of plant pathogens by PGP Bacilli is based on two features: (1) production of antimicrobial secondary metabolites and siderophores ('direct antibiosis'), and (2) stimulation of induced systemic resistance (ISR), which activates the plant defense system against harmful microbes and viruses. According to latest results, it is likely that ISR is more important than direct antibiosis in suppressing plant pathogens under conditions of plant rhizosphere. It is very unlikely that concentration of antibiotics within the plant rhizosphere reach levels sufficient for direct antibiosis (Nihorimbere et al., 2012; Chowdhury et al., 2015a,b). Stimulation of ISR is a multifactorial process probably dependent on the presence of several compounds produced by the rhizobacteria, such as the c-LP surfactin and volatiles (Raaijmakers et al., 2010). A strong correlation between the amount of surfactin produced and the ability to elicit ISR was demonstrated (Cawoy et al., 2014). In order to combine both suppressive mechanisms (SR and direct antibiosis), it might be necessary that improved bioformulations contain living *Bacillus* spores and concentrated culture supernatants with antimicrobial metabolites. Besides the number of living spores, also concentration of the main antagonistic metabolite (e.g., bacillomycin D) should be indicated in such formulations (Borriss, 2015b).

It has been proposed early (Compant et al., 2005) to create transgenic PGPB strains that combine multiple mechanisms of action (Timms-Wilson et al., 2000; Chin-A-Woeng et al., 2001; Huang et al., 2004). For example, transforming the 1-aminocyclopropane-1-carboxylic acid deaminase gene, which directly stimulates plant growth by cleaving the immediate precursor of plant ethylene (Glick et al., 1998) into *P. fluorescens* CHAO, not only increases plant growth but can also increase BC properties of PGPB (Wang et al., 2000).

Some studies have demonstrated that the production of cLPs in *Bacillus*, for example, mycosubtilin and iturinA, representatives of the iturin family with antifungal action, and surfactin could be improved by applying promoter exchange strategies.

Promoter Modulation to Promote Antibiotic Production and ISR

Enhancement of mycosubtilin production in *B. subtilis* strain ATCC 6633 was obtained by replacement of the native promoter of the mycosubtilin operon by a constitutive promoter originating from the replication gene *repU* of the *Staphylococcus aureus* plasmid pUB110. The recombinant strain, designated BBG100, produced up to 15-fold more mycosubtilin than the wild type produced. When tested for its BC potential, wild type strain ATCC 6633 was almost ineffective for reducing a *Pythium* infection of tomato seedlings. However, treatment of seeds with the BBG100 overproducing strain resulted in a marked increase in the germination rate of seeds. This protective effect afforded by mycosubtilin overproduction was also visualized by the significantly greater fresh weight of emerging seedlings treated with BBG100 compared to controls or seedlings inoculated with the wild type strain (Leclère et al., 2005). Enhanced mycosubtilin production (880 mg L^{-1}) was also obtained by introducing the tightly regulated *xylA* promoter in front of the *myc* operon of *B. subtilis* ATCC 6633 (Fickers et al., 2009). The P_{repU} promoter was previously reported to enhance the biosynthesis of iturin A, by about threefold in *B. subtilis* RB14 (Tsuge et al., 2001).

The biosurfactant surfactin, a cyclic heptapetide containing four leucine moieties, is known as elicitor of the plant response against pathogens and for its antiviral and antimycoplasmic action (Jacques, 2011). The inducible promoter P_{spac} was used to enhance production of surfactin in *B. subtilis*. After IPTG induction the recombinant *B. subtilis* fmbR-1 produced about 10-fold more than the wild type strain (Sun et al., 2009). In a more sophisticated approach it was found recently, that *comQ* null mutant strains of *B. subtilis* impaired in a social process called quorum sensing (QS), were able to overproduce surfactin. However, overproduction of the secondary metabolite led to reduced fitness of the mutant strains (Oslizlo et al., 2014).

The volatile 2,3-butanediol is known to stimulate ISR in plants (see above). Enhanced production of the volatile in *B. subtilis* was recently demonstrated (de Oliveira and Nicholson, 2015). The genes *alsS*, *alsD*, and *bdhA* encoding acetolactate synthase, acetolactate decarboxylase, and butanediol dehydrogenase, respectively were engineered into a single tricistronic operon under control of the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter.

Modifying Precursor Production

Coutte et al. (2010) hypothesized that precursors supply is one of the main parameters to optimize surfactin production. In fact, overproduction of surfactin was obtained by replacing the native promoter of the surfactin operon *(srfA)* by a constitutive one and disrupting the plipastatin (fengycin) operon *(ppsA)* to save the precursor availability. In order to enhance production of the surfactin precursor leucine, six knockouts were introduced in *B. subtilis* leucine metabolism to verify their effects on surfactin production. For all generated mutants, the specific surfactin production was found increased from 1.6- to 20.9-fold during the exponential growth phase, depending on the medium composition (Coutte et al., 2015). The highest increase

in surfactin production was obtained in *codY* mutant strains. This is feasible, since the expression of the *ilv-leu* operon is regulated by CodY in the presence of branched chain amino acids (Ratnayake-Lecamwasam et al., 2001).

RECONSTITUTION OF PRODUCT PRODUCTION

The lanthionine containing bacteriocin mersacidin is not synthesized in FZB42, but an incomplete *mrs* gene cluster presumably directing immunity against the bacteriocin was detected in the genome (**Table 1**). By contrast, the mersacidin producer strain *B. amyloliquefaciens* subsp. *plantarum* HILY harbors the complete *mrs* gene cluster including the genes for synthesis, modification and regulation. In a first step in order to achieve mersacidin production in FZB42, genomic DNA of *Bacillus* HILY mutant strain Rec1 was used to transform competent FZB42 cells. The resulting FZB42 mrs1 strain contained the complete *mrs* gene cluster except the essential genes *mrs*A and *mrs*R1. The completion of the mersacidin gene cluster in FZB42 was achieved *in trans* by transformation with the plasmid pPAR1, carrying the structural gene *mrs*A and *mrs*R1, yielding *B. amyloliquefaciens* mrs1 pPAR1. This surrogate FZB42 derivative was shown to produce active and fully modified mersacidin suggesting that FZB42 can be exploited as an appropriate *in vivo* expression system for the construction and expression of mersacidin analogs (Herzner et al., 2011).

MODIFICATION OF GLOBAL REGULATOR

In the following we describe examples for obtaining more powerful strains by applying genetic engineering techniques in the plant-growth-promoting strain FZB42. This work has been performed in the laboratory of Xuewen Gao, Nanjing Agriculture University, China. We have to acknowledge, that at present, use of such engineered PGPR strains under field conditions is refused by the public, at least in Europe. However in light of a steadily increasing world's population growing from 7 billion now to 8.3 billion in 2025 (Lugtenberg et al., 2013), innovative approaches for getting higher harvest yields without using increasing amounts of agrochemicals should not longer be excluded, given that their use is safe and without harmful consequences for human beings and nature. Careful environmental studies are a precondition before releasing genetic engineered bacteria into the environment (Broer, 2015).

We showed that bacilysin production is strictly controlled by the global regulator DegU (Mariappan et al., 2012). Although bacilysin has potential applications (see above), its use is restricted by low productivity of the producer strains including FZB42. To date, there have been some attempts to increase bacilysin production. However, most experimental approaches were primarily focused on optimizing culture conditions and did not affect basic genetic structures. Ozcengiz et al. (1990) reported that the nitrogen source controls bacilysin biosynthesis, and aspartate was better than glutamate as the sole nitrogen source. Inaoka and Ochi (2011) showed that addition of scandium to the growth medium stimulated the production of bacilysin at the transcriptional level.

In order to improve the production of bacilysin by genetic engineering, Wu et al. (2015b) developed a fast and accurate approach by combining the Cre/*lox* site-specific recombination system with PCR for replacement of the native bacilysin operon promoter with constitutive promoters P_{repB} and P_{spac} from plasmids pMK3 and pLOSS, respectively. In this system, cre-mediated recombination leads to excision of any DNA (e.g., an antibiotic resistance cassette) in between two distant intramolecular lox sites with a collinear orientation, leaving only one lox site behind and reinstating the antibiotic sensitivity of the respective strain. The engineered markerless strains FZBREP and FZBSPA, expressing the *bac* cassette under the control of the constitutive promoters P_{repB} and P_{spac}, significantly increased expression of the *bac* genes, as shown by RT-PCR and qRT-PCR. HPLC confirmed that FZBREP and FZBSPA strains produced up to 170.4 and 315.6 % more bacilysin than wild type, respectively. At 4 days after the *M. aeruginosa* culture had been treated with FZBREP and FZBSPA culture filtrates, the bactericidal activity was >95%, while that of FZB42 was just 56.9% (**Figure 4**). Bacilysin overproduction was also accompanied by enhancement of the antagonistic activities against *S. aureus* (an indicator of bacilysin) and *Clavibacter michiganense* subsp. *sepedonicum* (the causative agent of potato ring rot) (Wu et al., 2015b).

FOREIGN PROTEIN EXPRESSION IN *B. amyloliquefaciens* FZB42

The harpin protein group, which is secreted by many plant pathogenic bacteria during infection, elicits multiple plant responses, resulting in multiple beneficial effects on crop improvement. The *hrp* ("harp") genes encode type III secretory proteins enabling many phytopathogenic bacteria to elicit a hypersensitive response (HR) on non-host or resistant host plants and induce pathogenesis on susceptible hosts. The HR is a rapid localized death of the host cells that occurs upon pathogen infection and, together with the expression of a complex array of defense-related genes, is a component of plant resistance. The plant genes create a cascade of effects which promote a Systemic Acquired Resistance (SAR) throughout the plant. Beneficial effects on plant growth and health have been reported (Alfano and Collmer, 2004). The protein $HrpN_{Ea}$ produced in *E. amylovora* was the first found and identified in bacteria (Wei et al., 1992). Another hrp gene product, $HpaG_{Xooc}$, from rice pathogenic bacterium *X. oryzae* pv. *oryzicola*, contains two glycine-rich motifs and one cysteine residue (Wu et al., 2009). Despite there are some differences in the sequence and component of amino acids, it exhibits similar biological functions as $HrpN_{Ea}$ protein (Qiao et al., 2014).

The gene $hpa1_{Xooc}$ encoding protein $HpaG_{Xooc}$ had been cloned onto the expression plasmid pM43HF in *B. subtilis* OKB105, a derivative of *B. subtilis* 168 which is able to produce surfactin and to colonize plant roots (Wu et al., 2009). The

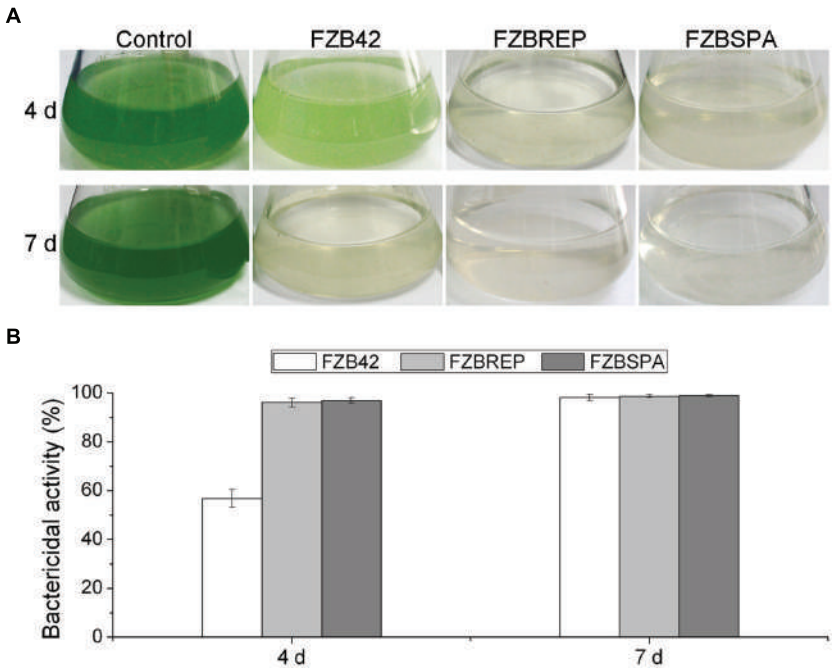

FIGURE 4 | Suppression of *Microcystis aeruginosa* by transgenic strains FZBREP and FZBSPA. (A) The antagonistic effect of *B. amyloliquefaciens* strains against a liquid *M. aeruginosa* culture after 4 and 7 days. **(B)** The bactericidal activity of *B. amyloliquefaciens* strains FZB42, FZBREP and FZBSPA. Note that suppressive effect of FZBREP and FZBSPA is enhanced compared to wild type FZB42.

engineered strain OKBHF expressing $HpaG_{Xooc}$ protein caused plants to have less severe disease symptoms after inoculation with *Ralstonia solanacearum*, suggesting that $HpaG_{Xooc}$ improves BC efficiency of *B. subtilis* (Gao et al., 2013). However, after 100 generations, the $HpaG_{XooC}$ expression plasmid pM43HF is segregationally unstable in *B. subtilis* under the non-selective condition, thus affecting the continuing expression of $HpaG_{XooC}$ and finally fails to secrete the protein. Transgenic tobacco plants expressing the $hpa1_{Xooc}$ gene were constructed, but were found unable to induce hypersensitive cell death (HCD) (Peng et al., 2004).

In order to overcome these difficulties, we decided to use FZB42 as the host strain (Qiao et al., 2013). Two copies of the $hpa1_{Xooc}$ genes were introduced into the two main extracellular protease genes *apr* and *npr* located on the FZB42 chromosome for avoiding proteolytic destruction of the recombinant harpin gene product (**Figure 5**). RT-PCR analysis showed that the $hpa1_{Xooc}$ was transcribed. Supernatant of the resulting recombinant strain FZBHarpin caused a hypersensitive response (HR) reaction on tobacco leaf, suggesting biological active Harpin protein is secreted into the medium. The *in vivo* effect of FZBHarpin on plant growth was tested by soaking rice and tobacoo seeds in the suspensions. A significant increase in shoot fresh weight and root length was observed compared to the untreated control and FZB42. Moreover, greenhouse experiments revealed that the control efficacy of FZB42Harpin against rice bacterial blight was 56.9%, showing significant improvement in resistance to *X. oryzae* pv. *oryzicola* relative to FZB42. In addition, a PGP effect by FZB42Harpin exceeding that of FZB42 was also detected (Qiao et al., 2014). However, before applying the recombinant FZB42Harpin strain in field trials, removal of the two resistance markers flanked by the cre *lox* recombinase recognition sites *via* site-directed recombination has to be performed.

MARKER REMOVAL STRATEGIES IN *Bacillus*

Classical chromosomal modification is connected with the insertion of a selectable marker, usually a drug resistance gene, into the chromosome of a bacterial strain. Using this strategy, a second selective marker gene is required to introduce another chromosomal modification, so the number of available selection genes limits the feasibility of multiple chromosomal modifications. Moreover, the selectable gene should be removed by single-crossover recombination if the strain is used for further genetic manipulation. In addition, the chance of obtaining a positive strain is relatively low, and the selection process is laborious. To overcome these problems, methods that can eliminate marker cassettes in the primary transformants are needed (Dong and Zhang, 2014). More important, in order to obtain a higher acceptance for genetic engineered strains in agriculture using markerless transgenic strains is a *conditio sine qua non*. Construction of a bacilysin overproducing FZB42 strain described above is an example for successful application of this technique in plant-associated *Bacillus* strains.

Today there are several methods for marker removal available. Site-specific recombination (SSR) systems are capable

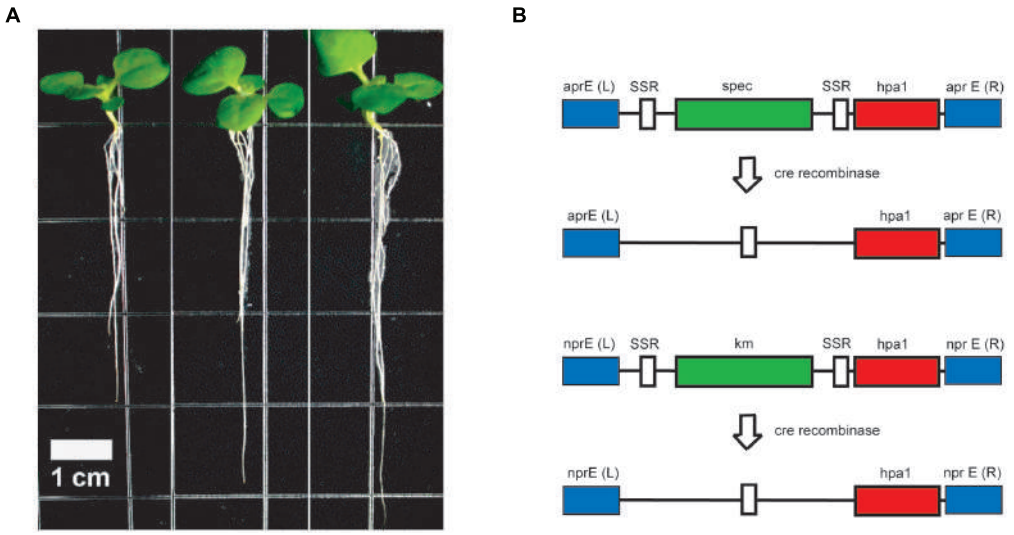

FIGURE 5 | Strain construction and enhanced root development of tobacco plants by FZBHarpin. (A) Root development of tobacco seedlings is enhanced after treatment with FZB42 and FZBHarpin. From left to right: control, FZB42, FZBHarpin. **(B)** Integration of two copies of the *hpa1* gene (shown in red) into the chromosome of FZB42 via homologous recombination leads to disruption of the genes encoding alkaline (top) and neutral (bottom) protease. The selectable genes, shown in green, are the resistance genes for spectinomycin (spec) and kanamycin (km). White regions represent recognition sites of site-specific recombinases, and blue regions represent sequences homologous between the genome and the integration cassette. Removal of the selectable marker genes is performed with site specific Cre recombinase at the SSR recognition sites.

of eliminating antibiotic resistance markers, if they are flanked by recombinase recognition sites. SSR systems that are used in *B. subtilis* are Cre/loxP from bacteriophage P1, and Xis/attP from bacteriophage λ.

In a previous study (Leibig et al., 2008), a Cre-*lox* setting was established that allowed the efficient removal of resistance genes from the genomes of *S. carnosus* and *S. aureus*. Two cassettes conferring resistance to erythromycin or kanamycin were flanked with wild type or mutant *lox* sites, respectively, and used to delete single genes and an entire operon. After transformation of the cells with a newly constructed *cre* expression plasmid, genomic eviction of the resistance genes was observed in approximately one out of ten candidates analyzed and subsequently verified by PCR. Due to its thermo-sensitive origin of replication, the plasmid can be eliminated at non-permissive temperatures and marker-less deletion mutants can be obtained.

Although Cre-mediated recombination and excision of the chromosomal sequence between two lox sites is efficient, it does not occur in all cells. To address this, Wang et al. (2012) developed a simple and efficient *B. subtilis* genome editing method in which targeted gene(s) could be inactivated by single-stranded PCR product(s) flanked by short homology regions and in-frame deletion could be achieved by incubating the transformants at 42°C. In this process, homologous recombination was promoted by the lambda beta protein synthesized under the control of promoter PRM in the lambda cI857 PRM–PR promoter system on a temperature sensitive plasmid pWY121. The hen egg white lysozyme gene is placed after promoter PR, which is effective in *B. subtilis*, and is precisely regulated by the CI857 repressor protein (Breitling et al., 1990). The efficiency of inframe deletion using this method can reach 100%. As hen egg white lysozyme is active against *Bacillus* species, and its encoding gene is distantly related to *Bacillus* genes, it could also be effective in other *Bacillus* species.

CONCLUSION

Due to increasing environmental problems caused by the exaggerated use of chemicals in agriculture, further improvement of BC agents is a timely task. Possibilities for developing more efficient bioagents include comparative genomic analysis in order to detect specific features unique for plant-associated bacteria and their improvement by applying genetic methods. Due to their ability to produce durable endospores plant-beneficial *Bacillus* strains offer perfect possibilities for stable bioformulations, which are competitive to the widely used agrochemicals. In order to enhance progress in getting highly efficient bioformulations, we have proposed to focus further research about plant-bacteria interactions on the model bacterium *B. amyloliquefaciens* FZB42, which is genetic amenable, widely used in practice, and in which a huge knowledge base already exists.

In this review we present examples for engineering several features, important for suppression of plant pathogens by direct antibiosis and ISR. Strategies applied include (1) modulation of promoter activity, (2) modification of precursor production, (3) reconstitution of product production, (4) control of metabolite production by global regulators, and (5) expression of foreign proteins. Although examples for applying such genetic engineering strategies in spore-forming Bacilli are relatively scarce, it is to expect that they will become in future a powerful tool for further improvement of biopesticides and biofertilizer,

given that the public will change their behavior against use of engineered environmental bacteria.

OUTLOOK

Today, applying and release of genetic engineered bacteria directly in the environment is not accepted by the public and governmental regulations are contradictory for use of such microorganisms in enhancing crop yield. One reason is the presence of resistance genes in transgenic strains, which have been introduced in the bacteria during the allelic replacement process, and methods avoiding use of such marker genes are therefore highly desirable.

Of course, marker removal is not the only precondition for improved acceptance of genetically engineered strains when released into the environment. As stated above, careful case studies demonstrating that no harmful effects caused by genetic engineered strains are urgently needed. In applying genetic engineered plant growth promoting bacteria we have to distinguish two different levels:

(1) Engineered strains without foreign genes but containing useful mutations in genes affecting the beneficial effect of the bacterium in terms of plant growth-promotion and BC of pathogens. Given that no resistance marker has been introduced, it might be unimportant whether the useful mutation has been introduced by a targeted allele exchange or has been evolved after applying a natural selection procedure. We believe, that such strains will be accepted in future when their improved action has been convincingly demonstrated.

(2) Engineered strains containing genes from other bacteria. Such bacteria will be considered as "recombinant," also when the donor bacteria occur in the same natural environment. This was the case in the example described here. Ironically, the harpin gene isolated from a pathogen bacterium was shown to act beneficial when cloned and expressed in FZB42. However, long-term environmental studies are necessary to demonstrate that such recombinant bacteria do not harm environment by novel recombination events with other microorganisms occurring in the same environment.

In summary, genomic analysis is already an important tool in characterizing of beneficial microbes. Moreover, we believe on the prospect of genetic engineering for obtaining improved bioformulations in future. This development should contribute to a more sustainable agriculture, and enabling us to save considerable amounts of agrochemicals, such as chemical fertilizers and chemical pesticides.

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REVIEW
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Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes

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Arbuscular Mycorrhizal Fungi (AMF) constitute a group of root obligate biotrophs that exchange mutual benefits with about 80% of plants. They are considered natural biofertilizers, since they provide the host with water, nutrients, and pathogen protection, in exchange for photosynthetic products. Thus, AMF are primary biotic soil components which, when missing or impoverished, can lead to a less efficient ecosystem functioning. The process of re-establishing the natural level of AMF richness can represent a valid alternative to conventional fertilization practices, with a view to sustainable agriculture. The main strategy that can be adopted to achieve this goal is the direct re-introduction of AMF propagules (inoculum) into a target soil. Originally, AMF were described to generally lack host- and niche-specificity, and therefore suggested as agriculturally suitable for a wide range of plants and environmental conditions. Unfortunately, the assumptions that have been made and the results that have been obtained so far are often worlds apart. The problem is that success is unpredictable since different plant species vary their response to the same AMF species mix. Many factors can affect the success of inoculation and AMF persistence in soil, including species compatibility with the target environment, the degree of spatial competition with other soil organisms in the target niche and the timing of inoculation. Thus, it is preferable to take these factors into account when "tuning" an inoculum to a target environment in order to avoid failure of the inoculation process. Genomics and transcriptomics have led to a giant step forward in the research field of AMF, with consequent major advances in the current knowledge on the processes involved in their interaction with the host-plant and other soil organisms. The history of AMF applications in controlled and open-field conditions is now long. A review of biofertilization experiments, based on the use of AMF, has here been proposed, focusing on a few important factors that could increase the odds or jeopardize the success of the inoculation process.

Keywords: arbuscular mycorrhizal fungi (AMF), abiotic and biotic stress, plant nutrition, inoculation, transcriptomics

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INTRODUCTION

Soil microorganisms such as arbuscular mycorrhizal fungi (AMF or AM fungi) represent a key link between plants and soil mineral nutrients. Thus, they are collecting growing interest as natural fertilizers. AMF are obligate symbionts, belonging to the phylum Glomeromycota (Schüßler et al., 2001), which form mutualistic symbioses with about 80% of land plant species, including several

agricultural crops. They provide the host plant with mineral nutrients and water, in exchange for photosynthetic products (Smith and Read, 2008). The AMF mycelium that emerges from the root system can acquire nutrients from soil volumes that are inaccessible to roots (Smith et al., 2000). Furthermore, fungal hyphae are much thinner than roots and are therefore able to penetrate smaller pores (Allen, 2011). Carbohydrates and mineral nutrients are then exchanged inside the roots across the interface between the plant and the fungus. AM fungal hyphae exclusively colonize the root cortex and form highly branched structures inside the cells, i.e., arbuscules, which are considered the functional site of nutrient exchange (Balestrini et al., 2015). Thus, AMF can alleviate the limitation in plant growth caused by an inadequate nutrient supply (Nouri et al., 2014). It has recently been suggested that, in natural environments, a non-mycorrhizal condition should be viewed as abnormal for the majority of species (Smith and Smith, 2012), although there is a marked diversity among AM fungal communities belowground, depending on plant species diversity, soil type, and season, or a combination of these factors (Smith and Smith, 2012).

In addition to an improved nutritional supply, AM interactions provide other benefits to plants, such as improved drought and salinity tolerance (Augé, 2001, 2004; Porcel et al., 2011; Augé et al., 2015) and disease resistance (Pozo and Azcón-Aguilar, 2007). Although several works have been devoted to studying the influence of AM symbiosis on the plant response to abiotic stress (such as drought, salinity, and flooding) in the last few years, the mechanisms responsible for the increased plant tolerance to stress have yet to be fully elucidated (Augé, 2001; Ruiz-Lozano, 2003; Ruiz-Lozano and Aroca, 2010; Bárzana et al., 2012, 2015; Ruiz-Lozano et al., 2012; Calvo-Polanco et al., 2014; Saia et al., 2014; Augé et al., 2015; Sánchez-Romera et al., 2015). Metals such as Fe, Cu and Zn play essential roles in several subcellular compartments, but they constitute a highly reactive group of elements that are toxic at high concentrations (Tamayo et al., 2014, and references therein). AM fungi are known to alleviate heavy metal toxicity in the host plants and to tolerate high metal concentrations in the soil (Göhre and Paszkowski, 2006; Lingua et al., 2008; Cornejo et al., 2013; Tamayo et al., 2014; Meier et al., 2015). Metal transporters play a central role in heavy metal homeostasis. A Zn transporter has been identified in *Glomus intraradices* (*GintZnT1*) (González-Guerrero et al., 2005) and, more recently, through a genome-wide analysis of the recently published *Rhizophagus irregularis* (formely *Glomus intraradices* DAOM-197198) genome (Tisserant et al., 2013), several putative genes coding for Cu, Fe, and Zn transporters have been identified (Tamayo et al., 2014). The next steps will involve the functional characterization of these transporters and the identification of their roles in the symbiosis.

Furthermore, AM fungi can also have a direct effect on the ecosystem, as they improve the soil structure and aggregation (Rillig and Mummey, 2006; Leifheit et al., 2014, 2015; Rillig et al., 2015) and drive the structure of plant communities and productivity (van der Heijden et al., 1998). The influence of AM symbiosis on greenhouse gas (GHG) emissions has recently been investigated (Bender et al., 2014; Lazcano et al., 2014). Bender et al. (2014) have demonstrated that AM fungi contribute to reducing emissions of N_2O, which is an important greenhouse gas, thus suggesting that they could play a role in the mitigation of climate change. AM fungi could regulate N_2O emissions by enhancing plant N uptake and assimilation, which results in the reduction of soluble N in the soil, and, consequently, in a limitation of denitrification (Bender et al., 2014). Furthermore, the correlation between the key genes involved in N_2O production (*nirK*) and consumption (*nosZ*) and AM fungal abundance suggests that the regulation of N_2O emissions is caused by changes induced by AM fungi in the soil microbial biomass and in the community composition. AM fungi could have an indirect influence on GHG emissions, and also change the physical conditions of soil, i.e., moisture, aggregation, and aeration, all of which influence the production and transport of GHG in soil. Lazcano et al. (2014) have reported that AM symbiosis helps to regulate N_2O emissions at high soil moisture levels and suggested that the control of N_2O emissions by AM plants could be driven by a higher use of soil water rather than by increased N uptake.

Thus, AM fungi are primary biotic soil components which, when missing or impoverished, e.g., due to anthropic input, can lead to a less efficient ecosystem functioning. The process of re-establishing the natural level of AMF richness can represent a promising alternative to conventional fertilization practices, with a view to sustainable agriculture, a key target for growers facing the global recession and having to deal with a more environmentally aware clientele. The main strategy adopted to achieve this goal is the direct re-introduction of AMF propagules (inoculum) into a target soil. However, the exploitation of these fungi in applicative programs requires the knowledge of how AMF adapt and react to the target ecosystem and soil management and of the events that lead to the establishment of a functional symbiosis, including the mechanisms involved in nutrient transfer. In this review, after a brief mention of the most recent results on the nutritional aspects of AM symbiosis and a quick overview of the challenges involved in AMF inoculum production, examples of AM fungi application, in controlled and open-field conditions, are reported and discussed, with particular attention being paid to identifying the factors that led to successful outcomes in the biofertilization experiments.

NEW INSIGHTS INTO MINERAL NUTRITION IN AM SYMBIOSIS

Since the characterization of a high-affinity phosphate transporter (PT) in an AM fungus (Harrison and van Buuren, 1995), the nutritional aspects of AM symbiosis have been studied extensively from both a physiological and a molecular perspective (Harrison et al., 2002; Paszkowski et al., 2002; Nagy et al., 2005; Bucher, 2007; Smith and Smith, 2011, 2012). AM fungi are capable of significantly improving plant mineral nutrient acquisition, mainly in low-nutrient conditions, and it has clearly been demonstrated that plants possess a symbiotic Pi uptake pathway (Harrison et al., 2002; Paszkowski et al., 2002; Nagy et al., 2005; Bucher, 2007; Smith and Smith, 2011). Radiotracer experiments have made it possible to verify the

relative amount of Pi that enters a plant *via* the AM fungus and directly through the root transport system, and have revealed that the fungus can transfer the Pi to the plant even without an evident growth effect (Pearson and Jakobsen, 1993; Smith et al., 2003, 2004). It is well-known that AM symbiosis specifically induces the expression of plant Pi transporters (Harrison et al., 2002; Paszkowski et al., 2002; Nagy et al., 2005; Xie et al., 2013; Walder et al., 2015). In addition to the increase in plant Pi acquisition, a role in regulating arbuscule morphogenesis and in maintaining symbiosis has been demonstrated (Javot et al., 2007, 2011; Yang et al., 2012; Xie et al., 2013). Recently, the expression of the *Medicago truncatula* and *Lotus japonicus* AM-induced Pi transporters *MtPT4* and *LjPT4* has also been found in the root tips of non-colonized plants, and a role of *PT4* genes as components of the Pi-sensing machinery in root tips has been suggested (Volpe et al., 2015). Three PT genes in tomato are up-regulated in AM-colonized roots (*LePT3, LePT4, LePT5*) (Nagy et al., 2005), and the functional characterization of LePT4 has been reported, thus showing its pivotal role during symbiosis (Xu et al., 2007). Evidence of the role of AM symbiosis in the transfer of several mineral nutrients has been obtained in studies on several plant species (Casieri et al., 2013, for a review on the "transportome" in AM symbiosis; Paszkowski et al., 2002; Nagy et al., 2005; Guether et al., 2009a; Hogekamp et al., 2011). In addition to the Pi transporters specifically involved in the uptake from the arbuscules (Harrison et al., 2002; Paszkowski et al., 2002; Nagy et al., 2005), mycorrhiza-inducible ammonium transporters (AMT) have also been identified (Gomez et al., 2009; Guether et al., 2009b; Kobae et al., 2010; Koegel et al., 2013). The periarbuscular membrane, which is the plant-derived membrane that envelops the arbuscule, is considered the site in which the last stages of the symbiotic mineral nutrient transfer occur: plant transporters located in this membrane can capture mineral nutrients from the periarbuscular apoplast and transfer them to the cortical cell (Javot et al., 2011; Bapaume and Reinhardt, 2012). AMTs have been located in the periarbuscular membrane of soybean and *Medicago*, as previously demonstrated for the Pi transporter MtPT4 in *Medicago truncatula* (Harrison et al., 2002), thus suggesting a role in ammonium transport to the cortical cells (Kobae et al., 2010; Breuillin-Sessoms et al., 2015). Taken together, the analyses on Pi and ammonium transporter Medicago mutants have demonstrated that the Pi and AMT symbiotic transporters (i.e., PT4 and AMT2;3) have an influence on the arbuscule lifespan (Javot et al., 2007; Breuillin-Sessoms et al., 2015). It has been speculated that the transport of Pi or ammonium through these transporters not only delivers nutrients to the root cells but also triggers signaling that enables conditions for arbuscule maintenance (Breuillin-Sessoms et al., 2015).

In addition to Pi and N, sulfur (S) can also be transferred to plants through AM fungi (Allen and Shachar-Hill, 2009; Sieh et al., 2013). In fact, AM symbiosis improves the S nutritional status of the host plant, affecting the expression of plant sulfate transporters (Casieri et al., 2012; Giovannetti et al., 2014). In this context, the application of laser microdissection technology has allowed the expression of Pi and NH_4 tansporters in arbusculated cells to be verified (Balestrini et al., 2007; Gomez et al., 2009; Guether et al., 2009b) and, more recently, a sulfate transporter specifically involved in the uptake from the arbuscules has also been identified (Giovannetti et al., 2014). On the fungal side, a phosphate (Balestrini et al., 2007; Tisserant et al., 2012; Fiorilli et al., 2013) and an ammonium transporter (Pérez-Tienda et al., 2011) have been found to be expressed in arbuscules, which would seem to suggest that the fungus may reabsorb nutrients released at the periarbuscular interface, thus exerting a control over the amount of nutrients delivered to the host (Balestrini et al., 2007). In spite of the importance of potassium (K^+) for the plant cell machinery, the contribution of AM symbiosis to plant K^+ nutrition has rarely been studied (Garcia and Zimmermann, 2014). This element is very abundant in soil, but its availability is very low due to its strong mineral adsorption. The accumulation of this element in AM fungi has been reported in spores (Pallon et al., 2007), hyphae (Olsson et al., 2008), and vesicles (Olsson et al., 2011). Furthermore, the up-regulation of a plant K^+ transporter has been reported in mycorrhizal *Lotus japonicus* roots (Guether et al., 2009a). Interestingly, the K^+ derived from AM symbiosis can be correlated to an improved plant tolerance to abiotic stress, e.g., salinity and drought (Garcia and Zimmermann, 2014 and references therein). Recently, two meta-analysis studies, focused on the contribution of AM symbiosis to different micronutrient concentrations in crops, have been published (Lehmann et al., 2014; Lehmann and Rillig, 2015). AM fungi could be used as a sustainable tool to improve micronutrient concentrations in crops, as an alternative to, or in addition to genetic and agronomic biofortification (i.e., the increase in the concentrations and/or bioavailability of mineral elements in plant products). Besides crop productivity, AM symbiosis, due to its role in plant nutrition, could also have a positive impact on crop quality, thanks to the enrichment in both macro and micronutrients (Antunes et al., 2012; Hart and Forsythe, 2012; Pellegrino and Bedini, 2014). Focusing on Zn, Lehmann et al. (2014) concluded that AM symbiosis positively affected the Zn concentration in various crop plant tissues under distinct environmental conditions. Soil texture, pH, and soil nutrient concentration (i.e., Zn and Pi deficiency) have in fact an influence on the AM fungus-mediated Zn content in different plant tissues (Lehmann et al., 2014). Moving attention to copper (Cu), iron (Fe), and manganese (Mn), the study by Lehmann and Rillig (2015) has shown that there is a positive impact of AM symbiosis on crop plant Cu and, for intermediate experimental duration (lasting 56–112 days), Fe nutrition, while a benefit for plant Mn nutrition has only been observed in herbs. Pellegrino and Bedini (2014) have demonstrated that AM fungal field inoculation could be an effective tool to improve the cultivation of chickpea as it can improve productivity, but also the grain nutritional content in protein, Fe and Zn.

Interestingly, it has been proposed that the nutrient-dependent regulation of AM colonization provides an important feedback mechanism for plants to promote or limit fungal colonization according to their needs (Nouri et al., 2014). It has already been demonstrated that phosphorous availability represents an environmental factor that can disturb the symbiotic interaction of AM. In fact, the suppression of AM colonization due to high Pi levels has been reported in several experiments

(Breuillin et al., 2010; Balzergue et al., 2013; Bonneau et al., 2013). Recently, in order to evaluate which nutrients, together with phosphorous, influence AM development, several elements have been tested to verify the inhibitory effects on AM colonization, using *Petunia hybrida* and *R. irregularis* as AM systems (Nouri et al., 2014). The results have shown that Pi, in agreement with previous data on the same AM system (Breuillin et al., 2010), and nitrate can potentially exert negative regulation on AM, while sulfate, Mg^{2+}, Ca^{2+}, and Fe^{3+} have no effect. Furthermore, the starvation of several nutrients, in particular of nitrate, has been shown to reverse the inhibitory effect of Pi on AMF, thus suggesting that nutrient starvation triggers a dominant AM-promoting signal that counteracts the effects of high Pi levels (Nouri et al., 2014). However, considering recent evidence on nutrient exchange in AM symbiosis, Walder and van der Heijden (2015) have reported that the cooperation in AM interactions is related to the partners involved in the symbiosis, and depends on several factors, including environmental conditions, acquisition of surplus resources and functional diversity.

CHALLENGES RELATED TO AMF INOCULUM PRODUCTION AND APPLICATION

The need to benefit from AMF as a biofertilizer, with a view to sustainable agriculture, is becoming increasingly urgent since the appropriate management of these symbiotic fungi could potentially decrease the use of agrochemicals. The main strategy adopted to achieve this goal is the inoculation of AMF propagules (inoculum) into a target soil. Unfortunately, AMF are obligate symbionts and cannot be cultivated in pure cultures, away from their host plants. This constraining feature makes the large-scale production of AMF inocula very challenging and complex. There are three main types of AMF inocula. First, soil from the root zone of a plant hosting AMF can be used as inoculum as it normally contains colonized root fragments, AMF spores, and hyphae. However, unless precise information about the propagule abundance, diversity, and infectivity are available, soil inocula can be unreliable and carry the possible risk of transferring weed seeds and pathogens. Spores extracted from soil can instead be used as starters for crude inoculum production. Crude inoculum can be obtained after a known isolate of AMF and a host trap plant (i.e., a plant that can be massively colonized by many AMF species) are grown together in an inert medium optimized for AMF propagation. This is the most commonly used type of inoculum for large-scale crop inoculation as it usually contains a more concentrated set of the same kind of propagules found in soil inocula. Finally, infected root fragments alone of a known AMF host that have been separated from a trap plant culture can also serve as a source of inoculum.

The production of AMF crude inoculum on a large-scale remains very challenging even though new methods for massive production (IJdo et al., 2011) and seed coating technology (Vosátka et al., 2013) have been developed in recent years (van der Heijden et al., 2015). The main obstacle to the production of an AMF inoculum lies in the obligate symbiotic behavior of AMF, that is, their need to have a host plant for growth and completion of their life cycles. This means that the propagation step must include a phase of cultivation with the host plant that is usually time and space-demanding. As a consequence, the setting up of AMF reference collections also requires methodologies that are rather different and more binding than those used for other microbial collections. Moreover, the absence of a prompt method for assessing whether and to what extent the host plant is colonized by AMF also contributes to making AMF agricultural usability challenging. The management of the high amount of inoculum necessary for large-scale application is also a demanding process. However, AMF inoculation is carried out more easily for plant production systems that involve a transplant stage, since smaller amounts of inoculum are needed.

At a first glance, carrying out an open-field, extensive inoculation treatment could seem technically impractical and economically prohibitive. However, once AMF biodiversity is restored and well-established, and if an AMF-friendly management, such as fall cover cropping (Lehman et al., 2012) and conservation tillage (Säle et al., 2015) is put in place, the AMF community will persist. If no detrimental practices are carried out before and after cultivation, it is known that the biodiverse mycorrhizal hyphal network will remain unaltered and infective in the future. As an alternative to large-scale inoculation, a small-scale approach is also feasible. Taking inspiration from the idea of creating the so-called "fertility islands" (Allen, 1987), AMF inoculation could be limited to small portions of a field, and this would gradually lead to the establishment of a healthy AMF mycelial network, but with reduced costs. This technique would be particularly indicated when the AMF inoculation is aimed at assisting the revegetation of a degraded land, since inoculated fertility islands likely allow native plant species to recover the nutrient impoverished land faster.

Hence, AMF restoration only represents an initial cost that, if the persistence of AM fungi is favored in the soil, could be prorated over the years. As already demonstrated (Gulati and Cummings, 2008; Barr, 2010), AMF inoculation can be economically profitable, in comparison to conventional fertilization, providing substantial savings for growers and for degraded land recovery projects. In order to provide further data to assess AMF inoculation attractiveness, it is important that the end-users should also cultivate an uninoculated portion of their crop, so as to be able to evaluate the cost-effectiveness and beneficial effects on plant fitness due to AMF (Dalpé and Monreal, 2004).

The global economic crisis is now forcing growers to try to understand the potential of sustainable agricultural systems, and of reducing the input of phosphorus using AMF inocula. Unfortunately, solid inoculation practices have yet to be implemented, and applied research focused on defining the best inoculum formulation strategies (Verbruggen et al., 2013) should be encouraged. The potential of AMF has drawn the attention of the commercial sector, and several companies nowadays produce and sell AMF-based inocula. The general tendency is to formulate inocula with only a few AMF species as components. Some manufacturers have chosen the single formulation approach, but

others produce different products that are supposedly targeted for end-users who are willing to apply the formulation to a range of environmental conditions and host-plant groups. The few species that are used can easily be routinely propagated and are normally generalist, as they are found in association with a large variety of host plants in different biomes. Although commercial inocula are often advertised as suitable for a wide range of plants and environmental conditions, the real benefits are not always positive (Corkidi et al., 2004; Faye et al., 2013). For this reason, in order to promote AMF inoculum market development and improvement, scientists should strengthen the link between research and companies and introduce a series of "best practices" that could be adopted to solve issues related to the functioning of commercial inocula. One of these issues arises from the need to control the biological composition of a product, for example, due to the possible presence of pathogens and weeds (Douds et al., 2005; Tarbell and Koske, 2007), but above all to the need to assess its purity in terms of AMF composition. In fact, the species list declared in a commercial inoculum label does not always correspond accurately to the actual inoculum composition (Berruti et al., 2013a). In addition, being obligate symbionts, AMF inocula are mostly produced using a containerized-culture, either in greenhouses, growth chambers, or in fields, and, as a result, cannot possibly be free from external microorganisms. Owing to the increased awareness of the risk of pathogens, many concerned manufacturers are now applying agrochemicals in order to avoid contamination of their product (Douds et al., 2005). In order to reduce pathogen carry-over, it is possible to opt not to include host root residues in the inoculum while, as an alternative, the incorporated root fragments can be surface sterilized without jeopardizing the viability of the AMF propagules (Mohammad and Khan, 2002). Over the last few decades, several techniques have been applied to molecularly characterize entire AMF communities in complex matrices, such as soil (Hempel et al., 2007; Lumini et al., 2010; Borriello et al., 2012; Davison et al., 2012) and AMF inocula (Berruti et al., 2013a,b). These methodologies also allow the inoculated AMF to be monitored inside the host plant during the cultivation cycle (Alkan et al., 2006; Farmer et al., 2007; Pellegrino et al., 2012; Thonar et al., 2012; Werner and Kiers, 2015). High-throughput next generation sequencing (NGS) potentially offers the most powerful and sensitive technique to trace the introduced fungus, both temporally and spatially. This set of techniques also makes it possible to verify whether the inoculated AMF favor significant levels of colonization, although this may not necessarily be important if the effects on crop production and quality are indirect via the resident AMF community (Rodriguez and Sanders, 2015). Finally, NGS also leads to the understanding of how the introduced AMF interact and coexist with the local AMF community (Rodriguez and Sanders, 2015).

LESSONS FROM PAST SUCCESSES AND FAILURES OF AMF INOCULATION

In order to capitalize the effort that has been made in the past years by researchers in an attempt to deliver a sustainable cropping system based on AMF inoculation, a large number of studies published over the last 15 years have been reviewed in detail in this review. In other words, 127 studies (**Supplementary Material S1**) were randomly selected from an article list obtained from a search on Google Scholar using the keywords "arbuscular mycorrhizal fungi" and "inoculation," with a publication date from 2001 to 2015. Overall, articles from 47 different scientific journals have been considered in this review. The most important information and factors featured in the reviewed articles are shown in **Figure 1**. Most of the studies were published in 2015 and in the 5 year window from 2011 to 2015, thus suggesting that researchers have been dedicating a great deal of effort into trying to implement agriculture with the potential advantages conferred by AMF. These 127 articles deal with 164 inoculation experiments aimed at determining the effect of AM fungi inoculation in different conditions on 43 plant families (mainly Fabaceae, Asteraceae, Poaceae, and Solanaceae) often subjected to abiotic stress. Biotic stress has received much less attention, and many experiments have not included any stress application. This could corroborate the fact that AMF-mediated pathogen protection is still poorly studied and that this aspect deserves more attention. The characteristics of these studies have been described in a table (**Supplementary Material S2**), in which the main factors involved in each experiment are categorized and the main results that have been obtained are schematically outlined. This table includes information on the experimental condition in which the inoculation study was carried out (field, outdoor pot, greenhouse, or growth chamber), the stress type (biotic or abiotic) against which AMF application was investigated, the host-plant species and family, the inoculant origin, the used inoculum propagation method, the type of AMF propagule applied, the method of application (monospecies, multispecies, or both) and the tested AMF species list. The main results of these inoculation studies are also reported. The results section of the table focuses on whether there was a significantly positive effect ($\alpha = 0.05$, regardless of which statistical test was applied) or not of the inoculation treatment vs. the respective non-inoculated control. In particular, the table reports six parameters that were considered to assess the AMF inoculation effect in most of the experiments considered in this review. These parameters are the root fungal colonization gain, root and shoot biomass increase, yield and plant nutrition improvement, and plant resistance to a given pathogen. Unfortunately, not all of the studies provided measurement results and statistical analyses for all of the six parameters that were chosen to describe the effects of AM fungi on the plant, but this was to be expected.

Overall, AMF inoculation has appeared to be highly positive for plant development and production in the experiments considered in this review (**Supplementary Material S2**). A significant root colonization gain, comparing to non-inoculated controls, was registered in 93.8% of the 130 experiments that provided this measurement. Root and shoot biomass was significantly increased by inoculation in 73.6 and 80.8% of 91 and 146 experiments, respectively. Yield and plant nutrition were improved by inoculation in 84 and 92% of 81 and 112 experiments, respectively.

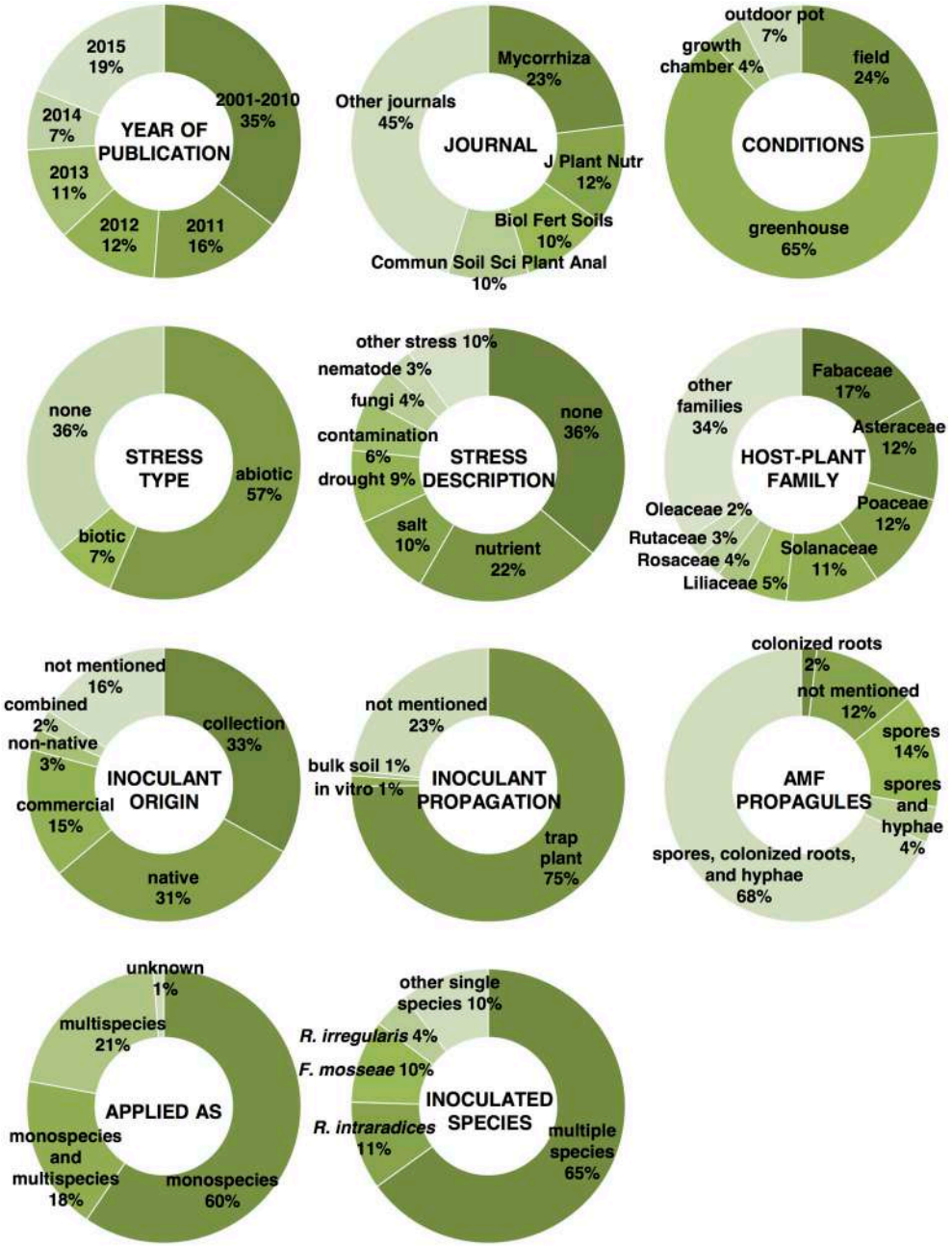

FIGURE 1 | Percentage of reviewed articles according to the year of publication and to the scientific journal, and percentage of experiments illustrated in the reviewed articles according to the cultivation conditions, stress type, and description, mycorrhizal host-plant family, origin of the AMF inoculants, method of AMF propagation used prior to inoculation, type of AMF propagule, method of application, and inoculated AMF species.

In an effort to define whether some of the most important factors considered in the reviewed studies have the potential to determine the success or the failure of inoculated AMF on plant productivity, the proportions of experiments showing a significant increase in colonization, biomass, yield, and nutrition following AMF inoculation was calculated for three important factors (**Supplementary Material S3**), i.e., the experimental condition (levels = greenhouse or open-field), the inoculant origin (levels = native origin or other origin), and the method of application (levels = as monospecies inoculum or multispecies inoculum). In order to statistically support the data interpretation, a 2-sample *z*-test was performed in which the percentage values of the two factor levels were compared. In addition, asymptotic confidence intervals were calculated for each percentage value. Providing sample proportions (the percentage of experiments resulting in a significant increase in

a given parameter divided by 100) and sample sizes (the number of experiments considered for the calculation of the percentage value), the *z*-test is able to return a *p*-value that can be used to either accept or reject the null hypothesis that the sample proportions are equal.

Inoculation Success in Greenhouse and Open-Field Conditions

Most of the experiments (65%) were carried out in greenhouses, while 24% were conducted in open-field conditions (**Figure 1**). As expected, the fungal colonization gain in inoculated plants, compared to non-inoculated controls, was significantly more frequent in the greenhouses than in the open-field conditions (*z*-test $p < 0.01$, **Figure 2**). This is most likely due to the fact that the non-inoculated control portion of a field often contains AM fungal propagules, while control pots in greenhouses are usually filled with sterilized substrates that are free of AM fungal propagules or highly reduced in AMF diversity. Interestingly, it has been observed that the root biomass benefits more from inoculation in field conditions than in greenhouse conditions, as can be seen from the results of a *z*-test *p*-value in the near-significant range (0.179). This is probably due to the fact that containerized roots stop growing because of constraints imposed by pot boundaries at a certain point in time during cultivation. In addition, the inoculated plants might sometimes be less prone to invest in root growth in pots. This could be due to the fact that containerized inoculated plants are likely to rely massively on fungal-mediated uptake (Smith et al., 2011) and can reach a maximum level of exploration of the substrate sooner than non-inoculated plants, without increasing the root biomass. Conversely, the effectiveness of AMF inoculation on shoot biomass, yield, and plant nutrition does not seem to be affected by the experimental conditions, and has been shown to be equally successful in greenhouse and open-field conditions.

Inoculum Propagation Method and Source

Among the reviewed studies, it has been found that the most widespread method for AMF propagation prior to inoculation is by using trap plants (75%), and, interestingly, only marginal use is made of other methods. A few other alternatives to the use of potted trap plants are in fact available. Soilless culture systems, such as aeroponics and hydroponics, lead to the production of pure clean spores and the maximization of growing conditions for the host plant (IJdo et al., 2011), and could soon reach massive use for large-scale production. The root-organ monoxenic culture is another method that allows the successful large-scale propagation of AMF that can be used directly as an inoculum. Unfortunately, the protocol has only been implemented for a reduced number of AM fungal species. The method consists in culturing inoculated excised roots (the so-called hairy roots) that have acquired the ability to proliferate without growing any epigeous portion, after transformation with *Agrobacterium rhizogenes* (Bécard and Fortin, 1988), which is a soil-borne bacterium containing the Ri (root-inducing) plasmid. A massive number of spores, mycelium, and colonized roots are obtained from one Petri dish in just a few months (Declerck et al., 1998). As AMF can use a number of different types

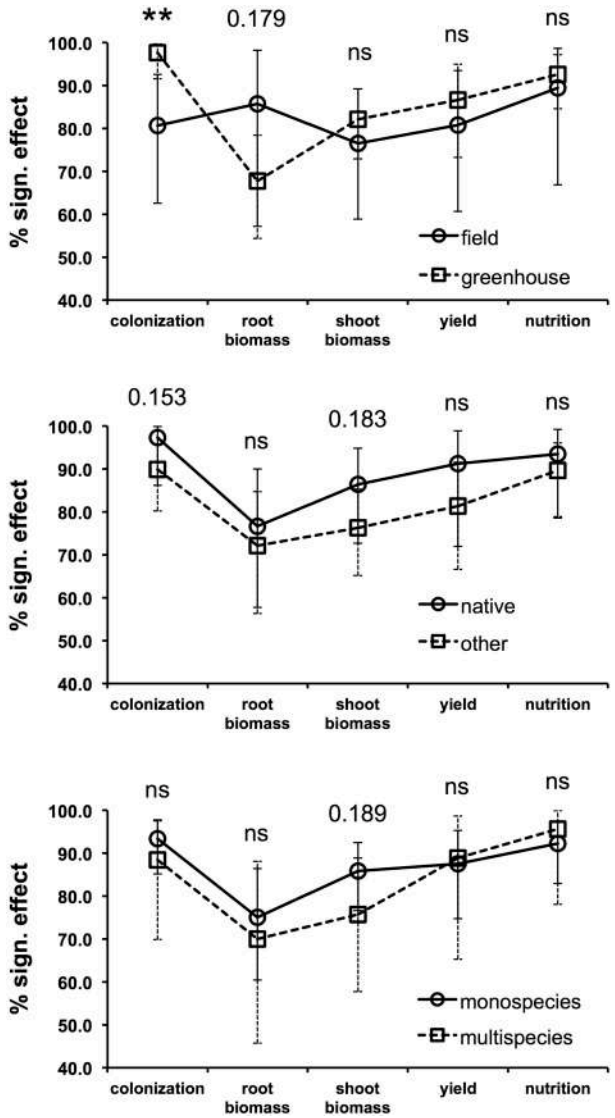

FIGURE 2 | Percentage of experiments showing significant increases in fungal colonization, root biomass, shoot biomass, yield, and plant nutrition comparing field and greenhouse conditions (upper graph), inoculation with native AMF and AMF of different origin (center graph), and inoculation with only one species and multiple species at the same time (lower graph). The statistical significance of the difference between the two proportions (percentage value divided by 100) calculated for each factor (i.e., cultivation condition, inoculant origin, and method of application) was computed with a series of 2-sample *z*-tests. The *z*-test *p*-value is reported for each of the five parameters in each graph (**$p < 0.01$; ns = $p > 0.20$). The asymptotic confidence intervals are reported for each percentage value.

of propagules in order to grow and colonize new roots with different degrees of efficiency (Klironomos and Hart, 2002), the choice of the inoculum source (described above) is a factor of primary importance for a successful colonization. Components of the extraradical and intraradical phase of AM fungi include spores, mycelium pieces fragmented from the belowground hyphal network, and several structures inside both living and dead root fragments. Intraradical vesicles, in particular, have been

shown to be a primary source of regrowth for certain AM fungal species (Biermann and Linderman, 1983). Various AM fungal taxonomical ranks differ in their ability to propagate from a given propagule. Propagation through mycelial fragmentation seems more important for species of the Glomeraceae family, whereas spore germination is the preferential type of propagation for members of other families (e.g., Gigasporaceae, Acaulosporaceae, and Scutellosporaceae; Brundrett et al., 1999). Hence, when wishing to apply a multispecies inoculum, the most eligible and user-friendly solution, since propagation via trap plant is the most commonly used method, is to sieve the substrate and finely chop the roots of the trap plant in order to retrieve all the different types of fungal propagules (crude inoculum). This solution was used in 68% of the reviewed experiments. Spores alone or mixed with hyphae were used in 14 and 4% of the cases, respectively. Mycorrhizal root fragments alone (root inoculum) were used in 2% of the cases.

Origin of the Fungal Inoculant

Another important factor involved in the success of the inoculation process is the choice of the AM fungal inoculants. The applied inoculants mostly came from culture collections or were isolated from the same types of soils used in the experiment. A moderate number of experiments featured the use of commercially available AMF-based inocula. Some AMF species are commonly recognized to be more stress tolerant than others, and are usually found in stressed and polluted soils (Leyval et al., 2002; Hildebrandt et al., 2007). Native AMF from areas affected by osmotic stress can potentially cope with salt stress in a more efficient way than other fungi (Ruiz-Lozano and Azcón, 2000). Thus, it is preferable to take this into account when "tuning" an inoculum to a particular kind of degraded/stressed soil and/or in order to avoid failure of the revegetation process (Vosátka et al., 1999; Oliveira et al., 2005). Overall, the reviewed studies point out the higher efficiency of native AMF. For example, indigenous AM fungi resulted in a better plant protection against root-knot nematode (Affokpon et al., 2011), higher growth in Mn contaminated soil (Briccoli Bati et al., 2015), and in a higher shoot biomass in highly calcareous soil (Labidi et al., 2015), than commercial inoculants. In addition, Estrada et al. (2013) demonstrated how, under saline stress, plants inoculated with native AMF had a higher shoot dry weight, efficiency of photosystem II, stomatal conductance, and accumulation of glutathione than those inoculated with AMF from culture collections. As a whole, the success of AMF application is always more frequent when native species are inoculated, although never in the *z*-test significance range (**Figure 2**). However, the more frequent occurrence of root colonization gain and shoot biomass increase in response to inoculation with native species are supported by the lowest *z*-test *p*-values, both of which are within the near-significance range (0.153 and 0.183, respectively). Most manufacturers advertise their commercial inocula by pointing out their suitability for a wide range of plants and environmental conditions. Unfortunately, the promises made about these products and the obtained results are sometimes far apart. Examples of ineffective or badly formulated inocula can be found in the literature (Corkidi et al., 2004; Garmendia and Mangas, 2014). For example, Corkidi et al. (2004) described an experiment in which commercial inocula that did not promote mycorrhizal colonization were the only ones that were able to improve the growth response of potted corn plants. Hence, the authors hypothesized the presence of other growth promoting additives in the tested inocula. Similarly, Garmendia and Mangas (2014) attributed the positive effect on lettuce growth and nutrition to the high mineral content included in a commercial inoculum. Optimal benefits are therefore more likely to be obtained from inoculation after a careful selection of the favorable host/niche/fungus combinations.

Composition of the Inoculum

The current general trend is to try one or more species of AM fungi for individual inoculation (monospecies inoculum), as seen in 60% of the reviewed experiments. Single species inoculation experiments tend to be more successful for a shoot biomass increase (*z*-test $p = 0.189$, **Figure 2**) than inoculation experiments with more than one species applied at the same time. Accordingly, Gosling et al. (2015), after assessing there was no beneficial effect on plant growth after inoculating diverse communities of AM fungi with functionally different traits, argued that when the host plant is exposed to a single factor, such as during greenhouse experiments, fewer fungal species able to alleviate that stress are likely to provide maximal benefit to the host, while a more diverse community would be required under multiple stress field conditions. Another greenhouse study has suggested that the composition rather than the diversity of species within a partnership could be more influential in determining how species function (Wagg et al., 2015). Many experiments have been limited to the single inoculation of one of the following three species: *Rhizophagus intraradices*, *Funneliformis mosseae*, and *R. irregularis* (**Figure 1**). These are very generalist symbionts that can colonize a large variety of host plants, survive long-term storage, are geographically distributed all over the world (Öpik et al., 2010), and can be easily and massively propagated. The aforementioned characteristics have made these species suitable for premium inoculum components. Several studies have highlighted that different isolates within the same species, rather than different species, can cause larger variations in plant response (Munkvold et al., 2004; Gai et al., 2006; Angelard et al., 2010). This suggests that the widespread use of single AM fungal species, such as *R. intraradices*, *R. irregularis,* and *F. mosseae*, in inoculation trials should not be considered a flaw as these species can contain considerable functional heterogeneity. In this context, in the presence of the *R. irregularis* reference genome (Tisserant et al., 2013; Lin et al., 2014), the partial genome re-sequencing of multiple isolates from different geographic origins will pave the way toward the study of the functional implication of genetic diversity in AMF populations as it may be possible to breed and select more effective AMF for crop plants (Rodriguez and Sanders, 2015). Another related aspect that has to be considered is the fact that plant species, including crops, vary greatly in their responsiveness to AMF inoculation (Johnson et al., 1997; Smith and Smith, 2011; Smith et al., 2011). In modern agriculture, plant breeding programs, which result in varieties or cultivars with a range of genetic differences, should consider the plant response to AM fungi as a selection trait.

CONCLUDING REMARKS AND PERSPECTIVES

It is currently estimated that the world's population will exceed nine billion by 2050 (Rodriguez and Sanders, 2015). Thus, global agriculture will have to face the task of almost doubling food production but also of reducing the dependence of producers on agrochemicals (for the EU, see Directive 2009/128/EC regarding the sustainable use of pesticides), in order to safeguard human and environmental health. The forecasted necessary yield increase exceeds the current global capacity to produce food (Rodriguez and Sanders, 2015), thus highlighting the need to implement or revitalize eco-friendly technologies, such as AMF-based biofertilization. Despite its enormous potential, the application of AMF has not been fully adopted by farmers so far.

In this review, it has been pointed out that AMF inoculation overall produces positive outcomes on plant production in both controlled and open-field conditions, mainly due to the several nutrition-related benefits that this class of soil fungal symbionts is able to provide to their host-plant. In particular, AMF inoculation in the field has proven to be as effective as inoculation in the greenhouse, where non-inoculated controls are normally free of AMF, unlike in open-field conditions. For these reasons, the next significant step toward the stable use of AMF in agriculture is to carry out large-scale multi-location field trials and conduct cost-benefit analyses, such as that presented in Ceballos et al. (2013), in order to increase awareness among the potential end-users of the benefits of AMF inocula. In addition, since indigenous AMF have been demonstrated to be equally or even better performing than commercial or culture collection isolates, farmers are encouraged to autonomously produce their AMF inocula, starting from native soils. This makes the biofertilization technology more likely to be affordable for farmers, including those in developing countries who need their cropping system to be as highly sustainable as possible.

AUTHOR CONTRIBUTIONS

AB, EL, RB, and VB contributed to the conception and drafting of the review. All the authors critically revised and approved the manuscript before submission.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01559

Supplementary Material S1 | The list of publications reviewed in Supplementary Material S2.

Supplementary Material S2 | The table reports the authors of the study, the count of articles that group more than one experiment, the year of publication, the scientific journal, the cultivation condition in which the experiment was carried out (field, greenhouse, outdoor pot, or growth chamber), the stress type applied (abiotic, biotic, or none), the stress description, the list of host-plant species included in the experiment, the plant family they belong to, the origin of the inoculants (native, collection, commercial, non-native, or combined), the type of AMF propagule applied (spores, hyphae, or colonized roots), the application method (monospecies, multispecies or both), the tested AMF species list, the list of other microorganisms combined to the inocula tested in the experiment and the outcome of the inoculation process in terms of the presence/absence of a positive significant effect (sign. or non-sign., respectively) on root colonization, root and shoot biomass, yield, plant nutrition, and plant defense from a given pathogen. As far as root colonization is concerned, any positive effect recorded in the experiment, regardless of whether it was found for the frequency, the intensity or the presence of arbuscules, was considered as significant. As for yield, any significant improvement, in terms of biomass or quality, was considered as significant. Plant defense from pathogen was considered as significant when plant survival, biomass and/or production were improved after AMF inoculation.

Supplementary Material S3 | The table reports the proportions of experiments showing a significant increase (sign./N) in colonization, biomass, yield, and nutrition following AMF inoculation for three factors, i.e., the experimental condition (levels = greenhouse or open-field), the inoculant origin (levels = native origin or other origin) and the method of application (levels = monospecies inoculum or multispecies inoculum). The number of experiments considered (N) for the calculation of each proportion is also reported.

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REVIEW
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Arbuscular Mycorrhizal Fungi for the Biocontrol of Plant-Parasitic Nematodes: A Review of the Mechanisms Involved

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Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts that can protect their host plant against biotic stress factors such as plant-parasitic nematode (PPN) infection. PPN consist of a wide range of species with different life styles that can cause major damage in many important crops worldwide. Various mechanisms have been proposed to play a role in the biocontrol effect of AMF against PPN. This review presents an overview of the different mechanisms that have been proposed, and discusses into more detail the plausibility of their involvement in the biocontrol against PPN specifically. The proposed mechanisms include enhanced plant tolerance, direct competition for nutrients and space, induced systemic resistance (ISR) and altered rhizosphere interactions. Recent studies have emphasized the importance of ISR in biocontrol and are increasingly placing rhizosphere effects on the foreground as well, both of which will be the focal point of this review. Though AMF are not yet widely used in conventional agriculture, recent data help to develop a better insight into the modes of action, which will eventually lead toward future field applications of AMF against PPN. The scientific community has entered an exciting era that provides the tools to actually unravel the underlying molecular mechanisms, making this a timely opportunity for a review of our current knowledge and the challenges ahead.

Keywords: arbuscular mycorrhizal fungi, biocontrol, cyst nematodes, induced systemic resistance, plant-parasitic nematodes, migratory nematodes, mycorrhiza induced resistance, root-knot nematodes

INTRODUCTION

Nematodes form a highly diverse group comprising free-living nematodes as well as plant and animal parasites that can be found worldwide in various habitats (Ferraz and Brown, 2002). Many species of plant-parasitic nematodes (PPN) can act as pests on a wide range of important agricultural crops. They mostly live in the soil, but some species such as several *Ditylenchus* spp. can act as aboveground pests. PPN show a wide array of life styles, but all have a usually hollow, retractable, needle-like mouth spear called the stylet for feeding. They are classified into different groups based on their feeding strategy (Perry and Moens, 2011). Ectoparasitic nematodes remain in the rhizosphere

during feeding, using their stylet to acquire food from the epidermal or outer root cortex cells. Endoparasitic nematodes on the other hand completely enter the root and feed inside the root. Migratory endoparasitic nematodes (e.g., *Radopholus* spp. and *Pratylenchus* spp.) migrate inter-or intracellularly while feeding on root cortex cells, thus causing damage to the plant along their migration path (Jones et al., 2013). Endoparasitic sedentary nematodes display the most complex feeding strategy of PPN, selecting cells in the vascular cylinder to be converted into a feeding site and then becoming sedentary with the onset of feeding (Gheysen and Mitchum, 2011). This last group includes the cyst and root-knot nematodes, which are considered to be the most damaging pests of agricultural crops worldwide (Jones et al., 2013; Bartlem et al., 2014). The sedentary endoparasitic *Meloidogyne* spp. such as *M. incognita* and *M. javanica*, can result in complete crop losses in tobacco and tomato or sunflower and pepper, respectively (Wesemael et al., 2011). The direct damage caused by PPN can be aggravated by secondary infections of the wounded plant tissues by other pathogens, while some PPN, such as the migratory ectoparasitic *Xiphinema* spp., can transmit plant viruses (Hao et al., 2012). Yield losses caused by PPN are expected to rise in the near future as a result of climate change and cropping systems intensification (Nicol et al., 2011). The use of nematicides is being limited, given the increasing concern for human health as well as the environment, which has led to their ban. Alternative nematicides are being sought (Oka and Mizukubo, 2009; Wesemael et al., 2011). Scientists are also looking for other nematode management strategies that fit into the recently launched framework of the Integrated Pest Management (IPM) directive of the European Union (EU directive 2009/128/EC), stating that member states have to implement IPM from 2014 onward, with the aim to reduce pesticide use and to promote non-chemical management practices as much as possible. One of the proposed environmentally friendly options to manage PPN is the use of biological control organisms, such as arbuscular mycorrhizal fungi (AMF).

Arbuscular mycorrhizal fungi are obligate root symbionts, estimated to colonize more than 80% of all land plant species. They improve plant growth through increased nutrient uptake in exchange for photosynthetic carbon from their host (Smith et al., 2010). Also, they can alleviate plant stress caused by abiotic as well as biotic factors, including PPN (Gianinazzi et al., 2010; Singh et al., 2011; Vos et al., 2012a). The biocontrol effect of AMF has been observed in a wide range of plant species and against many pathogens, most of them soil-borne fungal pathogens causing root rot or wilting, though successful biocontrol has also been observed against aboveground pathogens such as *Alternaria solani* in tomato (Harrier and Watson, 2004; Whipps, 2004; Fritz et al., 2006; Pozo and Azcón-Aguilar, 2007; Jung et al., 2012). Both necrotrophic and biotrophic pathogens have been reported to be suppressed by AMF, either directly or indirectly (Veresoglou and Rillig, 2012). AMF can also suppress PPN, as has been previously reviewed by Pinochet et al. (1996) and Hol and Cook (2005). *In vitro*, greenhouse as well as field experiments indicated protective effects against PPN by AMF in plants such as banana, coffee and tomato (Calvet et al., 2001; Vos et al., 2012b; Alban et al., 2013; Koffi et al., 2013). These protective effects ranged from a reduction in infection and reproduction to an enhanced tolerance. But though there are many reports on the biocontrol effect of AMF, their actual use as biological control agents in the field is still not a routine agricultural practice (Salvioli and Bonfante, 2013). This is partially due to variability in performance, depending on the AMF isolate, pathogen, plant species and environmental conditions (Dong and Zhang, 2006; Veresoglou and Rillig, 2012; Salvioli and Bonfante, 2013). An increased insight into their modes of action will therefore help to increase the efficacy of these biocontrol agents.

Several mechanisms can be involved in the AMF-mediated biocontrol; direct effects of AMF on the pathogen, involving competition for space or nutrients, or indirect, plant-mediated, effects. The latter can further be divided into the effects of AMF on plant tolerance, plant defense induction and altered plant exudation leading to altered rhizosphere interactions (**Figure 1**). The different mechanisms cannot be considered as completely independent from each other and biocontrol probably results from a combination of different mechanisms (Vierheilig et al., 2008; Cameron et al., 2013). In addition, the relative importance of a specific mechanism can vary depending on the specific AMF-pathogen-plant interaction. In recent years, much progress has been made, especially in the domains of induced systemic resistance (ISR; Pieterse et al., 2014) as well as on the role of the rhizosphere in biological control (Cameron et al., 2013), which will be the focal points of this review. We will present an overview of the different mechanisms that have been proposed to play a role in the AMF-mediated biocontrol, and we will discuss more into detail the possible significance of the different mechanisms for plant infection by PPN.

ENHANCED PLANT TOLERANCE

Higher Nutrient Uptake

Arbuscular mycorrhizal fungi are known to be able to increase the uptake of water and mineral nutrients for their host plant, such as phosphate and nitrogen (Parniske, 2008; Baum et al., 2015) but probably also micro-elements such as zinc (Smith and Smith, 2011a,b). In return, they receive photosynthetic carbon from their host (Gianinazzi et al., 2010). Similar to the protection of the plant by AMF against various abiotic stress factors such as drought, cold or heavy metal toxicity (Singh et al., 2011), AMF could also compensate for damage caused by pathogens. Although higher uptake of phosphate has been proposed as a mechanism for the AMF-mediated biocontrol, addition of phosphate to non-mycorrhizal plants did not result in a similar reduction of pathogen infection (Bodker et al., 1998). Fritz et al. (2006) showed that tomato plants colonized by *Rhizophagus irregularis* showed significantly less symptoms caused by *A. solani* than non-mycorrhizal plants, while no increase in phosphate uptake was observed. An additional phosphate supply even resulted in higher disease incidence. There is thus not always a positive correlation between increased phosphate uptake and plant growth promotion in mycorrhizal plants, as in some cases plant growth suppression resulted as a consequence of AMF colonization, even when phosphate transport from the AMF to the host plant was taking place (Smith and Smith, 2011a).

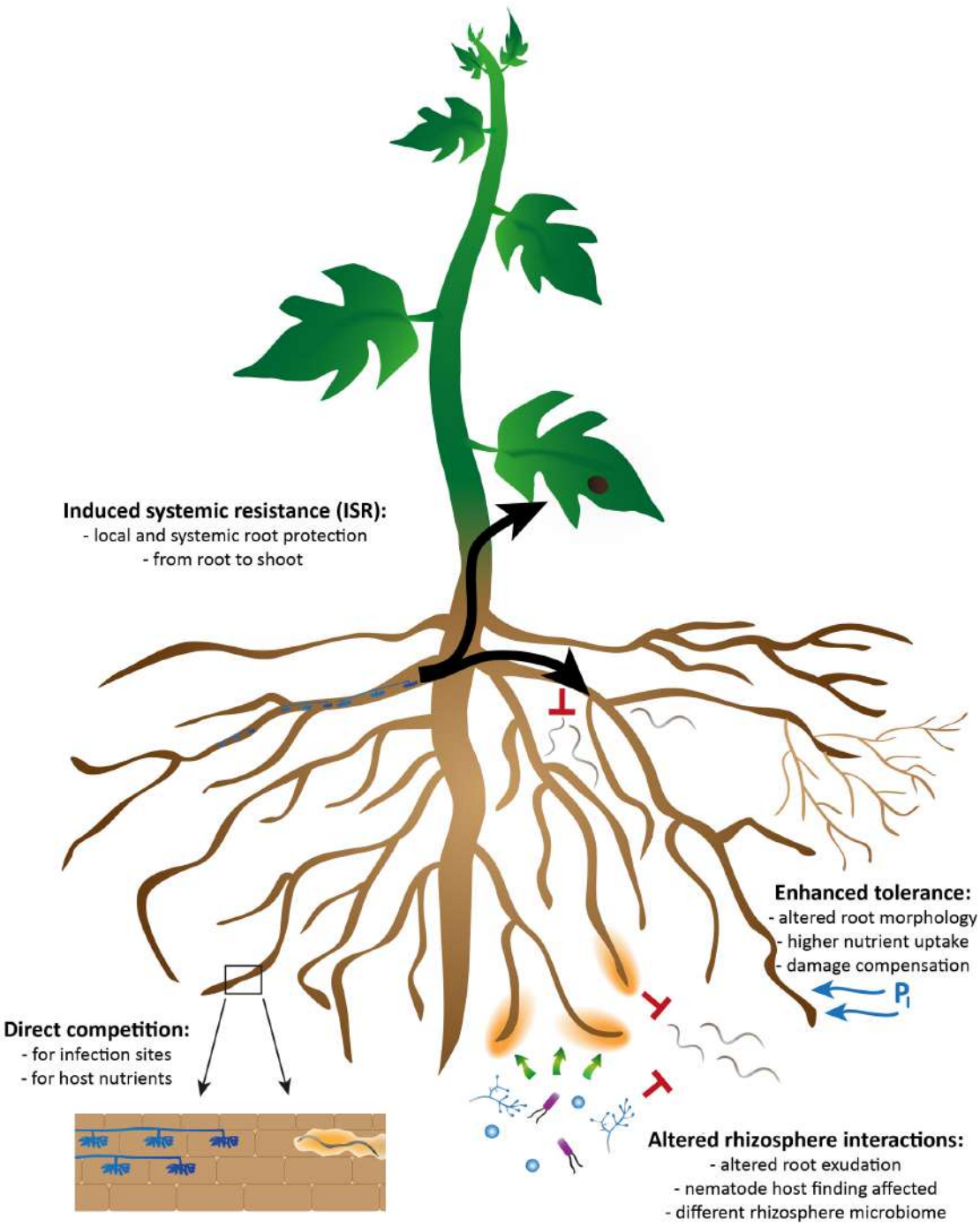

FIGURE 1 | Overview of the possible mechanisms by which arbuscular mycorrhizal fungi can exert biocontrol against plant-parasitic nematodes. They consist of direct effects of AMF on the pathogen, involving competition for space and nutrients (bottom left) and indirect plant-mediated effects, involving damage compensation and enhanced tolerance (top right). The latter can be further divided into the effects of AMF on plant defense induction (ISR; top left) and altered rhizosphere interactions through changes in plant root exudation (bottom right). The different mechanisms cannot be considered as completely independent from each other and biocontrol probably results from a combination of different mechanisms.

Plants with a better nutrient status are able to tolerate higher PPN population densities in their roots, as observed in cotton fields infested with the sedentary semi-endoparasitic nematode *Rotylenchulus reniformis* (Pettigrew et al., 2005). Regression analysis of nematode population densities against the mineral content in rice also revealed a positive correlation between migratory ectoparasitic *Helicotylenchus* spp. and Mg, however, a negative correlation was observed between the migratory

endoparisitic nematode *Pratylenchus zeae* and Zn or Fe, and between *M. incognita* and Mg and Ca (Coyne et al., 2004). These observations indicate that the nutrient status of the host plant can affect PPN population densities in both a positive and negative way. But so far, no solid data are available that prove that the AMF-enhanced nutrient status is a causal agent of a higher resistance against PPN.

Altered Root Morphology

Apart from an increased nutrient status, mycorrhizal plants often show increased root growth and branching (Gamalero et al., 2010; Orfanoudakis et al., 2010; Gutjahr and Paszkowski, 2013). The root morphology responses resulting from AMF colonization seem to depend on plant characteristics, with tap roots for example appearing to profit more from AMF than fibrous roots in terms of gained biomass and nutrient acquisition (Yang et al., 2014).

Increased root branching observed in mycorrhizal plants has been suggested to have implications for pathogen infection as well (Vos et al., 2014), although a clear correlation could not been found (Vierheilig et al., 2008). Positive effects could result from an increase in root vigor, due to a higher nutrient uptake capacity. It might even counterbalance the suppressed root growth caused by PPN. For example, decreased root branching caused by the migratory endoparasitic nematodes *Radopholus similis* and *P. coffeae* in banana was counterbalanced by the increased branching due to colonization by the AMF *Funneliformis mosseae* (Elsen et al., 2003a). Increased root branching can, however, also have a negative impact on the host plant by an increase in potential infection sites, depending on the PPN and plant species. Migratory endoparasitic nematodes, such as *R. similis*, have a preference for primary roots (Stoffelen et al., 2000; Elsen et al., 2003b). For the sedentary endoparasitic root-knot and cyst nematodes, the root elongation zones and sites of lateral root formation are preferred penetration sites, probably because of increased leakage of exudates in these zones (Wyss, 2002; Curtis et al., 2009). However, increased root branching and root length did not alter plant susceptibility to cyst nematodes, as it was observed that the number of penetrating *Heterodera schachtii* juveniles in transgenic *Arabidopsis thaliana* plants with long- or short-root phenotypes was similar to that in wild type plants (Hewezi and Baum, 2012).

The possible role of altered root morphology in AMF-mediated biocontrol has been investigated against several other pathogens. In an earlier study, Norman et al. (1996) investigated the consequences of a highly branched root system of strawberry on *Phytophthora fragariae* infection, a pathogen that mainly infects through the root tips. For non-mycorrhizal root systems infection was indeed higher on the more highly branched roots, but this was not observed in mycorrhizal root systems. Similar findings in different AMF-pathosystems were also reported later (Fusconi et al., 1999; Gange, 2000; Vigo et al., 2000; Gamalero et al., 2010), pointing toward other mechanisms involved in the AMF-mediated biocontrol. Although such experiments have not been performed specifically with PPN, similar mechanisms can be expected to play a role as well.

DIRECT COMPETITION FOR NUTRIENTS AND SPACE

Although no antibiotic production or mycoparasitism potential has so far been shown in AMF species, direct effects of AMF on pathogen infection through competition have been proposed. Competition for nutrients or for space and infection sites do occur between micro-organisms with the same physiological requirements in an ecological niche, especially where resources such as carbon might be limited (Vos et al., 2014).

Nutrient competition, with emphasis on competition for carbon, has been suggested as a mechanism of the AMF-mediated biocontrol though not much evidence is found in the literature (Jung et al., 2012). The carbon transfer from the host plant to the AMF is estimated to range from 4 to 20% of the total assimilated carbon (Hammer et al., 2011). It thus seems plausible that AMF compete with pathogens for this resource (Vos et al., 2014). As there is a difference in carbon sink strength between different AMF species, according to the hypothesis of carbon competition it could thus be expected that different AMF species mediate different levels of biocontrol (Lerat et al., 2003). Thus far, however, this hypothesis is not supported by experimental evidence (Vierheilig et al., 2008; Jung et al., 2012). For example, the AMF *R. irregularis* could not exert a stronger biocontrol effect on *R. similis* and *P. coffeae* in banana nor on *M. incognita* in tomato despite its higher carbon sink strength compared to *F. mosseae* (Vos, 2012).

Competition for space could also be involved in AMF-PPN interactions since they both reside in roots (Jung et al., 2012). Negative effects due to space constriction can be exerted on PPN as mycorrhizal arbuscules exclusively form in the cortex, where also migratory endoparasitic nematodes feed. Space competition between AMF and sedentary endoparasitic nematodes could be brought into play in case the feeding cells extend into the cortex (**Figure 2**). Cyst nematode feeding cells, the so-called syncytia, are confined within the endodermis and should therefore be less affected by AMF. Through PCR and DNA sequencing, del Mar Alguacil et al. (2011) reported that galls produced by *M. incognita* in *Prunus persica* roots could be colonized by AMF. However, as mycorrhizal arbuscules are short-lived structures (Parniske, 2008; Javot et al., 2011), it is difficult to distinguish whether AMF or PPN colonized the same root part first.

Competition for space also implies that a higher AMF colonization degree of the root should lead to a higher level of AMF-mediated biocontrol (Vierheilig et al., 2008). This hypothesis, however, holds only true to a certain degree. A mature AMF colonization, characterized by the presence of arbuscules, seems to be a prerequisite for biocontrol (Khaosaad et al., 2007; Pozo and Azcón-Aguilar, 2007). Dos Anjos et al. (2010) also concluded that when the symbiosis was well established prior to *M. incognita* inoculation, *M. incognita* reproduction was reduced, whereas co-inoculation had no effect. However, when co-inoculating native AMF together with *Meloidogyne exigua* in coffee plants, a biocontrol effect was observed (Alban et al., 2013). The competition for nutrients and space also implies that AMF could be affected by PPN infection as well. Hol and Cook (2005) concluded following a meta-analysis of the available

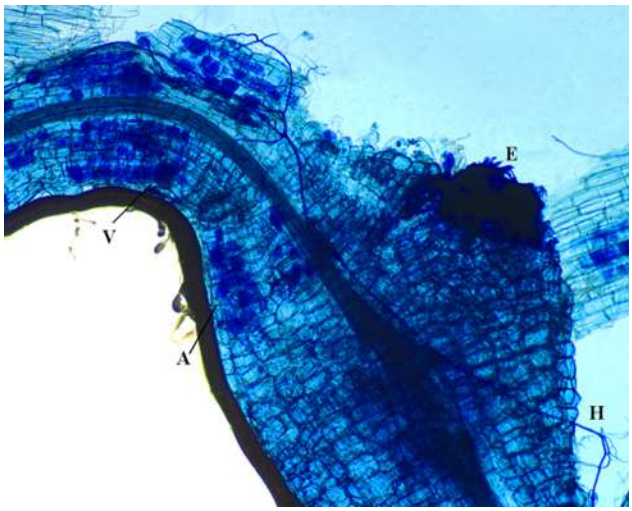

FIGURE 2 | Tomato roots stained with ink-vinegar solution to observe mycorrhizal colonization by *Funneliformis mosseae* (in blue). This root fragment shows typical mycorrhizal structures such as hyphae (H), arbuscules (A) and vesicules (V), showing the mature stage of the colonization along with the gall induced by *Meloidogyne incognita,* of which the egg mass (E) is also visible. As such, direct competition for space between arbuscular mycorrhizal fungi and sedentary endoparasitic nematodes probably can take place. Bar size = 100 μm.

literature that AMF colonization was reduced by ectoparasitic, migratory endoparasitic and sedentary endoparasitic nematodes. In greenhouse experiments, *R. similis* and *P. coffeae* in banana affected the frequency of *F. mosseae* colonization, but not the intensity (Elsen et al., 2003a,b). Contrastingly, root colonization by *R. irregularis* in *in vitro* banana plantlets was not affected either by *R. similis* (Koffi et al., 2013) or by *P. coffeae* in transformed carrot roots (Elsen et al., 2003c). For sedentary endoparasitic nematodes, Dos Anjos et al. (2010) showed that *M. incognita* could negatively affect the sporulation of the AMF *Scutellospora heterogama* in sweet passion fruit, while Alban et al. (2013) found that pre-inoculation of *M. exigua* led to a significant increase in the subsequent colonization of AMF compared to uninoculated mycorrhizal plants. del Mar Alguacil et al. (2011) also reported that the highest AMF diversity was found in uninfected roots compared to *M. incognita* infected roots and galls, and that the composition of the AMF community was different between infected and uninfected roots. Their results indicate that AMF colonization might also be suppressed by PPN, depending on the AMF species as some AMF species were not affected by the PPN.

EFFECTS THROUGH INDUCED SYSTEMIC RESISTANCE

In many cases the above mentioned mechanisms are not able to explain the observed AMF-mediated biocontrol, as plant-mediated responses seem also to be involved. For example, using a split-root experimental set-up in tomato in which the root system of one plant was divided over two physically separated compartments with one side pre-inoculated with *F. mosseae*, Vos et al. (2012b) demonstrated that *M. incognita* or *P. penetrans* infection in the other root compartment was significantly reduced. De la Peña et al. (2003), however, did not find a systemic biocontrol effect on *P. penetrans* in the dunegrass *Ammophila arenaria* pre-colonized by different native AMF species. However, more reports highlight a systemic suppression of nematode infection in mycorrhizal roots. As, for example, systemic suppression was observed in split root experimental set-ups in *R. irregularis*-colonized banana against *R. similis* and *P. coffeae* (Elsen et al., 2008) but also in grapevine against the ectoparasitic nematode *X. index* (Hao et al., 2012). A systemic biocontrol effect of AMF has also been shown in interactions with several other pathogens (Cordier et al., 1998; Pozo et al., 2002; Zhu and Yao, 2004; Fritz et al., 2006; Khaosaad et al., 2007; Castellanos-Morales et al., 2011), supporting the importance of this mechanism in the AMF-mediated biocontrol.

Novel insights that are shaping our understanding of the induction of systemic plant defense responses by AMF include the notion that plants initially perceive beneficial organisms as putative pathogens, due to MAMPs (microbe-associated molecular patterns) being conserved between beneficial and pathogenic fungi (Zamioudis and Pieterse, 2012). With AMF being obligate biotrophs, it has indeed been shown that a significant overlap exists in the transcriptional profile of the plant response to AMF and a biotrophic pathogen, such as *Magnaporthe grisea* (Paszkowski, 2006). Then, upon MAMP-recognition by the plant's pattern recognition receptors, a MAMP-triggered immunity response (MTI) is activated, forming the first line of defense of the plant in an effort to limit further pathogen invasion (Jones and Dangl, 2006). Surprisingly, the presence of an MTI response in the plant roots has only recently been demonstrated (Millet et al., 2010).

Being initially perceived by the plant as a putative biotrophic pathogen, AMF thus also induce a MTI response, leading to transcriptional and hormonal changes in their host plant upon establishment of the symbiosis. Fiorilli et al. (2011) studied the transcriptome of tomato plants during the colonization process by *F. mosseae* and observed significant gene modulation in both roots and shoots, with the largest alterations in primary and secondary metabolism, as well as in defense and response to biotic stimuli. López-Ráez et al. (2010) compared the transcriptional response of tomato to two AMF differing in colonization pattern, namely *F. mosseae* and *R. irregularis*. Despite the common induction of jasmonate (JA)-biosynthesis and signaling-related genes, they only found 35% overlap in the overall transcriptional profiles of tomato roots colonized by either of these two AMF species. In the case of *F. mosseae*, a stronger induction of the largely root-specific 9-lipoxygenase (9-LOX) pathway was observed, as well as the induction of the isoleucine conjugate of JA (JA-Ile), several JA-dependent markers and increased salicylic acid (SA) levels, which could be linked to its lower degree of colonization compared to *R. irregularis*. The early MTI-response involving the jasmonate-linked 9-LOX-pathway could have an early effect on root-knot nematodes as well. In maize, the expression or presence of the 9-LOX gene (*ZmLox3*) proved to be essential for resistance against *M. incognita* (Gao et al., 2008). However, so far tripartite studies involving the plant, AMF and PPN have not reported similar results.

Typically, the induction of an MTI response in the early stages of AMF colonization is only weak and transient, so that a further successful establishment of the symbiosis becomes possible. AMF try to avoid their detection by the plant as much as possible, but also actively suppress the MTI response using effectors (Zamioudis and Pieterse, 2012). As a biotroph, AMF colonization is negatively impacted by SA, which is why they are thought to attempt to suppress the SA-mediated defense response in the plant (Hause et al., 2007; Miransari et al., 2014). In addition, it has been proposed that the establishment of the symbiotic program, activated upon perception of mycorrhizal Myc factors, also counteracts the MTI response (Zamioudis and Pieterse, 2012). So far, only one effector molecule has been described for AMF, being the SP7 effector of *R. irregularis* that interferes with ethylene (ET) signaling in the plant (Kloppholz et al., 2011). This can be associated with recent reports highlighting the role of ET in the MTI response (Millet et al., 2010).

The initial interaction phase between AMF and its host plant primes the plant for a faster and stronger induction of usually JA-dependent defense responses upon subsequent pathogen attack. This cost-effective phenomenon is described as ISR, and specifically in the case of AMF the term mycorrhiza-induced resistance (MIR) has been proposed (Pozo and Azcón-Aguilar, 2007; Pieterse et al., 2014). Exogenous JA-applications and study of mutants altered in the JA-pathway have proven that the JA-dependent pathway is indeed able to mediate resistance to PPN (Soriano et al., 2004; Cooper et al., 2005; Fujimoto et al., 2011; Fan et al., 2015). Traditionally, defense responses to microbes fall into two categories, being termed either ISR or systemic acquired resistance (SAR). SAR is typically SA-dependent and leads to the induction of pathogenesis-related (PR) proteins, while ISR is defined as being regulated by JA and ET, and not accompanied by major changes in PR protein expression (Vlot et al., 2008; Pieterse et al., 2009). Recent research efforts, however, prove that the overlap between SAR and ISR is much larger than originally thought, with substantial crosstalk taking place between the different pathways (Mathys et al., 2012; Pieterse et al., 2014). In this context, the MIR defense response against PPN is probably not solely linked to the JA-dependent pathway.

Some light has been shed on this topic by a few transcriptome studies performed in recent years, involving the tripartite interaction of plants, AMF and nematodes. Li et al. (2006) reported the primed transcriptional activation of a class III chitinase gene in *Glomus versiforme* colonized grapevine roots upon infection by *M. incognita*. Constitutive expression of this gene in transgenic tobacco plants enhanced the resistance against the RKN, but did not affect the AMF. This strongly suggests that the class III chitinase gene is involved in a protective mechanism against the PPN. Other recent studies also show that transgenic plants with higher chitinase activity were more resistant to RKN. Though this mostly affected the viability of the eggs which contain chitin, it also reduced the amount of egg masses and thus productivity of the females (Chan et al., 2010, 2015). The primed activation of several other plant defense-related genes was recently also reported in *R. irregularis* colonized grapevine (*Vitis* spp.) after infection by the ectoparasitic *X. index* (Hao et al., 2012). Expression analyses of expressed sequence tags (ESTs) generated by suppression subtractive hybridization (SSH) showed several plant genes that are upregulated during MIR. Interestingly, these genes were only upregulated when AMF and nematode were both present in the root, indicating a priming of these defense genes. The products of these genes include chitinase 1b, but also PR 10, which has RNase and antimicrobial activity. Interestingly, a PR10 protein purified from *Crotalaria pallida* shows nematostatic and nematicidal effects against *M. incognita*, targeting a digestive proteinase of the nematode (Andrade et al., 2010). Furthermore, the SSH-study by Hao et al. (2012) showed that glutathione S-transferase was upregulated, which is probably involved in the detoxification of reactive oxygen species (ROS) that can be imposed by the stress of the cell's hypertrophy and necrosis following nematode infection. Stilbene synthase 1 was also upregulated. It is a key enzyme in the phenylpropanoid pathway toward the phytoalexin resveratrol, of which the accumulation is a typical defense response by grapevine to biotic or abiotic stresses. However, in a previous research, resveratrol was not found to be effective *in vitro* on *R. similis*, *P. penetrans* nor *M. incognita* (Wuyts et al., 2006). Genes of 5-enolpyruvyl shikimate-3-phosphate synthase (ESPS) and a heat shock protein 70-interacting protein (HIP) were also primed (Hao et al., 2012). ESPS catalyzes the penultimate step in the shikimate pathway. The differential expression of shikimate pathway genes has been reported before in response to root-knot nematode infection of tomato (Schaff et al., 2007) and is thought to be related to the regulation of the auxin balance which is of importance for nematode feeding site formation and possibly location (Curtis, 2007; Gheysen and Mitchum, 2011).

More recently, Vos et al. (2013) found through SSH a clear primed defense response against *M. incognita* by *F. mosseae* in tomato. The identified differential expressed genes were mainly classified in the categories of defense, signal transduction and protein synthesis and modification. For example, there was a primed upregulation of chorismate synthase, which catalyzes the conversion of the ESPS product to chorismate, which is the last step in the shikimate pathway. The shikimate pathway thus seems to be implicated in AMF-mediated biocontrol in different plant species against several types of nematodes (Hao et al., 2012; Vos et al., 2013). Furthermore, the shikimate pathway produces precursors for various aromatic secondary metabolites which are produced through the phenylpropanoid pathway among which flavonol synthase has been reported to be primed (Vos et al., 2013). The detrimental role of several phenylpropanoid pathway products, including flavonols, on *M. incognita*, *R. similis*, and *P. penetrans* has already been demonstrated *in vitro* (Wuyts et al., 2006). Through SSH, also the ROS metabolism was linked to the reduction of root-knot nematode infection in mycorrhizal tomato roots (Vos et al., 2013). Similarly, Beneventi et al. (2013) suggested an important role for ROS generation in the resistance of soybean to *M. javanica* as they found through pyrosequencing an over–representation of genes containing various oxidase and peroxidase domains upregulated in the incompatible interaction. Other noteworthy genes found to be primed upon infection by *M. incognita* in mycorrhizal tomato plants by Vos et al. (2013) were sinapoylglucose:choline sinapoyltransferase, involved in the

FIGURE 3 | Visualization of the experimental set-up of a twin-chamber with tomato plants used by Dababat and Sikora (2007) and Vos et al. (2012b) to study the differential attraction of *Meloidogyne incognita* to mycorrhizal roots. The two plant compartments are connected by a bridge. The experiment consisted of three treatments: (i) control plants in both compartments (control treatment), (ii) mycorrhizal plants in both compartments (AMF treatment) and (iii) a control plant in the left compartment and a mycorrhizal plant in the right compartment (mixed treatment). Two days after transplanting, the compartments and bridge were watered to field capacity at time of inoculation and 2,000 freshly hatched *M. incognita* 2nd-stage juveniles were inoculated per twin chamber, exactly in the middle of the bridge. Twelve days after inoculation, nematode penetration was assessed in control and mycorrhizal root systems of all treatments.

biosynthesis of a lignin precursor and 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), catalyzing the final step in the biosynthesis of ethylene and related to the jasmonate-ethylene dependency of ISR (Verhagen et al., 2004; Schäfer et al., 2009). These transcriptome data point toward several candidate genes by which AMF can exert biocontrol against PPN through priming of effective plant defenses. However, future research should focus on the elucidation of their specific action toward PPN, with emphasis on the proteome and metabolome AMF-associated changes.

ALTERED RHIZOSPHERE INTERACTIONS

The plant-mediated effects of the AMF-mediated biocontrol go beyond MIR though: the AMF symbiosis also leads to an altered root exudation composition and level (Hodge, 2000; Jones et al., 2004), which can in turn impact the PPN in the rhizosphere in terms of hatching, motility, chemotaxis, and host location. Differences in root exudate quantity and quality between mycorrhizal and non-mycorrhizal plants have been reported for various compounds, including sugars and organic acids (Sood, 2003; Lioussanne et al., 2008; Hage-Ahmed et al., 2013), amino acids (Harrier and Watson, 2004), phenolic compounds (McArthur and Knowles, 1992), flavonoids (Steinkellner et al., 2007) and even for the plant hormone strigolactone (López-Ráez et al., 2011). The root exudation furthermore depends on the plant or AMF species involved (Badri and Vivanco, 2009; Kobra et al., 2009), as well as on the degree of symbiosis (Scheffknecht et al., 2006; Lioussanne et al., 2008).

Differential root exudation is an important instrument used by the host plant for autoregulation of the symbiosis (Pinior et al., 1999; Vierheilig et al., 2003; Vierheilig, 2004; Schaarschmidt et al., 2013). This phenomenon involves a differential root exudation depending upon the degree of colonization, and it has been proposed that the plant in doing so keep AMF colonization under a certain threshold, as well as deter pathogenic rhizospheric micro-organisms (Vierheilig et al., 2008). Vierheilig et al. (2000) first observed autoregulation of the AMF colonization in barley roots, and they hypothesized later that the AMF-mediated biocontrol was related to the autoregulation of the AMF symbiosis (Vierheilig et al., 2008). This hypothesis is supported by the findings of Pozo and Azcón-Aguilar (2007) that a critical degree of colonization is considered as a prerequisite for biocontrol, which is typically characterized by the presence of arbuscules. These structures are typically found in a mature symbiosis, and might thus coincide with the onset of the autoregulation process. Lioussanne et al. (2008) also observed that the attraction of *Phytophthora nicotianae* zoospores toward *R. irregularis* colonized root exudates changed to repellency, depending on the maturity of the AMF colonization. Vos et al. (2012b,c) investigated the effect of mycorrhizal root exudates on nematode behavior. Using a twin-chamber experimental set-up with tomato plants, *M. incognita* juveniles were inoculated onto a bridge connecting a tomato plant colonized by *F. mosseae* with a non-colonized plant (**Figure 3**). A few days later similar numbers of nematodes appeared to have moved from the bridge to either compartment, however, in the compartment with the mycorrhizal tomato, the nematodes appeared to have mainly gathered in the soil without penetrating the tomato roots, in contrast to the non-colonized compartment in which most nematodes had entered the plant roots. Further experiments indicated that *M. incognita* root penetration could even be further reduced by the additional application of mycorrhizal root exudates. Also a temporal paralysis of the second-stage infective juveniles (J2) in the presence of mycorrhizal tomato root exudates was observed in *in vitro* assays (Vos et al., 2012c). Moreover, mycorrhizal root exudates reduced host location and penetration by *R. similis* compared to non-mycorrhizal control banana plants (Vos et al., 2012c; **Figure 4**).

Altered root exudation can also cause a change in microbial diversity in the rhizosphere, and therefore affect plant-pathogen interactions (Bais et al., 2006; Lioussanne, 2010). Some reports show an increase in facultative anaerobic bacteria, fluorescent pseudomonads, *Streptomyces* species and chitinase-producing actinomycetes after AMF colonization (Marschner and Baumann, 2003; Wamberg et al., 2003; Harrier and Watson, 2004; Scheublin et al., 2010; Miransari, 2011; Nuccio et al., 2013; Philippot et al., 2013). These micro-organisms can also have antagonistic potential against PPN, either by direct effects such as by nematode-trapping or egg-parasitizing fungi, but also by

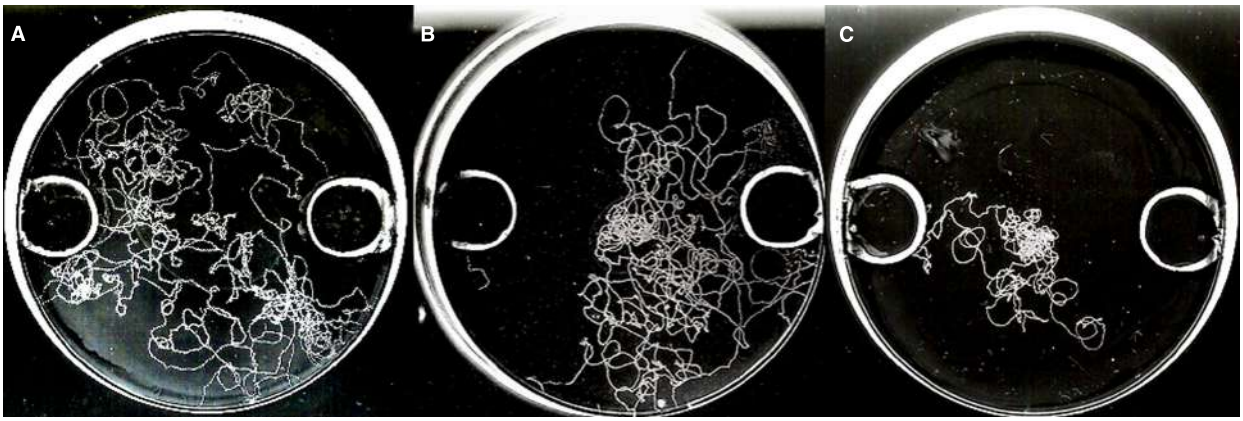

FIGURE 4 | *In vitro* chemotaxis bio-assay with visualization of nematode tracks on the medium. Pictures were taken 1 h after inoculation with 10 female *Radopholus similis* nematodes in the middle of the plate. Three hours before nematode inoculation, the opposite wells in the agar medium were filled with **(A)** water as control, **(B)** 1% acetic acid (left hole) *versus* 0,5M $CaCl_2$ (right hole) as repellent and attractive control, or **(C)** exudates of non-mycorrhizal roots (left) *versus* exudates of mycorrhizal roots (right) to observe chemotactic behavior of the nematode. Lower nematode movement can be observed in the plate with mycorrhizal root exudates, and the nematodes clearly show a chemotactic response away from them. For experimental set-up, see Vos et al. (2012c).

induction of the plant defense (Kerry, 2000; Tian et al., 2007; Zamioudis and Pieterse, 2012). Root exudates originating from mycorrhizal plants have, for example, been reported to attract plant growth promoting bacteria such as *Pseudomonas fluorescens* (Sood, 2003) and to affect beneficial soil micro-organisms such as *Trichoderma* spp. (Filion et al., 1999; Druzhinina et al., 2011) which also have biocontrol potential against PPN (Dong and Zhang, 2006; Sikora et al., 2008). In line with this, Cameron et al. (2013) recently presented an adaptation to the model of MIR in which the induction of systemic resistance by mycorrhizosphere bacteria is emphasized. MIR has also been shown to act against several PPN in dixenic *in vitro* culture (Elsen et al., 2001; Koffi et al., 2013), clearly suggesting that the AMF-mediated biocontrol does not act only through a change in soil biota, but it might be an important additional factor contributing to the biocontrol under field conditions.

CONCLUSION

In this review we provide an overview of the different mechanisms that have been proposed for AMF-mediated biocontrol, and specifically discuss their potential involvement in reducing PPN infections. The biocontrol effect depends on several factors such as the species involved, meaning that case-by-case studies will have to be carried out to result in field applications of AMF. Biocontrol effects of AMF on PPN have been reported since many years, but due to the technological progress that has been made in recent years we can now unravel the mechanisms behind. The sequencing of the genome of the root-knot nematodes *M. incognita* (Abad et al., 2008) and *M. hapla* (Opperman et al., 2008), the cyst nematode *G. pallida* (Cotton et al., 2014) and of host plants such as soybean (Schmutz et al., 2010), *Medicago truncatula* (Young et al., 2011) and several members of the *Solanaceae* family (Xu et al., 2011; Sato et al., 2012) are already contributing to increased insights. In addition, with the increase in microbiome studies, the importance of the mycorrhizosphere in the AMF-mediated biocontrol will be confirmed in the years to come. Progress will also be aided by the shift in attention to the plant root interactions with micro-organisms (De Coninck et al., 2015). This attention shift should already be taken into account in practice, as Castellanos-Morales et al. (2011) showed that older cultivars of wheat (before 1950) showed higher degrees of AMF root colonization compared to modern varieties (after 1950), also showing a higher bioprotective effect by AMF against the pathogen *Gaeumannomyces graminis*. Therefore, some consideration should be given to the trait of the ability to form symbiosis with AMF in future breeding efforts.

As the market for beneficial microbial inocula is growing steadily (Glare et al., 2012) and the development of beneficial microbial inocula for large-scale field application is moving forward quickly (Ijdo et al., 2011; Salvioli and Bonfante, 2013), field applications of AMF might be realistic. Thus far, few research about AMF-mediated biocontrol involved "omics" tools and systems biology approaches (Salvioli and Bonfante, 2013) but this will increase over time and provide more detailed insights in the complex mechanisms underlying AMF-mediated biocontrol. These insights might in turn lead to the effective application of AMF in the field.

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Root-associated bacterial endophytes from *Ralstonia solanacearum* resistant and susceptible tomato cultivars and their pathogen antagonistic effects

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This study was undertaken to assess if the root-associated native bacterial endophytes in tomato have any bearing in governing the host resistance to the wilt pathogen *Ralstonia solanacearum*. Internal colonization of roots by bacterial endophytes was confirmed through confocal imaging after SYTO-9 staining. Endophytes were isolated from surface-sterilized roots of 4-weeks-old seedlings of known wilt resistant (R) tomato cultivar Arka Abha and susceptible (S) cv. Arka Vikas on nutrient agar after plating the tissue homogenate. Arka Abha displayed more diversity with nine distinct organisms while Arka Vikas showed five species with two common organisms (*Pseudomonas oleovorans* and *Agrobacterium tumefaciens*). Screening for general indicators of biocontrol potential showed more isolates from Arka Abha positive for siderophore, HCN and antibiotic biosynthesis than from Arka Vikas. Direct challenge against the pathogen indicated strong antagonism by three Arka Abha isolates (*P. oleovorans, Pantoea ananatis,* and *Enterobacter cloacae*) and moderate activity by three others, while just one isolate from Arka Vikas (*P. oleovorans*) showed strong antagonism. Validation for the presence of bacterial endophytes on three R cultivars (Arka Alok, Arka Ananya, Arka Samrat) showed 8–9 antagonistic bacteria in them in comparison with four species in the three S cultivars (Arka Ashish, Arka Meghali, Arka Saurabhav). Altogether 34 isolates belonging to five classes, 16 genera and 27 species with 23 of them exhibiting pathogen antagonism were isolated from the four R cultivars against 17 isolates under three classes, seven genera and 13 species from the four S cultivars with eight isolates displaying antagonistic effects. The prevalence of higher endophytic bacterial diversity and more antagonistic organisms associated with the seedling roots of resistant cultivars over susceptible genotypes suggest a possible role by the root-associated endophytes in natural defense against the pathogen.

Keywords: 16S rRNA homology, bacterial wilt resistance, biological control, confocal microscopy, endophytic bacteria, *Ralstonia solanacearum, Solanum lycopersicum,* tomato

Introduction

Endophytic microorganisms colonize plants internally without any apparent adverse effects on the host (Hallmann et al., 1997; Gaiero et al., 2013). There is a growing interest in endophytic bacteria on account of their potential use in plant growth promotion, antagonistic effect on pests and pathogens, alleviation of abiotic stress and in phytoremediation (Compant et al., 2005; Ryan et al., 2008; Mercado-Blanco and Lugtenberg, 2014). Bacterial endophytes are generally known to enter the host from the surrounding soil through wounds in the roots (Hallmann et al., 1997; Compant et al., 2010) or through root hairs (Prieto et al., 2011; Mercado-Blanco and Prieto, 2012). They traverse the root cortex and reach various plant organs through the vascular system (Hallmann et al., 1997; Compant et al., 2010, 2011) while some use the apoplastic route (Sattelmacher, 2001; Reinhold-Hurek et al., 2007). Bacterial endophytes were earlier considered to be primarily colonizers in the inter-cellular or apoplastic spaces in the roots being present in relatively fewer numbers (Hallmann et al., 1997; Hallmann, 2001). Molecular studies have shown that there is considerable species diversity of bacterial endophytes albeit being present largely in a non-cultivable form (Lundberg et al., 2012; Sessitsch et al., 2012; Podolich et al., 2015). Intracellular colonization has also been documented in some plant systems (Pirttilä et al., 2000; de Almeida et al., 2009). A recent study employing banana shoot tissue has shown abundant endophytic bacteria in the two intracellular niches, namely in the cytoplasm and in the perispace between the cell wall and plasma membrane, and the terms 'Cytobacts' and 'Peribacts' have been coined to recognize the microorganisms in the respective intracellular niches (Thomas and Reddy, 2013; Thomas and Sekhar, 2014).

Bacterial wilt caused by the vascular pathogen, *Ralstonia solanacearum* (syn. *Pseudomonas solanacearum*) is a major constraint for tomato cultivation world over (Hayward, 1991; Genin and Denny, 2012). The wide host range covering major food and other economically important crops, broad geographic distribution, adaptation to survive in soil and water for long periods and the huge economic loss incited make the pathogen a very significant one worldwide (Genin and Denny, 2012; Mansfield et al., 2012). *R. solanacearum* invades the host through root injuries. The pathogen crosses the root cortex and overruns the xylem vessels leading to sudden wilting and plant death (Hayward, 1991; Genin and Denny, 2012). The similarities between bacterial endophytes and *R. solanacearum* in xylem colonization render the former as potential antagonistic and biocontrol agents against such vascular pathogens (Achari and Ramesh, 2014; Ting, 2014). Use of antagonistic bacteria for the biocontrol of bacterial wilt in tomato has been documented either as rhizospheric organisms (Vanitha et al., 2009) or as endophytes isolated from the same crop (Feng et al., 2013) or unrelated crops (Thomas and Upreti, 2014a).

Endophytic bacteria share an intimate symbiotic association with the host which makes them more valuable biocontrol agents (Compant et al., 2005; Bakker et al., 2013). Endophytes get an edge over their rhizospheric antagonist-counterparts on account of their ability to enter the host system without stimulating pathogen induced vulnerability responses but triggering host defense pathways (Conn et al., 2008; Gómez-Lama Cabanás et al., 2014; Podolich et al., 2015). Being internal colonizers, they could provide a barrier against the invading pathogens directly or through the production of bio-active compounds (Thomas and Upreti, 2014a; Podolich et al., 2015). Endophytes are better protected against abiotic stress and competing microbes compared with the rhizospheric counterparts (Hallmann et al., 1997; Ryan et al., 2008; Turner et al., 2013). While a vast majority of bacterial endophytes are known to be non-amenable for cultivation on common media (Lundberg et al., 2012; Sessitsch et al., 2012; Thomas and Sekhar, 2014), it entails that the organisms are easily cultivated to allow their agricultural exploitations. The present study was undertaken with a view to explore the extent of cultivable endophytic bacteria in transplantable-stage seedling roots of tomato cultivars that are either resistant or susceptible to *R. solanacearum*. Further, it was envisaged to evaluate the antagonistic and biocontrol features of the isolates to determine if the native endophytes played any role in governing the resilient property of the resistant cultivars.

Materials and Methods

Plant Material

Ralstonia solanacearum resistant (R) tomato (*Solanum lycopersicum* L.) cultivar Arka Abha and susceptible (S) cv. Arka Vikas (Thomas et al., 2015) were taken up as the primary test material in this study. In order to validate the findings, additional resistant (Arka Alok, Arka Ananya)/moderately resistant (Arka Samrat) and susceptible (Arka Ashish, Arka Meghali, and Arka Saurabhav) cultivars were employed. The names of genotypes are prefixed with R, MR, or S for easy recognition as resistant, moderately resistant or susceptible, respectively. Seedlings were raised in pasteurized organic cocopeat in protrays (Thomas et al., 2015) and used for the isolation of endophytes after $3^1/_2$–4 weeks which corresponded to the stage of transplanting to the field when seedlings normally get exposed to the field pathogen inoculum (Thomas and Upreti, 2014b).

Confocal Imaging of Seedling Roots

Seedling roots were examined for bacterial colonization through confocal laser scanning microscopy (CLSM) after SYTO-9 staining. For this, tender roots from 3 to 4 weeks-old cocopeat – grown seedlings were washed, cut to ∼1 cm segments and were treated with 1× SYTO-9 (12 μM) from the LIVE/DEAD BacLight® bacterial viability kit L13152 (Molecular Probes, Invitrogen) as per the kit instructions. After 10–15 min staining, the lateral roots and root hairs were examined using a LSM 5 LIVE confocal microscope and the images were processed as described elsewhere (Thomas and Reddy, 2013). Root tissues were also examined after surface sterilization which involved a quick dip in 90% ethanol, a rinse in sterile distilled water (SDW) and 1 min sodium hypochlorite (2% available chlorine) treatment followed by six SDW rinses.

Isolation of Endophytes from Seedling Roots

Twenty randomly picked seedlings from ^{R}Arka Abha and ^{S}Arka Vikas 4 weeks after sowing were lifted with the plug of cocopeat and washed under running water taking care to minimize root injury. Seedlings were excised below the cotyledonary node and surface-sterilized essentially as per Zinniel et al. (2002). This involved a quick dip in 90% ethanol, a rinse in SDW and 1 min NaOCl (2% chlorine) treatment as above. After three rinses in SDW, 2% $Na_2S_2O_3$ (10 min) was used to remove chloramine residues before finally rinsing the roots in SDW thrice. Root part was excised, blotted dry, weighed aseptically and macerated in a mortar employing 12.5 mM potassium phosphate buffer (Zinniel et al., 2002). After adjusting the volume to 10 ml g^{-1} tissue weight (10^0 stock), serial dilutions (10^1–10^5) were applied on NA through spotting- and tilt-spreading (SATS) approach (Thomas et al., 2012) with three replications per dilution. The plates were incubated at 30°C and the colony forming units (cfu) g^{-1} root tissue was determined on the third day. The NA plates used in this study were pre-monitored for absolute microbial sterility.

Identification of Organisms

Distinct bacterial colony types that emerged on NA from the root homogenate of ^{R}Arka Abha (Tm-Ab01 to Tm-Ab09) and ^{s}Arka Vikas (Tm-Av01 to Av05), serially numbered in the order of their relative abundance, were further purified through three rounds of streaking on NA. They were identified through partial 16S rRNA sequence homology analysis. For this polymerase chain reaction (PCR) was carried out with the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R-Y (5′-GGYTACCTTGTTACGACTT-3′; Y = C/T) with the thermocyling conditions as described elsewhere (Thomas et al., 2008). The identity of these organisms was established and validated through megablast analysis to the cultured organisms at the National Centre for Biotechnological Information (NCBI) and the Seqmatch analysis with the Type Strains at the Ribosomal Database Project (RDP), Michigan State University. Wherever the identification was inconclusive based on NCBI homologies in the case of less common organisms, the highest species homology from NCBI or the similarity score from RDP was adopted to suggest the identity at sequence data submission to NCBI. The final identity was fixed as per the genus/species assigned by the GenBank at the acceptance of sequence data.

Screening of Organisms for the Indicators of Biocontrol Property

The endophytic organisms were tested for siderophore production through chrome azurol S method (Schwyn and Neilands, 1987) and for HCN production as per Ahmad et al. (2008). The isolates were screened through PCR for functional genes involved in the biosynthesis of bacterial non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) as markers for antibiotic production as per Miller et al. (2012). The primers MTF2 (5′-GCNGGYGGYGCNTAYGTNCC-3′) and MTR2 (5′-CCNCGDAYTTNACYTG-3′) were employed for NRPS giving a PCR product of ∼1000 bp, and the primers DKF (5′-GTGCCGGTNCCRTGNGYYTC-3′) and DKR (5′-GCGATGGAYCCNCARCARMG-3′) for PKS yielding ∼650–700 bp PCR product.

Pathogen and Culture Media

Ralstonia solanacearum 'NH-Av01' strain (NCBI acc. no. KJ412034; biovar 3) isolated from the bacterial ooze of a wilted 'Arka Vikas' plant as described elsewhere (Thomas and Upreti, 2014b,c) was used in antagonistic assays. The culture was stored as glycerol stocks at −80°C and revived on Kelman (1954) medium containing 1.0 g l^{-1} casein hydrolysate (C), 10 g l^{-1} bacteriological peptone (P), 5 g l^{-1} glucose (G), and 15 g l^{-1} bacteriological agar (A) and was fortified with 0.005% 2,3,5-Triphenyltetrazolium chloride (KM-TTC). The media constitutes were sourced from Hi Media Biosciences, Mumbai, except for TTC (Sigma, St. Louis, MO, USA) employing P14 lot of Type-1 peptone as per Thomas and Upreti (2014c). This was based on the observation that the colony characteristics, lawn formation and inhibition zone development were significantly influenced by the type and batch of peptone. Other media employed included casein-peptone-glucose-agar (CPGA) or CPG broth. Three additional *Ralstonia* isolates, namely, NH-Av05, NH-Av07, and KAU-Av01 were also used in the antagonistic assays.

Antagonistic Assay

Antagonistic assays were set up essentially as described earlier (Thomas and Upreti, 2014a). Briefly, 200 µl of 2-days-old CPGA or KM-TTC culture of 0.1 OD at 600 nm (approximately cfu of 10^8 ml^{-1}) in peptone – salt (1 g l^{-1} each peptone and NaCl; Thomas et al., 2012) was spread over KM-TTC medium in 12 cm × 12 cm plates (Hi Media Biosciences, Mumbai) and wells of 6–7 mm diameter were prepared. After allowing *R. solanacearum* to establish at 30°C for 4 h, 50 µl of 0.2 OD endophytic bacterial inoculums in peptone – salt (approximately cfu in the range of 10^7–10^8 ml^{-1} for 0.1 OD culture depending on the organism) was applied in marked wells. After 20–25 min of surface drying, the plates were incubated inverted at 30°C. The antagonistic potential was rated based on the extent of clear zone formation, namely, strong (>20 mm; +++), medium (15–20 mm; ++), low (10–15 mm; +), or none.

Validation with Additional Tomato Cultivars

This included three additional resistant cultivars/F1 hybrids (^{R}Arka Alok, ^{R}Arka Ananya F1, ^{MR}Arka Samrat F1) and three susceptible cultivars (^{S}Arka Ashish, ^{S}Arka Meghali, ^{S}Arka Saurabhav; Thomas et al., 2015). Seedlings were grown in cocopeat in protrays and 5–10 surface-sterilized seedlings at 3½–4 weeks stage were employed for isolating the root endophytes. Tissue processing, culture purification, identification and assay for the antagonistic potential against the pathogen were undertaken as described earlier.

Nucleotide Sequences

The partial 16SrRNA gene sequences of the organisms have been deposited with the NCBI GenBank. The accession numbers are indicated in the Tables describing their identification.

Results

Confocal Imaging of Seedling Roots

The tender roots from 3 to 4 weeks-old ^{R}Arka Abha and ^{S}Arka Vikas seedlings showed green fluorescing bacterial cells on the root surface, inside the roots and in the surrounding film of water after SYTO-9 staining (**Figures 1A1,B1**). Root hairs showed abundant bacteria internally both along the cell periphery and in the cytoplasm (**Figures 1A2,B2**) confirming the endophytic colonization. Following surface sterilization, confocal imaging was impaired due to rapid signal bleaching (data not shown). However, it was possible to track the bacterial cells in both tender roots and root hairs with a notable reduction in the counts.

Isolation and Identification of Endophytes from ^{R}Arka Abha and ^{S}Arka Vikas

Root growth in ^{R}Arka Abha seedlings at endophyte isolation stage was relatively low compared with ^{S}Arka Vikas. However, both the genotypes showed similar cfu estimates per unit fresh tissue weight (3.9×10^4 and 4.3×10^4, respectively). A number of distinct colonies were picked up which were finally assigned to nine distinct species in ^{R}Arka Abha and five species in ^{S}Arka Vikas (**Table 1**). The organisms from ^{R}Arka Abha as per 16S rRNA gene sequence data accepted at NCBI GenBank included *Pseudomonas oleovorans*, *Pseudomonas plecoglossicida, Pantoea ananatis*, *Citrobacter freundii*, *Staphylococcus hominis*, *Sphingobacterium multivorum*, *Enterobacter cloacae*, *Arthrobacter globiformis,* and *Agrobacterium tumefaciens*. The isolates from ^{S}Arka Vikas constituted *P. oleovorans*, *Stenotrophomonas maltophilia*, *Bacillus pumilus, A. tumefaciens,* and *Microbacterium pumilum*. The resistant cultivar apparently displayed more endophytic bacterial diversity with two organisms (*P. oleovorans* and *A. tumefaciens*) common to both the cultivars. Both ^{R}Arka Abha and ^{S}Arka Vikas showed more of Gram-negative bacteria (78 and 60%, respectively) and γ-subclass of Proteobacterium formed the commonest single phylogenetic group in both the cultivars (56 and 40%, respectively).

Assessing the Endophytes for the Indicators of Biocontrol Property

Two of the ^{R}Arka Abha isolates (Tm-Ab01, Tm-Ab03) showed siderophore production, two isolates (Tm-Ab03, Tm-Ab07) HCN production and three isolates (Tm-Ab02, Tm-Ab06, Tm-Ab08) proved positive for NRPS/ PKS (**Table 2**). The respective numbers for ^{S}Arka Vikas were one, zero and one. Thus, the resistant cultivar harbored more organisms with biocontrol properties than the susceptible cultivar.

Screening of Endophytes for *Ralstonia* Antagonistic Activity

Seven isolates from ^{R}Arka Abha showed varying extents of antagonistic activity against *R. solanacearum* with Tm-Ab01 (*P. oleovorans*), Tm-Ab03 (*P. ananatis*), and Tm-Ab07 (*E. cloacae*) displaying significant effects, two isolates (Tm-Ab02, Tm-Ab08) offering medium activity and two others (Tm-Ab05, Tm-Ab06) showing low activity (**Table 2**). Among the ^{S}Arka Vikas isolates, Tm-Av01 (*P. oleovorans*) showed strong antagonism while Tm-Av02 and Tm-Av03 displayed low activity. This was found true in a repeat assay and with three other isolates of *R. solanacearum*, namely, NH-Av05, NH-Av07 and KAU-Av01 (**Figure 2**).

Validation with Additional Resistant and Susceptible Cultivars

^{R}Arka Alok, ^{R}Arka Ananya, and ^{MR}Arka Samrat yielded 8–9 distinct organisms each while ^{S}Arka Ashish, ^{S}Arka Meghali, and ^{S}Arka Saurabhav gave rise to four species each constituting a total of 37 isolates (**Table 3**). In general, there was a predominance of Gram negative bacteria in four cultivars (78, 62.5, 75, and 75%, respectively in ^{R}Arka Alok, ^{R}Arka Ananya, ^{S}Arka

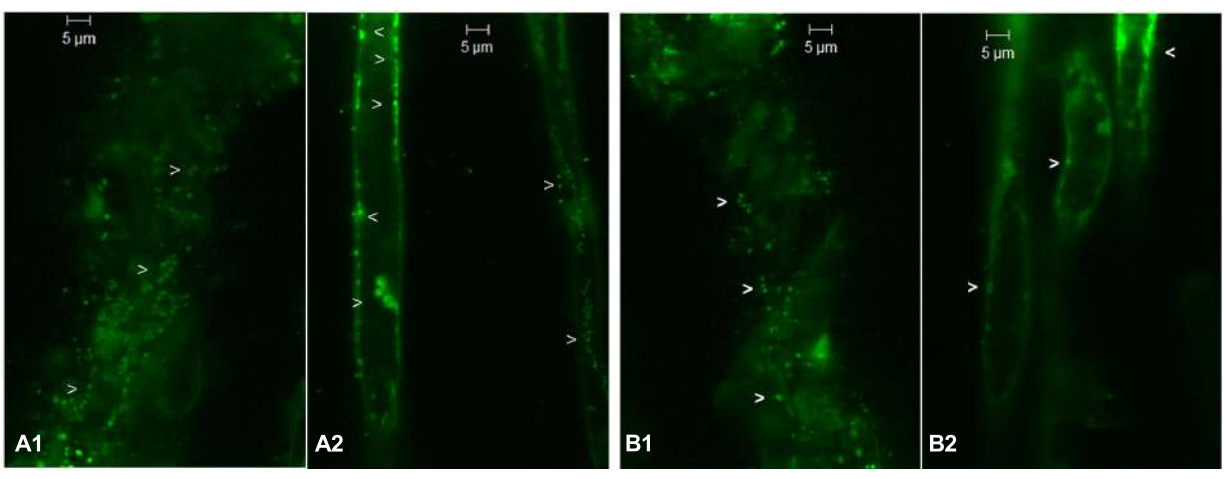

FIGURE 1 | Confocal laser scanning microscopy images from SYTO-9 treated non-surface sterilized roots of tomato ^{S}Arka Vikas and ^{R}Arka Abha showing green fluorescing bacteria (indicated by arrow heads) on the surface (A1,B1, respectively) and internally along the cell periphery and inside root hairs (A2,B2, respectively).

TABLE 1 | Identification of bacterial endophytes isolated from the seedling root tissue of tomato cvs. Arka Abha and Arka Vikas.

No.	Isolate ID	16S seq (bp) and NCBI acc. No	Identity based on closest species from NCBI/RDP (with acc. no and homology/similarity score)†	Phylogenic group and Gram reaction
Isolates from resistant cv. Arka Abha				
1	Tm- Ab01	770 (KM349750)	*Pseudomonas oleovorans* (HQ697330; 99%)	γ-Proteobacterium; −ve
2	Tm- Ab02	767 (KM349751)	*Pseudomonas plecoglossicida* (KJ395363; 99%)	γ-Proteobacterium; −ve
3	Tm- Ab03	711 (KM349752)	*Pantoea ananatis* (HQ683996; 98%)	γ-Proteobacterium; −ve
4	Tm- Ab04	793 (KM349753)	*Citrobacter freundii* (KF769539; 99%)	γ-Proteobacterium; −ve
5	Tm- Ab05	777 (KM349754)	*Staphylococcus hominis* (KJ018991; 100%)	Firmicute; +ve
6	Tm- Ab06	856 (KM349755)	*Sphingobacterium multivorum* (KF535161; 99%)	Bacteroidetes; −ve
7	Tm- Ab07	951 (KM349756)	*Enterobacter cloacae* (KF971358; 99%)	γ-Proteobacterium; −ve
8	Tm- Ab08	725 (KM349757	*Arthrobacter globiformis* (KJ124593; 99%)	Actinobacterium; −ve
9	Tm- Ab09	750 (KM349758)	*Rhizobium radiobacter* (S000721046; 0.967) #NCBI: *Agrobacterium tumefaciens*	α-Proteobacterium; −ve
Isolates from susceptible cv. Arka Vikas				
1	Tm-Av01	794 (KM349745)	*Pseudomonas oleovorans* (HQ697330; 99%)	γ-Proteobacterium; −ve
2	Tm-Av02	860 (KM349746)	*Stenotrophomonas maltophilia* (KM108534; 99%)	γ-Proteobacterium; −ve
3	Tm-Av03	810 (KM349747)	*Bacillus pumilus* (KC834607; 100%)	Firmicute; +ve
4	Tm-Av04	818 (KM349749)	*Rhizobium radiobacter* (S000721046; 1.0) #NCBI: *Agrobacterium tumefaciens*	α-Proteobacterium; −ve
5	Tm-Av05	662 (KM349750)	*Microbacterium pumilum* (KC213957; 99%)	Actinobacterium; +ve

†*As on 20 August 2014 at sequence submission to NCBI GenBank.*
#*Identity assigned by NCBI GenBank.*

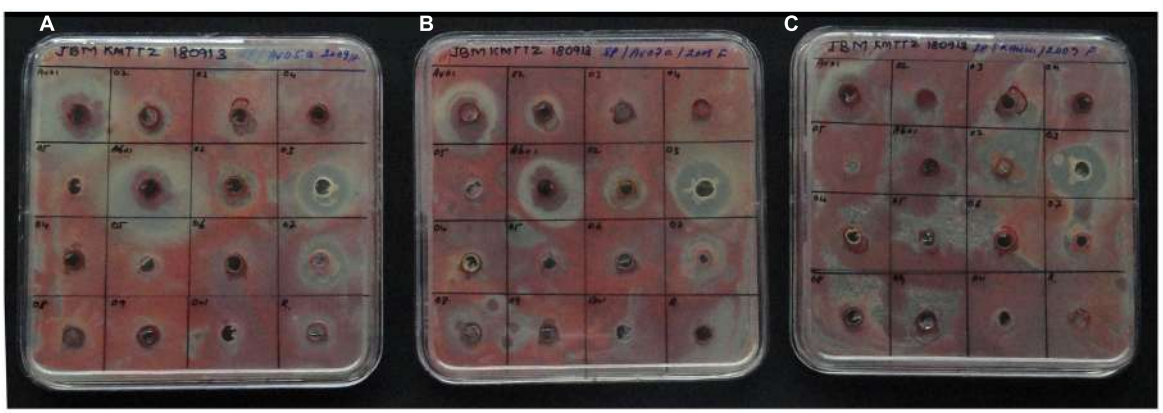

FIGURE 2 | Screening of bacterial endophytes from susceptible cv. Arka Vikas and resistant cv. Arka Abha for the antagonistic activity against *Ralstonia solanacearum* isolates NH-Av05 (A), NH-Av07 (B), and KAU-Av01 (C). Treatment order: Row 1: Tm-Av01 to Av04; Row 2: Tm-Av05, Tm-Ab01 to Ab03; Row 3: Tm-Ab04 to Ab07; Row 4: Tm-Ab08, Ab09, distilled water control, *Ralstonia* inoculum, respectively.

TABLE 2 | Screening of bacterial endophytes from *Ralstonia* resistant Arka Abha and susceptible Arka Vikas tomato cultivars for the indicators of bio-control property.

Isolate	Endophytic organism	Bio-control property indicator				Extent of inhibition zone
		Siderophore	HCN	Antibiotic markers		
				NRPS	PKS	
Isolates from resistant cv. Arka Abha						
Tm-Ab01	*Pseudomonas oleovorans*	×	_	_	_	+++
Tm-Ab02	*Pseudomonas plecoglossicida*	_	_	_	×	++
Tm-Ab03	*Pantoea ananatis*	×	×	_	_	+++
Tm-Ab04	*Citrobacter freundii*	_	_	_	_	-
Tm-Ab05	*Staphylococcus hominis*	_	_	_	_	+
Tm-Ab06	*Sphingobacterium multivorum*	_	_	×	_	+
Tm-Ab07	*Enterobacter cloacae*	_	×	_	_	+++
Tm-Ab08	*Arthrobacter globiformis*	_	_	×	_	++
Tm-Ab09	*Agrobacterium tumefaciens*	_	_	_	_	-
Isolates from susceptible cv. Arka Vikas						
Tm-Av01	*Pseudomonas oleovorans*	×	_	_	_	+++
Tm-Av02	*Stenotrophomonas maltophilia*	_	_	_	_	+
Tm-Av03	*Bacillus pumilus*	_	_	×	_	+
Tm-Av04	*Agrobacterium tumefaciens*	_	_	_	_	-
Tm-Av05	*Microbacterium pumilum*	_	_	_	_	-

_, Negative; × , positive; Antagonistic activity: none (–), low (+), medium (++), or high (+++).

Ashish, and ^{S}Arka Saurabhav). However, ^{MR}Arka Samrat and ^{S}Arka Meghali showed 88 and 50% Gram positive organisms, respectively. The resistant cultivars showed more organisms with antagonistic potential in comparison with susceptible cultivars (**Table 3**) as discussed below.

Endophytes in Resistant and Susceptible Cultivars in Relation to Pathogen Antagonism

When the whole spectrum of root-associated bacterial endophytes in the four resistant and four susceptible cultivars of this investigation is considered, γ-Proteobacteria formed the commonest group followed by Actinobacteria, α-Proteobacteria and spore-forming Firmicutes (**Figure 3A**). The four resistant cultivars together yielded 34 endophytic bacteria which belonged to five classes (Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Flavobacteria), 16 genera and 27 species while the isolates from susceptible cultivars represented three classes (Proteobacteria, Actinobacteria, and Firmicutes) including seven genera and 13 species (**Table 4**). The number of organisms displaying antagonistic activity during agar-well diffusion assay ranged from 4 to 7 in the former group while it was only one or two in the latter. Thus, among the R-cultivar isolates, 23 of them displayed varying levels of antagonistic effects while just seven from the S- category displayed such responses. Further, the extent of antagonistic activity as indicated by the diameter of clear zone was more with the isolates from R sources which included *P. oleovorans, P. ananatis,* and *E. cloacae* from ^{R}Arka Abha, *E. cloacae* and *P. otitidis* from ^{R}Arka Alok, and *E. ludwigii, P. otitidis,* and *Staphylococcus haemolyticus* from ^{R}Arka Ananya. Maximum organisms with the antagonistic activity was observed with the γ-Proteobacteria group constituted by the genera *Enterobacter, Pseudomonas,* and *Pantoea* spp. with 15 out of 17 isolates showing antagonistic effects (**Figure 3B**). The next most promising group included non-spore forming Firmicutes, namely *S. haemolyticus* and *S. hominis* with all three isolates displaying good antagonistic potential.

Discussion

Bacterial endophytes are known to confer protection against pathogens in a number of diseases (Compant et al., 2005; Mercado-Blanco and Lugtenberg, 2014) including *Ralstonia* wilt in tomato (Tan et al., 2011; Feng et al., 2013) and in related solanaceous crops (Ramesh and Phadke, 2012; Achari and Ramesh, 2014). Not many studies have addressed the diversity of endophytes or their possible involvement in offering a natural protection against this pathogen. The present study covering a number of tomato cultivars belonging to the resistant and susceptible categories enunciated the presence of greater cultivable endophytic bacterial diversity and more organisms with pathogen antagonistic potential in resistant cultivars. The isolates with antagonistic potential from resistant cultivars often showed accentuated pathogen inhibitory activity with one exception of Arka Samrat, which belonged to the moderately resistant category (Thomas et al., 2015). These observations suggested the possibility of an active role played by the endophytes in providing a natural protection against the pathogen in resistant cultivars. A recent study in tomato involving just one cultivar each from *Ralstonia* resistant and susceptible categories showed higher endophytic colonization, greater diversity and more pathogen antagonistic

TABLE 3 | Identification of bacterial endophytes from additional resistant and susceptible cultivars and their antagonistic activity against *Ralstonia solanacearum* NH-Av01 determined through agar-well diffusion assay.

Isolate	16S seq (bp) and NCBI acc. no	Identity based on closest species from NCBI/RDP (with acc. no and homology/similarity score)†	Phylogenic group and Gram reaction	Antagonistic effect
Arka Alok (Resistant) 6 × 10^5 cfu g^{-1} (nine isolates)				
Tm-Alk01	910 (KM603626)	*Bacillus megaterium* (KJ789369; 99%)	Firmicute; +ve	+
Tm-Alk02	822 (KM603627)	*Asticcacaulis benevestitus* (S000592821; 0.798)	α-Proteobacteria; −ve	+
Tm-Alk03	850 (KM603628)	*Microbacterium oleivorans* (KF307652; 99%)	Actinobacteria; +ve	-
Tm-Alk04	914 (KM603629)	*Hydrogenophaga intermedia* (FJ009392; 99%)	β-Proteobacteria; −ve	-
Tm-Alk05	892 (KM603630)	*Novosphingobium subterraneum* (FJ527720; 99%) #*Novosphingobium aromaticivorans*	α-Proteobacteria; −ve	+
Tm-Alk06	700 (KM603631)	*Pantoea ananatis* (HE716948; 98%)	γ-Proteobacteria; −ve	+
Tm-Alk07	950 (KM603632)	*Enterobacter cloacae* (KM077045; 99%)	γ-Proteobacteria; −ve	+++
Tm-Alk08	725 (KM603633)	*Pseudomonas taiwanensis* (S001095516; 0.918)	γ-Proteobacteria; −ve	+
Tm-Alk09	575 (KM603634)	*Pseudomonas otitidis* (KF699886; 99%)	γ-Proteobacteria; −ve	++
Arka Ananya (Resistant) 6 × 10^4 cfu g^{-1} (eight isolates)				
Tm-Ana01	750 (KM603635)	*Enterobacter ludwigii* (S000539659; 0.972)	γ-Proteobacteria; −ve	++
Tm-Ana02	925 (KM603636)	*Bacillus megaterium* (KJ789369; 99%)	Firmicute; +ve	-
Tm-Ana03	870 (KM603637)	*Chryseobacterium taiwanense* (KC122691; 99%)	Flavobacteria; −ve	-
Tm-Ana04	900 (KM603638)	*Rhizobium oryzae* (S001168838; 0.846)	α-Proteobacteria; −ve	+
Tm-Ana05	770 (KM603639)	*Staphylococcus hominis* (KJ197177; 99%)	Firmicute; +ve	+
Tm-Ana06	780 (KM603640)	*Pseudomonas otitidis* (LN558646; 99%)	γ-Proteobacteria; −ve	++
Tm-Ana07	900 (KM603641)	*Staphylococcus haemolyticus* (HG941667; 99%)	Firmicute; +ve	+++
Tm-Ana08	720 (KM603642)	*Pseudomonas taiwanensis* (S001095516; 0.918)	γ-Proteobacteria; −ve	+
Arka Samrat (Moderately resistant) 4.7 × 10^3 cfu g^{-1} (eight isolates)				
Tm-Sam01	920 (KM603643)	*Microbacterium lacticum* (S000013457; 0.947)	Actinobacteria; +ve	-
Tm-Sam02	895 (KM603644)	*Bacillus megaterium* (KF381342; 99%)	Firmicute; +ve	+
Tm-Sam03	555 (KM603645)	*Microbacterium pumilum* (LK391536; 99%)	Actinobacteria; +ve	-
Tm-Sam04	890 (KM603646)	*Bacillus safensis* (S000458519; 0.996)	Firmicute; +ve	+
Tm-Sam05	975 (KM603647)	*Bacillus soli* (S000323282; 0.948)	Firmicute; +ve	-+
Tm-Sam06	915 (KM603648)	*Bacillus bataviensis* (S000323277; 0.933)	Firmicute; +ve	-
Tm-Sam07	810 (KM603649)	*Corynebacterium amycolatum* (KF539917; 99%)	Actinobacteria; +ve	-
Tm-Sam 08	850 (KM603650)	*Rhizobium radiobacter* (S000721046; 0.987) #*Agrobacterium tumefaciens*	α-Proteobacteria; −ve	-

(Continued)

TABLE 3 | Continued

Isolate	16S seq (bp) and NCBI acc. no	Identity based on closest species from NCBI/RDP (with acc. no and homology/similarity score)†	Phylogenic group and Gram reaction	Antagonistic effect
Arka Ashish (Susceptible) 1.9 × 10^4 cfu g^{-1} (four isolates)				
Tm-Ash01	550 (KM603651)	*Microbacterium oleivorans* (KF777385; 99%)	Actinobacteria; +ve	-
Tm-Ash02	910 (KM603652)	*Pseudoxanthomonas mexicana* (KF358265; 99%)	γ-Proteobacteria; −ve	+
Tm-Ash03	905 (KM603653)	*Rhizobium pseudoryzae* (S002221791; 0.913)	α-Proteobacteria; −ve	-
Tm-Ash04	930 (KM603654)	*Acidovorax soli* (S001293324; 0.937)	β-Proteobacteria; −ve	-
Arka Meghali (Susceptible) 3.1 × 10^4 cfu g^{-1} (four isolates)				
Tm-Meg 01	968 (KM603655)	*Pseudomonas otitidis* (KF668329; 100%)	γ-Proteobacteria; −ve	+
Tm-Meg 02	690 (KM603656)	*Microbacterium oleivorans* (KF777385; 100%)	Actinobacteria; +ve	-
Tm-Meg 03	908 (KM603657)	*Bacillus megaterium* (S000979521; 0.961)	Firmicutes; +ve	+
Tm-Meg 04	865 (KM603658)	*Asticcacaulis benevestitus* (S000592821; 0.796)	α-Proteobacteria; −ve	-
Arka Saurabhav (Susceptible) 6.5 × 10^4 cfu g^{-1} (four isolates)				
Tm-Sau01	680 (KM603659)	*Microbacterium oleivorans* (KF777385; 100%)	Actinobacteria; +ve	-
Tm-Sau02	795 (KM603659)	*Pseudoxanthomonas mexicana* (KF135463; 99%)	γ-Proteobacteria; −ve	-
Tm-Sau03	905 (KM603661)	*Pseudomonas alcaliphila* (KC699534; 99%)	γ-Proteobacteria; −ve	+
Tm-Sau04	855 (KM603662)	*Acidovorax soli* (S001293324; 0.934)	β-Proteobacteria; −ve	-

†*As on September 2014 at NCBI Submission.*
#*Identity assigned by NCBI GenBank at sequence acceptance.*
Antagonistic activity: low (+), medium (++), or high (+++).

organisms in the former (Feng et al., 2013). Studies with other plant systems have also suggested the prevalence of a similar relationship (Araújo et al., 2002; Reiter et al., 2002). The endophytic communities perhaps are not random guests but essential associates interacting with the hosts (Gaiero et al., 2013; Podolich et al., 2015). It is postulated that the endophytic bacteria, which are largely in non-cultivable form, perhaps play an active role in crop protection through their revival to active form in response to pathogen attack or environmental stress (Podolich et al., 2015).

It was significant to note that several of the endophytes from ^{R}Arka Abha were positive for biocontrol properties compared to ^{S}Arka Vikas. The promising antagonistic organisms *P. oleovorans* and *P. ananatis* were siderophore producers while *E. cloacae* and *P. ananatis* showed HCN production indicating a relationship between antagonistic ability and siderophore/HCN production. On the other hand, no clear relationship between antibiotic (NRPS/PKS) biosynthesis capability and antagonistic property was observed. Therefore, it was imperative to undertake direct pathogen challenge assays to determine the antagonistic potential of the organisms.

Past investigations that reported elucidation of wilt-disease resistance mechanisms against *R. solanacearum* often laid emphasis on tissue-structural (Rahman and Abdullah, 1997; Rahman et al., 1999), genetic (Wang et al., 2000; Yang and Francis, 2006), or molecular attributes (Jacobs et al., 2012; Coll and Valls, 2013). It is generally concluded that the resistance trait of different cultivars is under genetic control. A perusal of reports on genetic basis of *Ralstonia* wilt resistance in tomato, however, showed considerable variations in the inheritance of this trait depending on the test hybrid combinations or the pathogen-isolate employed. This varied from monogenic to digenic dominant or recessive, or polygenic inheritance (Grimault et al., 1995; Mohamed et al., 1997; Hanson et al., 1998). The resistant cultivars have shown considerably low internal colonization by this pathogen than susceptible genotypes (Grimault et al., 1994; Rahman and Abdullah, 1997). The observations documented in this study raise a query whether the bacterial endophytes play either a direct active part or a supportive role in governing the resistance feature of a cultivar synergistic with the current concept of genetic inheritance of resistance.

Generally it is believed that the endophytes are recruited from the soil environment by the host influenced by the soil type where the host genotype is also known to have a significant influence (Compant et al., 2010; Lundberg et al., 2012; Mueller et al., 2015). It is difficult to visualize selective acquisition/recruitment of endophytes to take place from the soil in

TABLE 4 | Extent of diversity of endophytic bacteria in *Ralstonia* resistant and susceptible cultivars of tomato in relation to pathogen antagonistic effect.

S. no	Phylogenetic group	Resistant cultivars				Susceptible cultivars			
		Arka Abha	Arka Alok	Arka Ananya	Arka Samrat†	Arka Vikas	Arka Ashih	Arka Meghali	Arka Saurabhav
	α-Proteobacteria								
1	*Asticcacaulis benevestitus*		•/+					•/−	
2	*Agrobacterium tumefaciens*	•/−			•/−	•/−			
3	*Rhizobium oryzae*			•/−					
4	*Rhizobium pseudoryzae*						•/+		
5	*Novosphingobium aromaticivorans*		•/+						
	β-Proteobacteria								
6	*Acidovorax soli*						•/−		•/−
7	*Hydrogenophaga intermedia*		•/−						
	γ-Proteobacteria								
8	*Enterobacter cloacae*	•/+++	•/+++						
9	*Enterobacter ludwigii*			•/++					
10	*Pseudomonas alcaliphila*								•/+
11	*Pseudomonas oleovorans*	•/+++				•/+++			
12	*Pseudomonas otitidis*		•/++	•/++				•/+	
13	*Pseudomonas plecoglossicida*	•/+							
14	*Pseudomonas taiwanensis*		•/+	•/+					
15	*Pseudoxanthomonas mexicana*						•/−		•/−
16	*Pantoea ananatis*	•/+++	•/+						
17	*Stenotrophomonas maltophilia*					•/+			
	Bacteroidetes								
18	*Sphingobacterium multivorum*	•/+							
	Flavobacteria								
19	*Chryseobacterium taiwanense*			•/−					
	Actinobacteria								
20	*Arthrobacter globiformis*	•/+							
21	*Citrobacter freundii*	•/−							
22	*Corynebacterium amycolatum*				•/+				
23	*Microbacterium lacticum*				•/−				
24	*Microbacterium oleivorans*		•/−				•/−	•/−	•/−
25	*Microbacterium pumilum*				•/−	•/−			
	Firmicutes – non-sporulating								
26	*Staphylococcus haemolyticus*			•/+++					
27	*Staphylococcus hominis*	•/+		•/+					
	Firmicutes – sporulating								
28	*Bacillus bataviensis*				•/−				
29	*Bacillus pumilus*					•/+			
30	*Bacillus safensis*				•/+				
31	*Bacillus megaterium*		•/+	•/−	•/+			•/+	
32	*Bacillus soli*		•/+	•/−	•/+			•/+	
	Isolates showing antagonistic effect/Total	7/9	7/9	5/8	4/8	3/5	1/4	2/4	1/4

•, *Presence in the cultivar; –, no antagonistic activity; +, ++, +++: low, medium, or strong Ralstonia solanacearum antagonistic activity, respectively.*
†*Moderately resistant.*

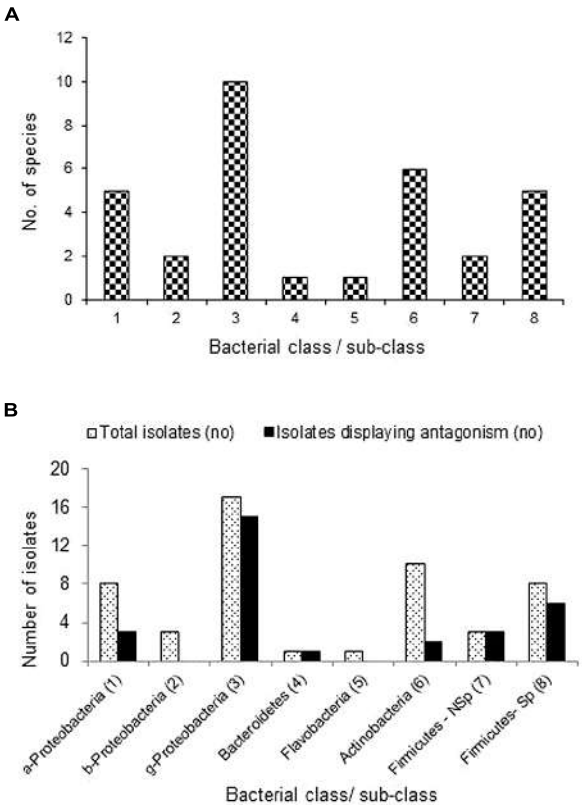

FIGURE 3 | Extent of cultivable bacterial endophytes belonging to different bacterial classes and sub-classes across four *Ralstonia* resistant and four susceptible tomato cultivars (A), and the extent of organisms belonging to different phylogenetic groups displaying antagonistic activity against the pathogen (B); a-, b-, g-Proteobacteria represents α, β, and γ sub-classes; Firmicutes-Nsp and Sp represent non-sporulating and spore-forming Firmicutes.

a resistant cultivar. The present study in which the seedlings were grown in pasteurized cocopeat ensured to be devoid of pathogenic *Ralstonia* leaves no room for such selective recruitment. The host genotype is known to play a significant role in governing the plant associated microorganisms, particularly endophytes (Hartmann et al., 2009; Lundberg et al., 2012; Bakker et al., 2013; Podolich et al., 2015). There are also reports on transmission of endophytes through seeds (Hardoim et al., 2012; Truyens et al., 2014) which would explain their possible integral association with a particular host cultivar. This study, supported by the recent reports on intracellular colonization by bacterial endophytes (Thomas and Reddy, 2013; Thomas and Sekhar, 2014), suggests the possibility of maternal inheritance of endophytes as seed colonizers. This hypothesis necessitates the isolation of same organisms from different batches of a cultivar. A subsequent trial with ^{S}Arka Vikas showed three of the five isolates same as the earlier set (*P. oleovorans, A. tumefaciens,* and *Microbacterium* sp.) while two isolates constituted different organisms (*Mitsuaria chitosanitabida* and *Kocuria palustris*) indicating vertical transmission as well as lateral recruitment of bacterial endophytes. Three repeat trials with ^{R}Arka Abha showed antagonistic *P. oleovorans* as a common associate. The current opinion on seed-transmission of endophytes appears divided with some in favor while others remaining inconclusive (Hallmann, 2001; Hardoim et al., 2012; Truyens et al., 2014). It now calls for more detailed investigations on seed colonization and vertical transmission of endophytes *vis-à-vis* genetic control of disease resistance. Observations with aseptically grown watermelon (Thomas and Aswath, 2014) and preliminary observations with papaya *in vitro* systems (Thomas, unpublished data) endorsed this possibility.

In this study, our main objective was to understand if the native endophytes in different tomato genotypes had any bearing on the inherent resistance characteristic of a cultivar. This study was confined to the natural endophytes without any external fortifications. It needs further investigations to elucidate how the organisms protect the crop in natural conditions; whether they act singly or synergistically, and their interactive action with other rhizospheric organisms. For instance, *P. oleovorans* constituted the most common endophyte in Arka Vikas, but this cultivar was susceptible to the pathogen (Thomas et al., 2015). It is possible that the population level of this antagonist in ^{S}Arka Vikas was low to offer any formidable protection against the pathogenic intruder. It is feasible to increase the population levels of this endophyte through seed/seedling fortification which perhaps may impart some pathogen resistance in this cultivar. There is a general criticism that the *in vitro* antagonism activity by the endophytes may not be translated into effective biocontrol strategies. Our preliminary trials also suggested that exploiting antagonistic agents as potential biocontrol agents has uncertain results. The biocontrol effects are influenced by various other factors. The significance of microbe–microbe interactions in antimicrobial activity among soil bacteria is being increasingly recognized now (Tyc et al., 2014). Therefore, additional trials are needed to work out the biocontrol strategy which forms the next action plan.

In this study, the identification of the organisms was determined based on 16S rRNA sequence homology to the sequences available at the NCBI GenBank and RDP databases, and the final identity was fixed as per the genus/species assigned by the GenBank. The identification of some of the organisms based on such single gene data may not be conclusive as demonstrated with *Pseudomonas* spp. (Hilario et al., 2004). Classification based on additional genes is envisaged as we proceed with the biocontrol studies in the case of promising organisms.

The isolates from ^{R}Arka Abha (*P. oleovorans, P. ananatis,* and *E. cloacae*) which showed strong antagonistic activity and that from ^{S}Arka Vikas (*P. oleovorans*) are now short listed for further biocontrol investigations. The two isolates of *P. oleovorans* (Tm-Av01 and Tm-Ab01) and one *A. tumefaciens* isolate (Tm-Ab09) also showed higher seedling vigor index over uninoculated control in both tomato cultivars offering scope for their exploitation in organic vegetable growing (Thomas and Upreti, 2015). The hallmark of this study has been the elucidation that the native endophytic bacterial floras associated with the seedlings in resistant cultivars perhaps play a role in natural defense against the pathogen which hypothesis goes synergistic with the current concept of genetic inheritance of disease resistance. The present

findings contribute to a better understanding of the basic aspects related to host - pathogen - endophyte interactions and open the scope for further explorations into the biological control of this pathogen.

Author Contributions

The experiments were planned together by the two authors. Bacterial isolation, PCR, and antagonistic assays were undertaken by RU. Bacterial identification, data interpretation, and manuscript preparation were done by PT. This work forms a part of the doctoral thesis of RU. The publication bears the Institute Contribution No. IIHR 92/2014.

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Colonization of lettuce rhizosphere and roots by tagged *Streptomyces*

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Beneficial microorganisms are increasingly used in agriculture, but their efficacy often fails due to limited knowledge of their interactions with plants and other microorganisms present in rhizosphere. We studied spatio-temporal colonization dynamics of lettuce roots and rhizosphere by genetically modified *Streptomyces* spp. Five *Streptomyces* strains, strongly inhibiting *in vitro* the major soil-borne pathogen of horticultural crops, *Sclerotinia sclerotiorum*, were transformed with pIJ8641 plasmid harboring an enhanced green fluorescent protein marker and resistance to apramycin. The fitness of transformants was compared to the wild-type strains and all of them grew and sporulated at similar rates and retained the production of enzymes and selected secondary metabolites as well as *in vitro* inhibition of *S. sclerotiorum*. The tagged ZEA17I strain was selected to study the dynamics of lettuce roots and rhizosphere colonization in non-sterile growth substrate. The transformed strain was able to colonize soil, developing roots, and rhizosphere. When the strain was inoculated directly on the growth substrate, significantly more t-ZEA17I was re-isolated both from the rhizosphere and the roots when compared to the amount obtained after seed coating. The re-isolation from the rhizosphere and the inner tissues of surface-sterilized lettuce roots demonstrated that t-ZEA17I is both rhizospheric and endophytic.

Keywords: biocontrol, *Lactuca sativa*, *Sclerotinia sclerotiorum*, streptomycetes, rhizosphere competence

INTRODUCTION

Roots anchor plants in soil, provide uptake of water and nutrients, and mediate numerous interactions with soil organisms. The interface between roots and soil – where most of these interactions take place – is called rhizosphere. This narrow and specific zone is distinct from bulk soil in terms of nutrient availability, pH and presence of a wide variety of microorganisms and invertebrates attracted and influenced by root exudates and rhizodeposits (Hinsinger et al., 2009; Compant et al., 2010; Philippot et al., 2013). Many microbes present in rhizosphere have neutral effect on plants, while others positively or negatively affect host development and health via complex interactions, which we are only beginning to understand (Raaijmakers et al., 2009; Compant et al., 2010; Glick, 2012). Some microorganisms are deleterious as they compete with plants for nutrients or cause disease (soil borne plant pathogens), while others support their hosts by mobilizing nutrients, stimulating growth, and increasing yield or reducing biotic and abiotic stresses, such as mycorrhizal fungi and plant growth promoting bacteria (PGPB; Compant et al., 2010; Aeron et al., 2011; Smith and Smith, 2011).

Plant growth promoting bacteria are gaining more and more attention in modern agriculture, where sustainable and environmentally friendly strategies of crop cultivation increasingly rely on their use as biofertilizers, phytostimulants, or biopesticides. They employ several mechanisms to improve the plant growth, such as synthesis of phytohormones, nitrogen fixation and increasing availability of nutrients by production of siderophores and solubilization of phosphates (Lugtenberg et al., 2002; Compant et al., 2010). Furthermore, special attention is dedicated to biological control agents (BCAs), a group of microorganisms producing a wide variety of biologically active molecules potentially able to inhibit plant pathogens. Antagonism is one of the most common modes of action; here the BCA inhibits or kills pathogens via production of diffusible or volatile antimicrobial compounds and cell wall degrading enzymes. Antagonism is widespread in *Bacillus*, *Pseudomonas*, and *Streptomyces* spp., from which a wide range of biologically active secondary metabolites were isolated (Raaijmakers et al., 2002; Compant et al., 2005).

Despite the optimal performance at laboratory-scale screening tests, PGPB often fail to demonstrate their potential or show inconsistent results in greenhouse and field trials. This variable performance may have different causes, such as reduced or delayed expression of bioactive molecules in the presence of competing microorganisms or lower rhizosphere competence, i.e., poor colonization of root tissues and rhizosphere of the host plant (Lugtenberg et al., 2001; Compant et al., 2010; Ghirardi et al., 2012). To overcome these obstacles, it is essential to understand how PGPB interact with the host plant and with other microorganisms present in soil. Several studies have demonstrated better plant protection when bioactive *Pseudomonas* spp. strains with improved rhizosphere-competence were used (Ghirardi et al., 2012). In recent years, several characters essential for rhizosphere colonization were identified in *Pseudomonas*

spp. (Latour et al., 2003; Lugtenberg and Kamilova, 2009), however, similar studies are missing for other beneficial genera of bacteria.

One of such genera is *Streptomyces*, filamentous Gram-positive bacteria commonly inhabiting soil and rhizosphere and renowned for the production of a variety of bioactive secondary metabolites (Loria et al., 2006; Hopwood, 2007). They have been largely exploited in pharmaceutical industry since 1940s (Watve et al., 2001; Lucas et al., 2013), whereas only a few have been developed as commercial products for plant application in agriculture (Yuan and Crawford, 1995; Minuto et al., 2006; Berg, 2009). Streptomycetes have been long considered simply as free-living soil inhabitants, but recently the importance of their complex interactions with plants and other organisms is being uncovered (Seipke et al., 2012). Some of them, such as *S. scabies* or *S. turgidiscabies,* are plant pathogens with broad host range, causing important economic losses especially on tap root and tuber crops, such as potatoes, sweet potatoes, carrots, or beet (Loria et al., 2006; Seipke et al., 2012). On the contrary, many others establish beneficial relationships with host plants as endophytes (Sardi et al., 1992; Coombs and Franco, 2003; Cao et al., 2004). Auxin production was described for endophytic and free living *Streptomyces* in rhizosphere (Coombs et al., 2004; Khamna et al., 2009), while *S. lydicus* augmented the nodulation by *Rhizobium* species in pea plants, increasing iron and molybdenum assimilation as well as root growth (Tokala et al., 2002; Seipke et al., 2012).

Several markers have been developed and adopted to study localization and quantification of PGPB in the rhizosphere; among these, antibiotic resistance has been widely used (Prosser, 1994; Gamalero et al., 2003). Because many of soil microorganisms produce a variety of different antibiotics, it is necessary to determine the specificity of the antibiotic marker selected for the identification of PGPB before its use. Currently, fluorescent markers are gaining increasing popularity for colonization studies (Lu et al., 2004; Cao et al., 2011; Krzyzanowska et al., 2012). Various derivatives of green fluorescent protein (GFP) have been engineered to increase the fluorescence and to overcome the variable expression of the original marker in different species (Errampalli et al., 1999; Gamalero et al., 2003). Enhanced GFP (EGFP) contains numerous silent nucleotide changes in comparison to GFP to maximize its expression in mammalian cells (Haas et al., 1996), and was adopted for use in *Streptomyces* spp., which have a similar codon usage (Sun et al., 1999).

Green fluorescent protein has been utilized to study PGPB colonization of roots and rhizosphere in sterile conditions (Coombs and Franco, 2003; Weyens et al., 2012). These studies provide a basic understanding of the interactions between PGPB and the host plant, but they do not consider the complex interactions *in vivo*. In non-sterile conditions with high microbial diversity, PGPB have to compete with other microorganisms present in the rhizosphere, and in some cases the competition reduced the colonization ability of PGPB (Cao et al., 2011; Hohmann et al., 2012; Weyens et al., 2012). Moreover, the activity and the fitness of the transformed strain need to be controlled following the transformation, as it has been observed that the presence of the transgene may interfere with the biological activity of the studied organism (Nigro et al., 1999; Lübeck et al., 2002; Weyens et al., 2012).

The objective of this work was to get insight into the localization and colonization of a genetically modified *Streptomyces* strain, selected as potential BCA, in lettuce roots and rhizosphere. First, we compared the fitness of the transformed and the corresponding wild-type strains, then we studied the colonization dynamics of the most promising transformed strain in rhizosphere and roots of lettuce plants in non-sterile growth substrate. Finally, we compared the effect of two inoculation methods on the ability of the *Streptomyces* strain to differentially colonize rhizosphere and roots.

MATERIALS AND METHODS

TRANSFORMATION OF *Streptomyces* spp.

Five *Streptomyces* strains, potential BCAs against *Sclerotinia sclerotiorum*, were used in this study: CX14W, CX16W, FT05W, SW06W, and ZEA17I. They were maintained at the Plant Pathology Laboratory, Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, and selected previously from a wide collection of actinomycetes isolated from roots of different plants (Sardi et al., 1992; Petrolini et al., 1996; Bonaldi et al., 2011, 2014). *Escherichia coli* strain ET12567 (harboring the helper plasmid pUZ8002), was provided by prof. Flavia Marinelli, University of Insubria, Italy, and was used as donor strain for conjugation. Plasmid pIJ8641, obtained from prof. Mervyn Bibb, John Innes Centre, UK, was maintained in *E. coli* strain DH5α. It carries the EGFP gene under the constitutive *ermE* promoter, an apramycin resistance marker [*aac(3)IV*], an *oriT*/RK2 region, and a lambda phage chromosomal integration sequence (IntC31; Sun et al., 1999). The strain *S. coelicolor* A3(2) was obtained from F. Marinelli, and used as a reference strain to evaluate transformation efficiency.

Plasmid pIJ8641 was transformed into the donor strain *E. coli* ET12567 (pUZ8002) by rubidium chloride method (Hanahan, 1983) and conjugated into recipient *Streptomyces* strains as previously described (Kieser et al., 2000). Prior to conjugation, the concentration of the *E. coli* donor strain ET12567 containing plasmid pIJ8641 was adjusted to 1×10^7 CFU/mL. The ex-conjugants were selected on the basis of apramycin resistance. The conjugation efficiency was calculated as number of ex-conjugant colonies per number of recipient spores.

Genomic DNA of wild-type and transformed (t-) *Streptomyces* strains was extracted by the CTAB method (Kieser et al., 2000). The amplification of 16S rDNA fragment (expected size 1500 bp) was used to evaluate the quality of DNA in all samples, using PCR primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and fD2 (5′-ACGGCTACCTTGTTACGACTT-3′; Weisburg et al., 1991), and the following thermal cycling conditions: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 92°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, a final extension at 72°C for 5 min. PCR primers rEGFP-N (5′-CTGGTCGAGCTGGACGGCGACG-3′) and rEGFP-C (5′-CACGAACTCCAGCAGGACCA TG-3′) were designed to amplify the EGFP gene fragment (expected fragment size 700 bp), using the following thermal cycling conditions: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 92°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 2 min, a final

extension at 72°C for 2 min. The DNA amplification was carried out using PCR thermal cycler (BioRad, USA), performed in a total volume of 25 μL containing 30–50 ng DNA, 0.25 μM each primer, 1 U/μL Go *Taq* DNA polymerase (Promega, USA), 5 μL of 5x Go Taq buffer (Promega, USA), and 0.2 mM of each dNTP. The PCR products were visualized under Gel Doc transilluminator (BioRad, USA) following electrophoresis in 1%(w/v) agarose gel.

For microscopic observations, the transformed *Streptomyces* strains were inoculated on Czapek yeast extract agar (CZY; 35 g/L Czapek-Dox Broth Difco, 2 g/L Yeast Extract Difco, 15 g/L agar). A microscopic cover slide was partially inserted in the medium at the edge of the inoculated strain under the 45° angle to allow the strain to grow on the cover slide. The plates were incubated at 24°C for 5 days. Subsequently, cover slides were removed from the medium and observed by brightfield and epifluorescence microcopy using Olympus BX51 with the FITC filter set (467–498 nm excitation and 513–556 nm emission) to confirm the expression of EGFP in transformants.

INHIBITION OF *Sclerotinia sclerotiorum* GROWTH *IN VITRO*

The antagonistic activity of wild-type and transformed (t-) *Streptomyces* strains against *S. sclerotiorum* was determined by dual culture assay on CZY agar as described (Bonaldi et al., 2014). Briefly, 10 μL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated on a 40 mm line two days prior to *S. sclerotiorum* inoculation. An agar-mycelium plug (5 mm diameter), obtained from the edge of an actively growing colony of *S. sclerotiorum* grown on Malt Extract Agar (MEA; 20 g/L Malt Extract, Difco, and 15 g/L agar), was placed at 25 mm distance from the growing *Streptomyces* colony and the plates were incubated for 72 h at 24°C. Plates inoculated with *S. sclerotiorum* only were used as a control. The antagonistic activity was determined by calculating the percentage of growth inhibition of *S. sclerotiorum* compared to the control. The experiment was repeated twice in three replicates.

MYCELIUM GROWTH AND SPORULATION

The mycelium growth curve of transformed and wild-type strains was determined daily as follows: 20 μL of *Streptomyces* spore suspension (1×10^7 CFU/mL) were transferred into a 50 mL tube containing 20 mL of CZY broth, and incubated at 30°C with 200 rpm constant shaking for 8 days. Each day, 2 mL of liquid culture were removed and spun at 10600 *g* for 10 min and the dry weight of the pellet was repeated twice in three replicates and expressed in g/L.

The sporulation of the strains was measured by plating 1 mL of spore suspension (1×10^7 CFU/mL) on a CZY agar plate and determining the number of spores produced after 6 days of incubation at 30°C (Grantcharova et al., 2005). Five mL of sterile water were added to the Petri plate and the surface of colonies was gently scraped to release the newly formed spores (Kieser et al., 2000). The spore suspension was filtrated through two layers of sterile gauze and the spore concentration (CFU/mL) was quantified by plating serial dilutions of the spore suspension and counting the number of colonies grown after 4 days of incubation at 30°C. The experiment was repeated twice in three replicates.

PRODUCTION OF SECONDARY METABOLITES

Siderophore production

Ten milliliter of Fe-free Czapek solution (300 g/L $NaNO_3$, 50 g/L KCl, 50 g/L $MgSO_4 \cdot 7H_2O$) were mixed with 15 g/L agar, 30 g/L sucrose, 1 g/L K_2HPO_4, and 5 g/L yeast extract to prepare the Fe-free Czapek agar medium. Ten microliter of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of a Fe-free Czapek agar plate and incubated at 30°C for two weeks. Subsequently, the *Streptomyces* colony was overlaid by 15 mL of the Chrome azurol S (CAS) agar (Schwyn and Neilands, 1987; Perez-Miranda et al., 2007). The siderophore production was determined as color change in the overlay medium (from blue to orange) after 24 h of incubation at room temperature. The experiment was repeated twice in three replicates.

Chitinase production

The colloidal chitin and the colloidal chitin agar were prepared as described previously (Saima et al., 2013). Ten microliter of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of colloidal chitin agar plate (chitin as single carbon sources) as a 40 mm line and incubated at 30°C for 10 days. The production of chitinase was determined based on the presence of a clear hydrolysis zone on the agar plate below the colony. The experiment was repeated twice in three replicates.

Phosphate solubilization

The phosphate solubilization activity of the *Streptomyces* strains was assessed using a plate assay with National Botanical Research Institute's Phosphate (NBRIP) medium (Nautiyal, 1999), in which $Ca_3(PO_4)_2$ is the only phosphate source. Ten microliter of *Streptomyces* spore suspension (1×10^7 CFU/mL) were inoculated in the center of a NBRIP-medium Petri plate and incubated at 30°C for 2 weeks. The phosphate solubilization was determined based on the presence of a clear hydrolysis zone on the agar plate below the colony. The test was repeated twice in three replicates.

Indole-3-acetic acid (IAA) synthesis

The IAA production was determined as described previously (Bric et al., 1991; Bano and Musarrat, 2003). In brief, 10 μL of *Streptomyces* spore suspension (1×10^7 CFU/ml) were incubated with constant shaking at 5 *g* in 5 mL CZY broth added with 500 μg/mL tryptophan (Sigma, USA) in the dark at 30°C for 10 days. Two mL of the liquid culture were centrifuged for 10 min at 18000 *g*. One mL of the supernatant was mixed with 50 μL 10 mM orthophosphoric acid and 2 mL of Salkowski reagent (1 mL of 0.5M $FeCl_3$ in 50 mL of 35% $HClO_4$). The tubes were incubated at room temperature for 30 min. The development of pink color indicated the IAA production, which was quantified by spectrophotometer at 530 nm. The experiment was repeated twice in three replicates.

SOIL, ROOT, AND RHIZOSPHERE COLONIZATION BY t-ZEA17I

The transformed ZEA17I strain (t-ZEA17I) was grown on CZY medium containing 50 mg/L of apramycin at 24°C for 10 days. Spores were collected in 10% sterile glycerol and filtered through two layers of gauze. The concentration was determined and the spore suspension was stored at −20°C.

Bulk soil colonization

Prior to colonization studies, the presence of naturally occurring apramycin-resistant streptomycetes in non-sterilized Irish and Baltic peat-based growth substrate (Vigorplant, Italy) was assessed. A portion of the substrate was resuspended in sterile water and plated on water agar medium (WA) containing 15 g/L agar, 25 mg/L nalidixic adic, 50 mg/L apramycin, 50 mg/L nystatin, and 50 mg/L cycloheximide. Plates were incubated for 7 days at 24°C and the presence of apramycin-resistant streptomycete colonies was visually checked.

The growth substrate was placed in a polystyrene seed tray (48 cm^3/cell) and watered with tap water. In every cell, t-ZEA17I was uniformly distributed on the top of the substrate adding 1 mL spore suspension (1×10^7 CFU/mL). The growth substrate was incubated in a growth chamber (24°C, 55% relative humidity and 15 h photoperiod) and watered every 2–3 days with tap water. t-ZEA17I was re-isolated 4 h (day 0), 10, 20, and 30 days after inoculation (dai) in six replicates. The entire amount of growth substrate in the cell was collected and weighed. The substrate was mixed to homogenize the inoculum and divided in two identical parts. One part was incubated in the oven at 50°C and the dry weight was determined. The other part was stirred in sterilized water (1:10 substrate fresh w/v) for one hour and serial dilutions were plated on WA. Plates were incubated for 7 days at 24°C and streptomycete colonies were counted. The t-ZEA17I concentration was expressed as CFU/g of growth substrate dry weight.

Plant inoculation

Ice queen lettuce seedlings (*Lactuca sativa* var. *capitata*, Iceberg group, Semeurop, Italy) were grown in polystyrene seed trays, as described previously. Seeds were surface sterilized in 0.7% sodium hypochlorite for 5 min and rinsed three times in sterile water. Two methods were used to inoculate the t-ZEA17I strain. In the growth substrate inoculation method, 1 mL spore suspension (1×10^7 CFU/mL) was uniformly distributed in every cell on the top of the growth substrate. In the seed coating method, 50 seeds were soaked in 1 mL of t-ZEA17I spore suspension (1×10^7 CFU/mL) and left to dry under the laminar flow hood. One seed for each cell of the tray was sown and the seedlings were incubated and watered as described previously.

To determine the inoculum load t-ZEA17I was re-isolated from seeds and growth substrate after inoculum application. In case of the growth substrate inoculation method, the t-ZEA17I strain was re-isolated four hours after soil inoculation as described above for bulk soil, and its amount was expresses as CFU/g of growth substrate dry weight. For the seed coating method, 10 randomly collected seeds were incubated for 30 min in 1 mL of sterile 0.9% NaCl and serial dilutions were plated on WA medium in six replicates. Following incubation at 24°C for 7 days the t-ZEA17I colonies were counted and the amount was expressed first as CFU/seed and then recalculated as CFU/g of growth substrate dry weight.

t-ZEA17I re-isolation from rhizosphere and root tissues

The t-ZEA17I strain was re-isolated 10, 20, and 30 days after sowing from rhizosphere and root tissues of six lettuce seedlings, equal to number of replicates. Seedlings with root system were carefully taken off the cell and the bulk soil was removed by gently shaking the plants (Bulgarelli et al., 2012).

For the rhizosphere analysis, each seedling was cut at base and the roots were vortexed two times for 15 s in 1–3 mL (volume varying according to period of sampling) of sterilized 0.9% NaCl and 0.02% Silwet L-77 (washing solution). The roots were removed and the suspension was filtered through a 300 μm nylon mesh to obtain the rhizosphere soil and its dry weight was determined. The suspension was centrifuged at 10600 *g* for 10 min and the pellet was resuspended in 0.5–1.5 mL of washing solution and plated in serial dilutions on WA medium. The plates were incubated at 24°C for 7 days. The t-ZEA17I colonies were counted and the concentration was expressed as CFU/g of rhizosphere dry weight.

For inner root tissues analysis, the roots were surface sterilized with propylene oxide for one hour (Sardi et al., 1992). Then, they were washed in washing solution and 1/10 of the total volume was plated on WA medium to verify the absence of contaminants. Subsequently the roots were finely homogenized in 1–3 mL washing solution, let to macerate for one hour and the suspension was plated in serial dilutions. The t-ZEA17I concentration was determined as described before and expressed as CFU/g of roots dry weight.

STATISTICAL ANALYSES

All analyses were done using R software, version R3.0.2. (R Core Team, 2013). The statistical differences between data of transformed and wild-type strains in *S. sclerotiorum* growth inhibition, sporulation, and IAA production were compared by a Student's *t*-test ($P = 0.05$). The percent data were arcsine root-squared transformed. The soil, root, and rhizosphere colonization data were submitted to ANOVA, followed by a Tukey *post hoc* test for multiple comparison ($P = 0.05$), using the TukeyC package (Faria et al., 2013).

RESULTS

TRANSFORMATION OF *Streptomyces* spp. WITH PLASMID pIJ8641

All six strains, including *S. coelicolor* A3(2) were transformed with the pIJ8641 plasmid harboring the EGFP gene under a constitutive promoter and apramycin resistance. Conjugation efficiency varied among strains: four strains, CX14W, SW06W, CX16W, and FT05W showed conjugation efficiency similar to *S. coelicolor* A3(2), while one strain, ZEA17I conjugated with lower efficiency (**Table 1**). The EGFP gene was detected in transformed strains (data not shown), and its expression was confirmed by fluorescence microscopy observing a strong green fluorescence in all transformants following exposition to fluorescent light (**Figure 1**), while the corresponding wild-type strains did not fluoresce.

EFFECT OF THE TRANSFORMATION ON STRAIN FITNESS

Following the transformation, the fitness of transformants was evaluated in terms of mycelium growth and sporulation, inhibition of *S. sclerotiorum* mycelium growth, and production of selected secondary metabolites. All the transformed strains showed similar mycelium growth and sporulation to their corresponding wild-type strains (**Figure 2** – for simplicity, the growth

Table 1 | Conjugation efficiencies of five *Streptomyces* spp. strains and *S. coelicolor* A3(2) with the pIJ8641 plasmid.

Strain	Recipient strain (CFU/mL)	Conjugation efficiency (CFU/recipient strain)
S. coelicolor A3(2)	1×10^{8}	9.10×10^{-6}
CX14W	1×10^{8}	4.64×10^{-5}
CX16W	1×10^{10}	6.88×10^{-6}
FT05W	1×10^{9}	1.60×10^{-6}
SW06W	1×10^{8}	3.13×10^{-5}
ZEA17I	1×10^{9}	5.81×10^{-8}

curves for only two strains were reported, **Table 2**). The transformants inhibited *S. sclerotiorum* radial growth from 66 to 81%, and no significant differences were observed between wild-type and transformed strains. Finally, no significant differences were detected in siderophore, auxin, chitinase production, and phosphate solubilization (**Tables 2** and **3**).

COLONIZATION DYNAMICS OF THE TRANSFORMED ZEA17I IN BULK SOIL

In this study, apramycin was used as a marker to identify the transformed t-ZEA17I strain. However, to be able to use it in greenhouse experiments, we first checked for the presence of naturally occurring apramycin-resistant *Streptomyces* spp. in the growth substrate, but none were detected. Therefore non-sterilized growth substrate was used in following experiments.

The colonization dynamics of t-ZEA17I in bulk soil showed that the initial inoculum, 1.16×10^{8} CFU/g dry weight added to the soil, was recovered at 3.85×10^{7} CFU/g dry weight four hours after inoculation (0 dai). The t-ZEA17I amount decreased significantly within the first 10 days and thereafter it remained stable up to 30 days at 2.18×10^{4} CFU/g dry weight (**Table 4**).

COLONIZATION OF LETTUCE RHIZOSPHERE AND INNER ROOT TISSUES BY THE TRANSFORMED ZEA17I

The *Streptomyces* strain t-ZEA17I was inoculated with two different methods: as a spore suspension distributed on soil surface and as seed coating. The colonization dynamics of rhizosphere and

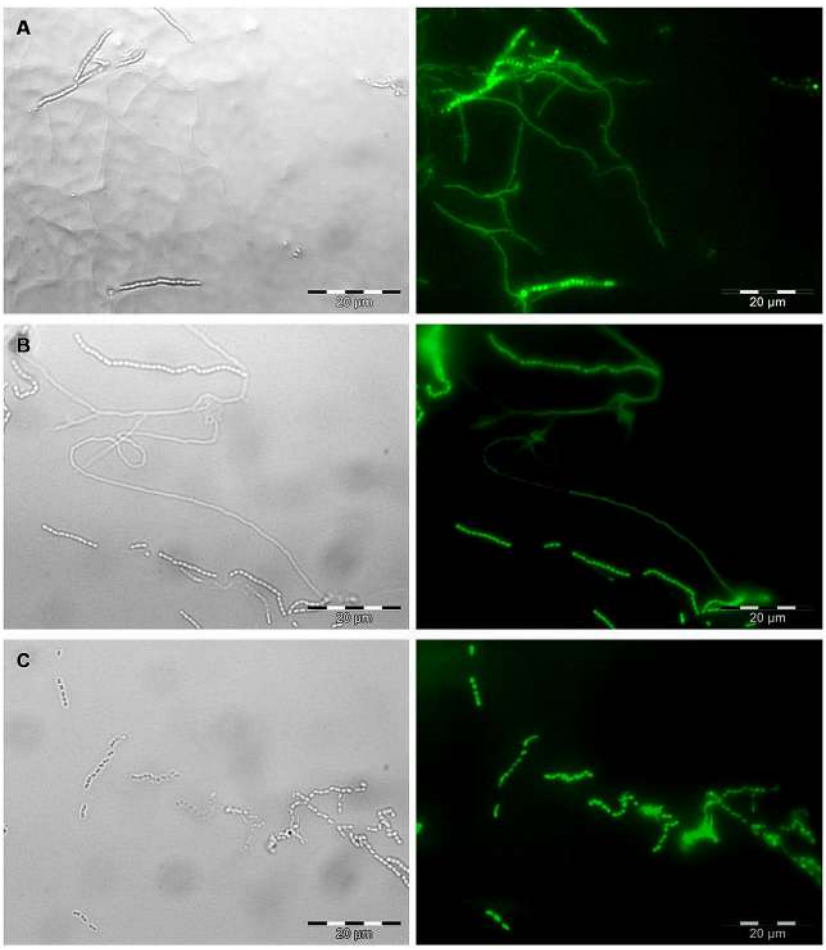

FIGURE 1 | Microscopic observation of EGFP expression in transformed *Streptomyces* strains by bright field (left) and epifluorescence microscopy (right). Mycelium and spore chains of **(A)** the strain t-ZEA17I, **(B)** the strain t-FT05W, and **(C)** spore chains of the strain t-CX16W. Scale bar, 20 µm.

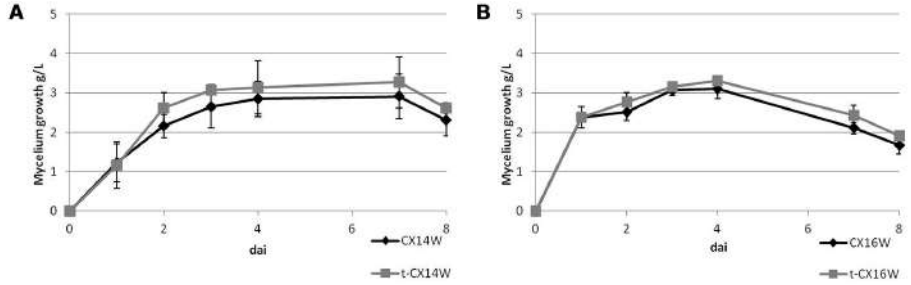

FIGURE 2 | Mycelium growth of the wild-type and transformed *Streptomyces* spp. strains, (A) CX14W and (B) CX16W. Vertical bars represent SE ($N = 3$).

Table 2 | Sporulation, auxin production, and inhibition of *Sclerotinia sclerotiorum* mycelium growth of the wild-type and transformed (t-) *Streptomyces* spp.

Strain	Sporulation (10^9 CFU/mL)		Auxin production (μg/mL)		*S. sclerotiorum* inhibition (%)	
CX14W	7.47 ± 3.22[1]	$P^2 = 0.229$	5.82 ± 0.44	$P = 0.278$	78.80 ± 1.70	$P = 0.072$
t-CX14W	6.80 ± 4.91		5.11 ± 0.35		73.00 ± 2.28	
CX16W	4.23 ± 6.58	$P = 0.178$	5.87 ± 0.86	$P = 0.872$	68.99 ± 0.98	$P = 0.091$
t-CX16W	5.55 ± 1.61		6.03 ± 0.24		65.87 ± 1.33	
FT05W	1.45 ± 3.69	$P = 0.242$	6.04 ± 0.60	$P = 0.207$	67.59 ± 0.68	$P = 0.684$
t-FT05W	1.37 ± 4.56		7.11 ± 0.21		67.13 ± 0.85	
SW06W	2.27 ± 1.15	$P = 0.205$	7.32 ± 0.66	$P = 0.588$	80.21 ± 1.34	$P = 1.000$
t-SW06W	0.78 ± 1.67		6.88 ± 0.28		80.21 ± 1.34	
ZEA17I	0.47 ± 0.23	$P = 0.417$	7.56 ± 0.59	$P = 0.690$	74.80 ± 1.76	$P = 0.069$
t-ZEA17I	1.63 ± 1.15		6.84 ± 0.38		80.75 ± 1.51	

[1] *Mean value followed by SE.* [2] *P-values of the Student's t-test pairwise comparisons.*

Table 3 | Phosphate solubilization, chitinase, and siderophore production of the wild-type and transformed (t-) *Streptomyces* spp.

Strain	Phosphate solubilization	Chitinase production	Siderophore production
CX14W	+[1]	+	+
t-CX14W	+	+	+
CX16W	+	+	+
t-CX16W	+	+	+
FT05W	+	+	−
t-FT05W	+	+	−
SW06W	+	+	−
t-SW06W	+	+	−
ZEA17I	+	+	−
t-ZEA17I	+	+	−

[1] *+ indicates activity. – indicates no activity.*

inner root tissues of lettuce seedlings differed between the two methods.

In the rhizosphere, the concentration of the t-ZEA17I strain remained similar to the inoculated amount during the first 20 dai with either method. When t-ZEA17I was distributed on top of the growth substrate, a significant increase in concentration 30 dai was observed. In the case of the seed coating, after a slight increase within the first 10 days, the final amount was not significantly different from the initial inoculum (**Table 5**).

Similarly, we studied the dynamics of t-ZEA17I colonization in the inner tissues of lettuce roots. First, we ruled out the possible external root contamination due to ineffective sterilization, and no *Streptomyces* colonies were detected. The t-ZEA17I strain was re-isolated from inner root tissues of surface-sterilized roots independently of the inoculation method, confirming its ability to endophytically colonize lettuce roots. The concentration of t-ZEA17I declined steadily through time, however, this reduction was not significant with either inoculation method (**Table 6**).

Finally, we compared the two inoculation methods to get a further insight into whether one of them could improve the survival and colonization rates of t-ZEA17I in lettuce rhizosphere and roots. In the rhizosphere, significantly more t-ZEA17I was re-isolated at all sampling times using the growth substrate inoculation rather than seed coating ($P = 0.0037$, 0.0389, and 0.0005, for sampling time 10, 20, and 30 dai, respectively). Similarly, in roots, significantly higher concentration of t-ZEA17I was re-isolated using the growth substrate inoculation at 20 and 30 dai

Table 4 | Colonization dynamics in bulk soil by transformed *Streptomyces* ZEA17I.

Inoculation method	Bulk soil (CFU/g dry weight)			
	0 dai[1]	10 dai	20 dai	30 dai
Growth substrate	3.85×10^7a[2]	1.25×10^4b	1.54×10^4b	2.18×10^4b

[1] *dai = days after inoculation.* [2] *Tukey post hoc test; means in a row with the same letters are not significantly different (P = 0.05).*

Table 5 | Colonization dynamics of *Lactuca sativa* var. *capitata* rhizosphere by transformed *Streptomyces* ZEA17I strain according to two inoculation methods.

Inoculation method	Rhizosphere (CFU/g dry weight)			
	0 dai[1]	10 dai	20 dai	30 dai
Growth substrate	2.51×10^6 b[2]	2.72×10^7 ab	3.07×10^7 a	3.80×10^7 a
Seed coating	1.28×10^6 ab	2.01×10^6 a	9.85×10^5 ab	1.19×10^5 b

[1] *dai = days after inoculation.* [2] *Tukey post hoc test; means in a row with the same letters are not significantly different (P = 0.05).*

Table 6 | Colonization dynamics of *L. sativa* var. *capitata* inner root tissues by transformed *Streptomyces* ZEA17I strain according to two inoculation methods.

Inoculation method	Roots (CFU/g dry weight)		
	10 dai[1]	20 dai	30 dai
Growth substrate	1.94×10^7 ns[2]	1.45×10^6 ns	2.36×10^5 ns
Seed coating	3.93×10^5 ns	2.23×10^5 ns	1.39×10^4 ns

[1] *dai = days after inoculation.* [2] *ns, ANOVA not significant (P > 0.05).*

($P = 0.0415$ and $P = 0.0604$, respectively). However, in spite of higher strain amounts present in roots using the growth substrate inoculation method, not all seedlings were colonized. Indeed, we failed to re-isolate t-ZEA17I from roots of three seedlings (one seedling at 20 dai and two seedlings at 30 dai), whereas, using the seed coating method, all roots were endophytically colonized.

DISCUSSION

Plant beneficial bacteria have a great potential in agriculture as PGPB and BCAs and reports about successful control of plant diseases are increasing. However, application of these microbial agents in field often fails to achieve the expected results, which could be due to lack of knowledge about their biology and interactions with the host plant, the pathogens, and other microorganisms in the rhizosphere. Therefore, there are increasing attempts to study these complex interactions that take place in the rhizosphere (Gamalero et al., 2003; Compant et al., 2010).

Our aim was to study spatio-temporal dynamics of colonization of lettuce roots and rhizosphere by *Streptomyces* spp. with biological control potential, to better understand if and how they inhabit the rhizosphere and colonize plant roots. We selected five *Streptomyces* strains on the basis of their strong *in vitro* antagonism against the major soil-borne pathogen of horticultural crops, *S. sclerotiorum* (Bonaldi et al., 2014), and we transformed them with the pIJ8641 plasmid harboring apramycin resistance marker and EGFP gene under a strong constitutive promoter (Sun et al., 1999). The conjugation efficiency varied, and for most strains it was comparable to the reference strain *S. coelicolor* A3(2). The pIJ8641 plasmid integrates at the chromosomal attachment site for the temperate phage φC31, which may result in disruption or alteration of fitness and biological activity of the transformed strains. Indeed, decrease or loss of biological activity was detected after GFP-transformation of various BCAs, e.g., *Pseudomonas putida*, *Metschnikowia pulcherrima,* or *Clonostachys rosea* (Nigro et al., 1999; Lübeck et al., 2002; Weyens et al., 2012). We compared several traits important for biological control and plant growth promotion of transformed and wild-type strains, before studying their interactions with the host plant. None of the transformed strains showed altered growth or sporulation, which could have conferred a disadvantage in plant root and rhizosphere colonization. All transformants retained the ability to suppress growth of *S. sclerotiorum in vitro*, therefore they will also be used for studying their interactions with the pathogen and the mechanisms of biological control *in vivo*. Moreover, we compared the expression of some of the most common traits involved in plant growth promotion and biological control (Brader et al., 2014), such as production of auxins, siderophores and lytic enzymes, and no change in performance between the wild type and the transformants was observed.

We chose the most promising transformed strain, t-ZEA17I, for pilot studies of lettuce roots and rhizosphere colonization. We intentionally used a non-sterile growth substrate to simulate competition with natural microflora and evaluate the competitiveness of the inoculated *Streptomyces* strain exploiting the apramycin resistance for its identification among soil microorganisms. In absence of the host plant, we confirmed that t-ZEA17I freely survives in soil, although we observed a sharp decrease in its density

within the first 10 days after bulk soil inoculation. Similar dynamics for introduced microbial population in non-sterile soil are already known, attributed to scarcity of available nutrients and adverse biotic and abiotic factors (van Veen et al., 1997). However, following the initial fall in population density, the t-ZEA17I population remained stable for up to 30 days, probably establishing an equilibrium with the indigenous microflora as described previously (Yuan and Crawford, 1995; Merzaeva and Shirokikh, 2006). In the presence of the lettuce plant, we did not detect in the rhizosphere the initial rapid decrease in t-ZEA17I amount that was observed in bulk soil. On the contrary, its concentration augmented when applied directly on the growth substrate. It is possible that t-ZEA17I was chemoattracted to the rhizosphere of the growing seedling, where it quickly established a stable interaction with the host plant roots. Indeed, the presence of a host plant may greatly affect the survival of PGPB, as was observed, i.e., for the sharp decline in *Azospirillum brasilense* population after removal of inoculated plants (Bashan et al., 1995).

Different strategies are being used for studying BCAs and PGPB in the rhizosphere. Their localization in roots and seeds rely on microscopic tools exploiting fluorescent markers, which give a fundamental insight into the spatial distribution of the microorganism along and inside the growing root, but do not quantify the microbial amounts and their dynamics (Coombs and Franco, 2003; Olivain et al., 2006; Compant et al., 2010). Additionally, studying the dynamics of colonization by beneficial microorganisms exploits the strain identification mostly by natural or introduced antibiotic resistance and its quantification by dilution plating (Gamalero et al., 2003, 2005). Here, we quantified the t-ZEA17I in roots and rhizosphere through the introduced antibiotic resistance for its identification, to understand if it can inhabit soil in competitive concentrations in comparison to the indigenous microflora. t-ZEA17I was detected in the inner root tissues of growing seedlings already 10 days after inoculation at high concentrations. Indeed, Coombs and Franco (2003) demonstrated that the EGFP-tagged endophytic *Streptomyces* sp. strain EN27 rapidly colonizes the wheat embryo, as it was detected in developing roots as early as 24 h after inoculation. Re-isolation of t-ZEA17I from the rhizosphere and the inner tissues of surface-sterilized roots indicates that it is both rhizospheric and endophytic, although it is not known if its localization affects its potential for biocontrol and plant growth promotion. It has been hypothesized that endophytic bacteria form more stable interactions with plants, rather than rhizospheric or epiphytic bacteria (Compant et al., 2010; Malfanova et al., 2011).

Finally, we tested how different methods of inoculation influence the t-ZEA17I colonization ability. When it was distributed directly on the growth substrate, higher concentration was detected in roots, however, we could not re-isolate the strain from all inoculated plants. On the contrary, when the seed coating method was used, less propagules were recovered but all plants were endophytically colonized. It is possible that in case of seed coating, t-ZEA17I is more closely associated with the growing seedling, which increases its probability to internally colonize root tissues. In roots, we observed progressive decline in its concentration using either inoculation method. Although the total amount of t-ZEA17I increased at different sampling times (data not shown), the increase in lettuce root biomass was probably higher than the strain growth, thus resulting in lower strain concentration per g of root. To ensure that t-ZEA17I colonizes roots in effective concentrations, and to prevent its decline to undetectable levels, studies assessing optimal amount of inoculum are needed. Moreover, it is possible that strains colonize only certain root zones (Gamalero et al., 2004, 2005). Therefore, further studies are needed to establish which zones of the plant roots are colonized by t-ZEA17I and ultimately how it interacts with the plant in presence of *S. sclerotiorum,* to evaluate its biological control activity *in vivo*.

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Advancing the science of microbial symbiosis to support invasive species management: a case study on *Phragmites* in the Great Lakes

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A growing body of literature supports microbial symbiosis as a foundational principle for the competitive success of invasive plant species. Further exploration of the relationships between invasive species and their associated microbiomes, as well as the interactions with the microbiomes of native species, can lead to key new insights into invasive success and potentially new and effective control approaches. In this manuscript, we review microbial relationships with plants, outline steps necessary to develop invasive species control strategies that are based on those relationships, and use the invasive plant species *Phragmites australis* (common reed) as an example of how development of microbial-based control strategies can be enhanced using a collective impact approach. The proposed science agenda, developed by the Collaborative for Microbial Symbiosis and *Phragmites* Management, contains a foundation of sequential steps and mutually-reinforcing tasks to guide the development of microbial-based control strategies for *Phragmites* and other invasive species. Just as the science of plant-microbial symbiosis can be transferred for use in other invasive species, so too can the model of collective impact be applied to other avenues of research and management.

Keywords: symbiosis, *Phragmites*, invasive species management, fungi, bacteria, collaborative, endophyte, Great Lakes Region

INTRODUCTION

Invasion of native ecosystems by non-native (i.e., exotic) plant species is a widespread problem. For example, Morse et al. (1995) estimated that more than 5000 exotic plant species have become established and displaced native plant species in the U.S. The problem continues to grow as over 700,000 hectares per year of wildlife habitat are invaded by invasive species (Babbitt, 1998). Invasive plants negatively impact both the ecosystems and the economy of the United States (Pimentel et al., 2000), where about 400 of the 958 species listed as endangered or threatened are considered to be at risk due to pressure from invasive species (Wilcove et al., 1998). Management and control of invasive plants is a priority for many agencies and organizations across the United States and entails a significant investment of resources. For example, the National Invasive Plants Council, composed of members of many federal agencies with a goal to provide high-level interdepartmental coordination of federal invasive species actions, estimated that $2.2 billion (U.S.) was spent during FY2012 on invasive species activities (National Invasive Species Council, 2014). The total control cost for exotic and invasive aquatic weeds in the United States is estimated at $100 million annually (Pimentel, 2005). In the State of Florida alone, $14.5 million is spent annually on aquatic hydrilla (*Hydrilla verticillata*) control, and *H. verticillata* infestations in only two Florida lakes have amounted to $10 million annually in recreational losses, including swimming and boating (Center et al., 1997). Similarly, state departments of natural resources, various collaboratives, and local watershed councils are also concerned with invasive species. In the Great Lakes region, the Great Lakes Restoration Initiative (GLRI), the largest U. S. investment in the Great Lakes in two decades, includes combating invasive species as one of its five urgent issues (Great Lakes Restoration Initiative, 2010, 2014).

Although extensive resources from state and federal agencies have been devoted to both management and control of invasive plant species across the U.S., there is evidence that this intensive investment may not be producing the intended management results (Reid et al., 2009; Martin and Blossey, 2013). There is a need for new, innovative tools to control invasive

species that address the drivers of invasion. A growing body of literature supports microbial symbiosis as a foundational principle for the competitive success of invasive species. Much of this insight has emerged from ecological studies of microbiomes (see Glossary for definitions of select terms) demonstrating that the health, productivity, and adaptive capacities of all organisms, whether they be humans (Pflughoeft and Versalovic, 2012), non-human mammalian species (Ley et al., 2008; Muegge et al., 2011), insects (Engel and Moran, 2013), amphibians (Kohl et al., 2013; Kueneman et al., 2014), birds (Kohl, 2012), fish (Wu et al., 2012; Ye et al., 2014), or plants (Bulgarelli et al., 2013; Berg et al., 2014; Rout, 2014) can be linked in various ways to their microbiomes (i.e., microbial communities). This new and growing understanding of the diversity, specificity, and wide-ranging function and impacts of host-associated microbiomes makes it clear that the behavior, dynamics, and interactions of organisms cannot be understood or predicted without a consideration of their associated microbiota (Gilbert et al., 2012). We believe, therefore, that a deeper understanding of the relationships between invasive species and their associated microbiomes, as well as the interactions with the microbiomes of native species, can lead to key new insights into invasive success and potentially new and effective control approaches. This approach is particularly promising for invasive plant species because of opportunities to target control efforts on the special dependence that all plants have on the recruitment of microbiota for growth, tolerance to stress, and resistance to disease. Potential control efforts could target the introduction of pathogenic microbes or inhibition of beneficial fungi (e.g., targeting microbial relationships that confer competitive benefits). In this manuscript, we review microbial (primarily endophytic) relationships with plants, outline steps necessary to develop invasive species control strategies that are based on those relationships, and use the invasive plant species *Phragmites australis* as an example of how development of microbial-based control strategies can be enhanced using a collective impact approach.

PLANT-MICROBIAL INTERACTIONS

As with humans and other animals, plants also interact symbiotically with microbes throughout their life history. These symbioses are initiated through vertical transmission to juveniles at the time of seed development or through continuous horizontal acquisition from the environment (**Figure 1**). This plant-associated microbiome (Turner et al., 2013; Rout, 2014) spans the diversity of microbial life residing either within the plant as endophytes or as epiphytes on foliar (Peñuelas and Terradas, 2014) and subterranean (Mendes et al., 2013) plant surfaces. Broadly, these associations may be either intimate or casual, yet many are

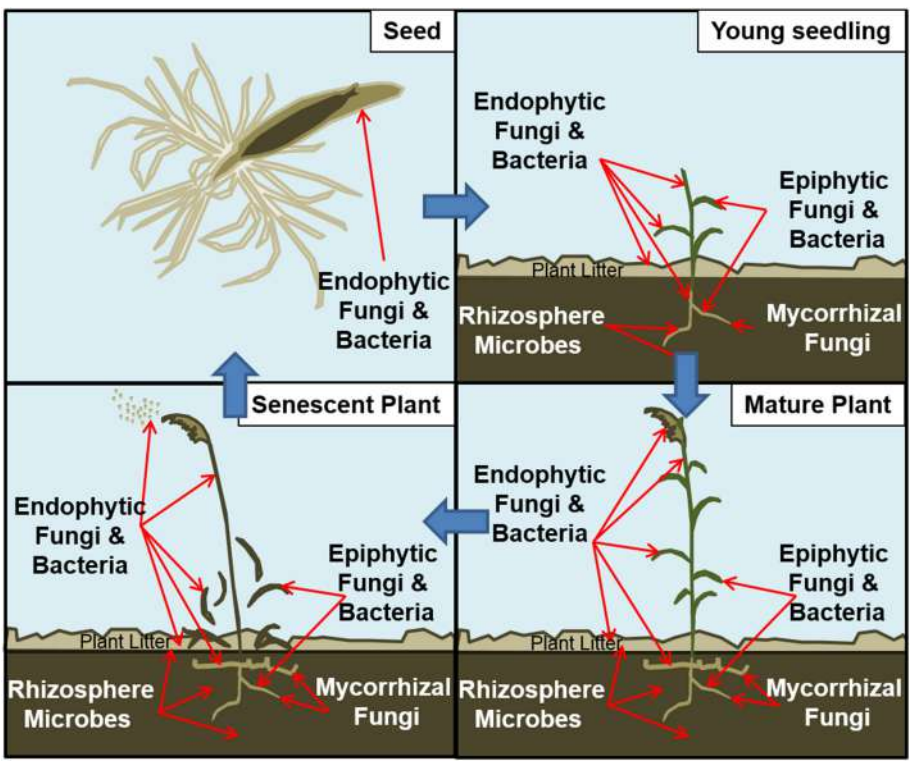

FIGURE 1 | Schematic of microbiome surrounding a plant throughout its life history. UL: Endophytic fungi and bacteria can be transmitted within the seed coats of certain plant species. UR: As seeds germinate, roots, stems, and leaves of seedlings can be inhabited by various microbes. Those microbes may have been transmitted through seeds, soil and plant litter on site, or airborne spores. LR: A mature plant may be thoroughly infected with microbes. LL: As perennial plants senesce, some endophytes are transmitted to the next generation through seeds or through living rhizomes. Other microbes may be transmitted through spores in the plant litter.

thought to contribute to plant health and development (Borer et al., 2013). Many plant-microbe associations may be commensal, for which no overt benefit or harm is observed, or mutualistic, in which plant growth and development are often promoted (Hirsch, 2004). Still other pathogenic symbioses may negatively impact plant growth and/or result in developmental deficiencies or mortality. These relationships also are not static and may vary from mutualistic to pathogenic during different stages of the microbial or plant life cycle (Kogel et al., 2006). Responses of plant populations to this range of symbiotic associations will directly reflect the net impacts of both mutualistic and pathogenic symbioses, as well as indirect impacts that may involve commensals (Bever et al., 2010).

Endophytic mutualists and pathogens

The endophytic habit is described as the internal colonization of a plant by a microbe. There are several variations to this endophytic life style. For example, endophytic microbes are often restricted to particular organs, usually roots, stems, or leaves. Some endophytes occupy only above-ground plant parts, whereas others are restricted to subterranean organs and tissues. Endophytic microbes most commonly live exclusively within a plant in a biotrophic mode, obtaining their nutrition solely from the plant. As a result, many endophytic microbes form obligate associations with plants, most often inside the plant host but occasionally outside of the host (e.g., arbuscular mycorrhizal fungi or AMF). Commonly, endophytic microbes that are systemically distributed in plants (Class 1 and 2 fungal endophytes) are vertically transmitted through successive generations of hosts in seeds or in rhizomes of clonal plants, whereas endophytes restricted to particular tissues or organs of hosts (e.g., class 3 endophytes) are generally transmitted horizontally (Rodriguez et al., 2009). Among the more commonly studied fungal endophytes are species of *Epichloë* (=*Neotyphodium* asexual stage) that are restricted to above-ground portions of cool-season grasses, including leaves and inflorescences (Tanaka et al., 2012), and broadly distributed root-infecting AMF that comprise species within the phylum Glomeromycota (Willis et al., 2013), commonly in the genera *Glomus* and *Gigaspora* (Dumbrell et al., 2010). Bacterial endophytes, however, may represent species spanning several bacterial phyla: Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes (Malfanova et al., 2013). Endophytic infections by either fungi or bacteria often lead to enhanced plant productivity, either by enhancing nutrient acquisition, producing plant growth hormones, synthesizing metabolites that restrict vertebrate or invertebrate herbivory, or also by reducing disease susceptibility (Rodriguez et al., 2009). However, under appropriate conditions, endophytic interactions may transform from a mutualistic association to a pathogenic association (Newton et al., 2010; Alvarez-Loayza et al., 2011), blurring the lines between species that are strict mutualists and those that are strict pathogens.

Most common epiphytic bacterial and fungal plant pathogens also have a significant endophytic phase to their life cycle. Latent infections are common, and pathogens may reside endophytically in plants for extended periods without causing any mortality, growth reductions, or reductions in fitness (Delaye et al., 2013; Malcolm et al., 2013). Many of these potential pathogens originate from epiphytic populations residing either in the rhizosphere (below-ground zone adjacent to plant roots) or phyllosphere (above-ground zone adjacent to leaves), although pathogen communities ultimately found in roots are more diverse than those found in leaves (Angelini et al., 2012). As with *Epichloë* and other fungal and bacterial endophytes, interactions with plants may switch from pathogenic back to mutualistic, reinforcing the importance of the dynamics of interacting factors associated with hosts, microbes, and the environment (Scholthof, 2007) that ultimately determine the nature of microbial interactions with plants at any given time.

Epiphytic microbial associations

The epiphytic lifestyle generally refers to microbial development directly on host surfaces. Yet, this development, driven by carbon release from plant parts (Hirsch et al., 2013), is often maintained within a spatially and temporally variable phyllosphere and the rhizosphere. These epiphytic symbionts originate from soil, water, seed, animal excrement, or the atmosphere and comprise the breadth of bacterial and fungal diversity (Vorholt, 2012). Different plant organs and tissues support different communities of microbes (Normander and Prosser, 2000), but in all cases, these epiphytic associations are driven largely by nutrients released from the plant into the adjacent soil (Dennis et al., 2010) or leached from foliar plant parts (Vorholt, 2012). Similar to endophytic associations, epiphytic associations span the range from mutualistic to pathogenic but often provide positive impacts on plant growth and health either through direct growth enhancements (Lugtenberg and Kamilova, 2009) or suppression of pathogens (Mendes et al., 2011).

PLANT-MICROBIAL SYMBIOSIS AND INVASION

Increasingly, it is recognized that microbial symbioses may be important determinants of plant invasiveness and can either exacerbate or inhibit invasive success, depending on origins of the symbiont (from the native or invasive range) and on the direction, prevalence, and strength of the symbiotic interactions (Richardson et al., 2000; Berg et al., 2014; Coats and Rumpho, 2014). However, the nature and magnitude of the role of microbial symbioses in biological invasions is not always clear (van der Putten et al., 2007). Therefore, a better understanding of general mechanisms of biological invasions as a whole will result in more effective management of invasive plant populations (Mack, 1996; Rejmanek, 2000; Richardson et al., 2000).

Invasive plants and native pathogens

Darwin (1872) observed that plant and animal species brought to new regions of the world often experienced dramatic population growth and surmised that these species escaped from regulation by "natural enemies." The enemy release hypothesis (ERH) predicts that plants introduced to a new region will benefit by encountering fewer specialist enemies compared to their native range and will be less affected by resident generalist enemies than resident plants. This escape from natural enemies would provide a competitive advantage over resident species (but see van Kleunen and Fischer, 2009). Many studies suggest that biological invasions are most likely to start in areas with low levels of

ecological resistance and by invaders largely free from their native natural enemies (Reinhart et al., 2003; DeWalt et al., 2004; Knevel et al., 2004; Vila et al., 2005) (but see Beckstead and Parker, 2003, for an exception). For example, correlative studies report that many invasive plants are associated with more foliar (Mitchell and Power, 2003) and root (van der Putten et al., 2005) pathogens in their native than non-native ranges. Further, plants categorized as harmful invaders experienced a greater decline in pathogen infection from native to invaded range than weak invaders. Because most natural plant communities have diverse resident pathogens, successful invaders are likely to encounter non-adapted pathogens that cause less damage relative to what they experienced in their native ranges. However, as the density, range, and time-since-invasion of invasive plants increase, interactions with pathogens are likely to change.

Invasive plants and novel pathogens

Native pathogens, which may be novel to the introduced species, may immediately prevent invasion (biotic resistance hypothesis) so that the invading species never becomes established or reaches such densities as to displace native species (Elton, 1958; Knevel et al., 2004; Parker and Gilbert, 2004). Outside of agricultural species, we have little knowledge of failed invasions (Scheffer, 1997). Biotic resistance may be more effective where invasive species are closely related to native species. For example, Parker and Gilbert (2004) found no difference in disease levels in native vs. introduced clovers occurring at the same site. Invasive species from an unrelated genus or family should be less likely to be colonized by novel pathogens than invasive species closely related to co-occurring native species. However, over time, the number of novel pathogens that accumulate on invasive species is likely to increase. For example, Strong and Levin (1975) found that introduced British trees support the same number of fungal parasites as native tree species 300 years following their introduction. As the success (i.e., high density) of an invasive species increases, the chance that a virulent pathogen will arise and lead to epidemics and major die-offs also increases. Negative effects of pathogen buildup have been demonstrated both theoretically and empirically (Hudson et al., 1998; Turchin et al., 1999; Hassell, 2000). Disease epidemics in native plant species (Rizzo and Garbelotto, 2003) and rapid control of invasive species by biocontrol efforts (Burdon et al., 1981; Cox and McEvoy, 1991) also point to the potential of enemies to regulate plant populations. For example, the weevil (*Euhrychiopsis lecontei*) colonized Eurasian watermilfoil (*Myriophyllum spicatum*) and greatly reduced populations across its invasive range (Creed and Sheldon, 1995; Creed, 2000). More recently, Flory et al. (2011) reported that a *Bipolaris* fungal pathogen greatly reduced the biomass and reproduction of invasive Japanese stiltgrass (*Microstegium vimineum)* in naturally-infected invasive populations. Over time, invasive plant species may become increasingly regulated by natural enemies (Flory and Clay, 2013).

Invasive plants and native mutualists

Most plant species form mutualistic symbioses with arbuscular mycorrhizal fungi (Allen, 1991), N-fixing bacteria (Huss-Danell, 1997; Parker, 2001), or endophytic fungi (Clay and Schardl, 2002; Angelini et al., 2012) or from simultaneous infection by multiple mutualists (Larimer et al., 2010). However, as in the case of the ERH, invasive species may often colonize new habitats without their native symbiont. If a microbial mutualist is obligate, invasions will fail in the absence of the symbiont. For example, early attempts to introduce pines into Australia failed until appropriate mycorrhizal fungi were introduced simultaneously (Allen, 1991). Similarly, Parker (2001) concluded that "legumes may often fail at colonization attempts within habitats where mutualist partners are scarce." However, this situation can also favor invasive species that are less dependent on mutualistic symbionts. For example, invasive St. John's wort (*Hypericum perforatum)* is less dependent on AMF compared to populations from its native range (Maron et al., 2004). More generally, colonizing species may be less dependent on symbiotic associations than non-colonizing species (Baker and Stebbins, 1965). On the other hand, if colonization by the plant and symbiont occur simultaneously, as in the case of seed-transmitted fungal endophytes of grasses (Clay and Schardl, 2002), then invasiveness may be enhanced by symbiosis. In experimental plots of non-native tall fescue grass (*Lolium arundinaceum*) where endophyte infection was experimentally manipulated, endophyte-infected plots had significantly greater biomass of tall fescue, less biomass of other species, and lower species richness (Clay and Holah, 1999; Rudgers and Clay, 2008). Non seed-transmitted mutualists may be widely dispersed and not limit invasions. For example, in Hawaii the invasive species faya (*Myrica faya)* fixes nitrogen via symbiosis with *Frankia* bacteria and greatly alters ecosystem nitrogen dynamics (Vitousek et al., 1987; Walker and Vitousek, 1991). However, Zimpfer et al. (1999) found that the density of infective *Frankia* decreased with distance from established invasive *Casuarina cunninghamiana* trees in Jamaica, suggesting strong spatial dependence of invasions on *Frankia* density associated with established host populations.

Invasive plants and novel mutualists

Invasive plants colonizing habitats in the absence of their native symbionts may become colonized by novel mutualists. The likelihood of this occurring may depend on the level of host-symbiont specificity and on the phylogenetic relationship of the invasive plant with native plant species. Some mutualistic interactions like pollination or seed dispersal may be fairly general and do not represent a strong barrier to invasion (Richardson et al., 2000). One example of an invasive plant that established a symbiotic association with a novel mutualist in its invaded range is purple nutsedge (*Cyperus rotundus*) infected by the fungal symbiont *Balansia cyperi*. The plant is native to Asia but has been widely introduced in agricultural areas outside its native range–to the extent that it is classified as the world's worst weed (Holm et al., 1977). *Balansia cyperi*, on the other hand, is native to the southeastern U.S., Central America, and South America, where it infected several native *Cyperus* species (Diehl, 1950). Invading purple nutsedge populations in the U.S. Gulf coast region were also infected by *B. cyperi*, which produced a large increase in bulbil production and overall plant reproduction (Stovall and Clay, 1988). The fungus likely jumped from a native *Cyperus* host to *C. rotundus* in this region, exacerbating its competitive ability and invasiveness. Host shifts of novel mutualists onto invasive plants

must certainly occur in other systems but have not been well-documented.

INVASIVE SPECIES MANAGEMENT THROUGH MICROBIOME MANIPULATION

Given the multitude of means by which microbes can impact host organisms and, ultimately invasion success, there is great potential for the management of invasive species through intentional manipulations of symbiotic relationships that result in either reduced competitiveness of invasive species or increased productivity and fitness of non-invasive plants (e.g., plants recruited after habitat restoration efforts). For example, if it is shown that fungal endophytes enhance the competitive capacities of an invasive plant species, encouraging the growth of antagonistic bacterial endophytes through exogenous applications may be explored as a way to truncate benefits stemming from fungal endophytes. This strategy is successfully being employed in crop plants to eliminate toxic endophytes (Bacon and Hinton, 1999).

Manipulation of the plant microbiome is a strategy that may be used to alter the competitive capacity of plants. Strategies to encourage or discourage specific microbes that impact plant performance may be employed, either to reduce competitiveness of the invader or to increase the resilience of native species. Such a microbiome manipulation strategy has been successfully explored with human health issues and serves to illustrate the promise of such an approach. Although many now recognize the importance of diet in directly manipulating the gut microbiome of humans and other animals (Muegge et al., 2011), other manipulation strategies with humans such as fecal transplants are gaining scientific credibility and public acceptance (van Nood et al., 2014). In fecal transplantation therapy, complex gut microbiomes from a healthy donor are introduced into the colons of patients suffering from intestinal infections. Often such probiotic manipulations reverse the trajectories of sick patients in a matter of days, restoring them to health (de Vrieze, 2013). Similar probiotic therapies involving plants have been used in agriculture for many years, whereby the introduction to soils of complex microbiomes from naturally disease suppressive soils (Chaparro et al., 2012) or from disease suppressive organic amendments (Hadar and Papadopoulou, 2012) have altered plant health trajectories by altering microbial species in the soil microbiome. These species are then recruited to the plant as endophytes and epiphytes. Similar plant and/or soil microbial manipulations could also be possible to alter invasion trajectories of introduced plant species.

We recognize that determining the role of the various microbial species in the success of invasive *Phragmites* or other plant species is complex, given the large number of biotic and abiotic variables involved. It is widely appreciated that beneficial symbionts such as rhizobia and mycorrhizae can enhance host nutrition, growth, and stress resistance, while pathogens have opposite effects. Beneficial plant-growth promoting bacteria, primarily found in the soil environment, are also known from many agricultural and natural systems where they help improve the growth and vigor of host plants (Compant et al., 2010). In agricultural systems, specific microbes are often used as bioinoculants to enhance crop productivity or to reduce pathogen and pest damage (Nelson, 2004; John et al., 2011). For example, plant growth-promoting rhizobacteria are applied directly to seed or to the soil when planting to ensure inoculation with the most beneficial strains (Kaymak, 2011). Plant-growth promoting fungi such as *Trichoderma* species can also have similar positive effects on plants distinct from other plant-symbiotic fungi such as mycorrhizal fungi and foliar fungal endophytes (Harman et al., 2004; Contreras-Cornejo et al., 2009). Colonization of roots by plant-growth promoting fungi can enhance resistance to pathogens and abiotic stresses, nutrient uptake and the productivity of crops. However, the biological roles of most host-associated microbes are unknown. Initial research to identify the most common and widespread microbial taxa found in the rhizosphere or within the target species can guide subsequent evaluation of microbial impact on host plants. Microbial taxa common among target plants growing within many populations throughout the landscape are more likely to influence landscape-scale competitiveness than taxa only observed in a limited number of plants. In the case of invasive plant species, the most prevalent and beneficial microbes could be targeted for control through chemical or biological treatments to reduce the growth and vigor of the invasive plant indirectly.

FORMING A SCIENCE AGENDA

There has been some recent work highlighting the role of the microbial community in invasion success (highlighted above), but significant information gaps need to be filled before microbial-based control measures can be developed. To accelerate this development, we propose that a series of strategic actions can be used to ensure that the correct microbes are being targeted and that the desired results are achieved. **Figure 2** provides a foundation of sequential steps to guide the development of microbial-based control strategies for invasive plant species.

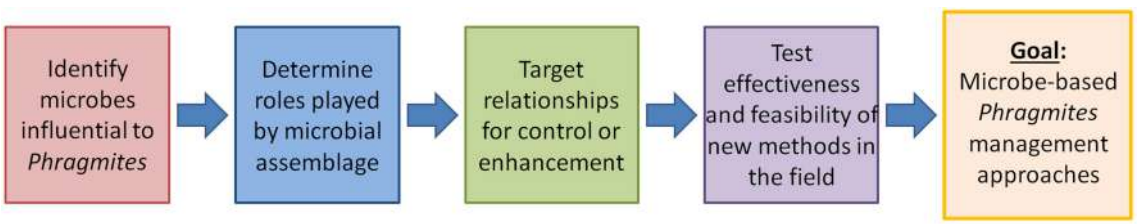

FIGURE 2 | Conceptual strategy for developing a microbial-based management approach to invasive plant species (e.g., *Phragmites australis*).

Similarly, it could be used as a guide to develop probiotics that promote the growth of native plant species.

1. ***Identify and characterize microbes influential to target invasive and non-invasive plants***—To design and implement an effective microbiome manipulation strategy, the microbial constituents relevant to the *invasive* plants of interest must be characterized. The host range, tissues colonized, mode of transmission (e.g., vertical, horizontal), assemblage diversity, temporal variability, and other criteria can help describe the local microbiome. A complete inventory will include documentation of any relevant rhizospere microbes, such as *Oomycetes* or plant growth promoting and endophytic *Bacillus* spp. (Gond et al., 2014), and whether significant interactions among endophytes exist. This step establishes the foundation on which the remaining steps are based.
   It is likely that *native* plant communities also are intrinsically linked to fungi and bacteria. Thus, it is also important to identify which endophytes are common in native species and initiate studies that will allow forecasting of possible behavior and outcomes from either a common species or an interaction of species during a specific growth phase. Results could guide targeting of specific life-stages, both pre-infection and post-infection, to maximize treatment response.
2. ***Determine roles played by the microbial community***—Once the target microbiomes are characterized, it is necessary to examine the benefits or other effects that they confer to the plants. Specifically, this step involves identifying the functional roles of identified microbes and exploring how they affect plant growth, development, and tolerance to extreme conditions (all characteristics that contribute to a plant's competitive ability). Similarly, examination of how identified microbes affect the function and competitiveness of native plants.
3. ***Target relationships for control or enhancement***—Once the microbial constituents and their roles are identified, the most influential relationships could be targeted for control or enhancement. Specifically, this stage will involve determining if endophytes can be controlled, how control treatments impact both target and non-target species, and how treatments alter competitive ability. Endophytes in target native plant species require a different approach focused on determining whether native species can be inoculated with beneficial endophytes (i.e., probiotics) and if inoculation will increase competitive abilities compared to invasive plants.
4. ***Test effectiveness and feasibility of new methods under field conditions***—After critical microbial assemblages are identified and targeted for control or enhancement, new management methods need to be developed and field-tested to characterize effectiveness, cost-efficiency, and risk through space and time. For example, tests of treatment specificity will characterize potential impacts of a control method on non-target organisms and environments. This step also involves examining the feasibility of scaling up to the landscape level and exploring the regulatory and financial aspects of new control (or enhancement) methods.

A CASE STUDY ON THE INVASIVE COMMON REED: CREATING A SCIENCE AGENDA FOR MANAGING INVASIVE *PHRAGMITES AUSTRALIS* THROUGH MICROBIAL INTERVENTION

ECOLOGY OF *PHRAGMITES*

The invasive form of common reed (*Phragmites australis*, hereafter referred to as *Phragmites*) is a tall non-native perennial grass often growing in dense clones throughout North American wetlands (**Figure 3**). Although a native subspecies of *Phragmites* (*Phragmites australis* spp. *americanus*; Saltonstall et al., 2004) has been present in North American wetlands for thousands of years, recent aggressive proliferation has been attributed to a non-native, invasive subspecies (*Phragmites australis* spp. *australis*), also known as haplotype M. The invasive *Phragmites* was introduced into North America from Europe near the beginning of the 1900s and has since been aggressively replacing the native type (Saltonstall, 2002; Mozdzer et al., 2013) and displacing native wetland plant assemblages. It is widely distributed and has been found in each state within the contiguous United States, is now established across the whole Great Lakes basin (Mal and Narine, 2004; Trebitz and Taylor, 2007; Tulbure et al., 2007; Bourgeau-Chavez et al., 2013), and can be found throughout southern Canada (Saltonstall, 2002).

This highly invasive plant spreads rapidly through seed dispersal, stolons, and rhizomes. *Phragmites* invasion displaces native plants and decreases wetland biodiversity, primarily because of its aggressive root system and tall, dense canopy that shades out other wetland plants (Chambers et al., 1999). It also may exude phenolic gallic acid as a form of allelopathy (Rudrappa et al., 2007; Bains et al., 2009), but the significance of that trait is not clear (see Weidenhamer et al., 2013). The presence of *Phragmites* is known to impair recreational use of wetlands and shorelines, decrease property values, increase fire risk, and reduce public safety when proximity to roads disrupts driver visibility (Warren

FIGURE 3 | Invasive *Phragmites australis* in a Great Lakes coastal wetland.

et al., 2001; Mal and Narine, 2004; Trebitz and Taylor, 2007; Kettenring and Adams, 2011). A few studies describe some positive effects of *Phragmites*, including improved oxidation of the substrate and quality of the sediments (Tulbure et al., 2012), filtration of nutrients from agricultural lands (Kettenring et al., 2012), and providing beneficial habitat for the American bullfrog population (Rogalski and Skelly, 2012). However, invasive *Phragmites* is considered a significant ecological and economic threat by the public, NGOs, and governmental agencies (Meyerson et al., 2000; Great Lakes Restoration Initiative, 2014).

CURRENT MANAGEMENT OF *PHRAGMITES*

Current management approaches can be effective in the short term, but there are currently no clear means to stop *Phragmites* invasions completely (Marks et al., 1994; Warren et al., 2001). Conventional *Phragmites* management typically involves the application of several strategies (chemical, mechanical (cutting and burning), and hydrologic) used in combination over a long period of time (Hazelton et al., 2014). This integrated approach is considered to be the most effective, yet when employed independently, these strategies may enhance *Phragmites* growth. Specific management protocols depend on many factors, including patch size and management agency capacity, but in general, repeated application of herbicides (glyphosate and imazapyr), followed by removal of biomass by burning or mowing is an effective *Phragmites* management approach (Carlson et al., 2009; Michigan Department of Natural Resources, 2010; Hazelton et al., 2014). While this protocol has been successful at reducing *Phragmites* in the short term, it is expensive, time-consuming, and generally not sustainable in the long term. Herbicides can also have negative impacts on the surrounding environment (Back and Holomuzki, 2008), and their application often draws negative social attention (Blossey, 1999). Furthermore, aerial and over-water application of herbicides is prohibited in Canada. Because current management methods are unsustainable and not available to all resource managers, new microbe-based strategies are being investigated.

THE MICROBIOME OF *PHRAGMITES*

Baseline assessments of *Phragmites*-associated endophytes offer a foundation for exploration of potential control methods based on microbiome manipulations. Commonly, the freshwater and saltwater wetlands invaded by *Phragmites* harbor high levels of microbial diversity and activity (Gutknecht et al., 2006; Stephenson et al., 2013). As a result, diverse symbiotic interactions of *Phragmites* with eukaryotic and prokaryotic microbes are likely to occur. Although not always easily detectable (Lambert and Casagrande, 2006), ample evidence exists that *Phragmites* harbors rich endophytic fungal (Angelini et al., 2012; Fischer and Rodriguez, 2013) and bacterial (Li et al., 2013; Ma et al., 2013) communities comprised of both mutualists and potential pathogens. Equally significant are the epiphytic prokaryotic (bacterial and archaeal) communities (Llirós et al., 2013; Zhang et al., 2013) and fungal communities (Wirsel et al., 2001; Van Ryckegem and Verbeken, 2005). In addition, *Phragmites* is known to support oomycete communities (water molds, Wielgoss et al., 2009; Nelson and Karp, 2013). However, despite the detection of many known mutualistic and pathogenic symbionts associated with *Phragmites*, the specific roles of nearly all of these *Phragmites*-associated microbes have not been evaluated. The exceptions are a few *Phragmites*-associated rhizosphere bacteria (Reed et al., 2005) and fungi (Ernst et al., 2003) that have been shown to enhance plant growth.

A diversity of known pathogenic fungi (Ban et al., 1998; Mazurkiewicz-Zapalowicz, 2010) and oomycetes (Nechwatal et al., 2008; Nelson and Karp, 2013) have also been described in *Phragmites* populations found in both Europe and North America. Yet, despite the fairly extensive list of putative foliar- and root-infecting pathogens, virtually nothing is known about their virulence to either native or non-native *Phragmites* haplotypes, and little mechanistic understanding is known about how they might influence invasive success. Therefore, screening for well-studied microbes, like *Bacillus* spp. that are known to promote growth and resistance to biotic and abiotic stresses in a range of plants (Gond et al., 2014; White et al., 2014) could provide initial insight into the relationship between *Phragmites* and pathogenic microbes. These microbes are better understood in terms of their mechanisms of activity in plants and therefore could provide important targets for altering the competitiveness of invasive *Phragmites*.

Detection and examination of mutualistic or pathogenic endophytes in *Phragmites* is complicated by the fact that plant-growth characteristics associated with host variation may be completely distinct from those influenced by environmental or physiological adaptations (Lissner et al., 1999; Meyerson et al., 2000; Saltonstall et al., 2004). Since microbiome associations are very genotype specific and there is a suite of *Phragmites* lineages in North America, host variation must be addressed in any investigation endophytic and epiphytic microbes and their effect on the invasive success of *Phragmites*.

MICROBIAL-DERIVED BENEFITS TO *PHRAGMITES*

Microbial interactions are thought to convey benefits to invasive *Phragmites* through enhanced nutrient processing capabilities and increased tolerance to environmental and habitat disturbances. *Phragmites* is well-adapted for growth in nutrient-rich habitats but is somewhat plastic in that it grows at low nutrient levels also (Mozdzer and Megonigal, 2012). Although *Phragmites* commonly can be found in low nutrient soils, it grows best at fertile sites (Romero et al., 1999). The capacity of *Phragmites* to cross a range of soil nitrogen concentrations could be related to maintenance of microbial functional diversity with respect to nitrogen processing in multiple parts of the nitrogen cycle (Li et al., 2013). A species of the fungus *Stagonospora*, for example, was found to be a common growth-promoting endophyte of *Phragmites* (Ernst et al., 2003), so it is possible that this fungus or other fungal species could effectively replace the nutrient absorption function of AMF. Because AMF have obligate associations with plants, the limited support provided to these fungi by *Phragmites* may also provide a possible explanation for the slow re-colonization by native plants in managed marshes that were previously dominated by *Phragmites* and have depleted levels of AMF in the soil (e.g., Tanner and Gange, 2013). Holdredge et al. (2010) found that native *Phragmites* was much more heavily colonized by AMF, suggesting that it would benefit more from increased abundance of AMF than would the invasive strain.

Table 1 | Specific tasks outlined by members of PSC to guide research to support *Phragmites* management using microbial symbiosis.

Science agenda	Tasks
Microbial inventory	(a) Gather data on the composition and transmission method of epiphytic and endophytic microbes associated with *Phragmites* populations (b) Determine the variation of the *Phragmites* microbiome in time and space (e.g., within a stand, by site) or time (e.g., over plant life cycle, age of *Phragmites* stand) (c) Explore the relevant pathogenic microbes in *Phragmites* communities and interactions that may exist with mutualistic microbes (d) Characterize the microbiomes of target native plant species to determine if there is a common core group of taxa from which to explore their significance in a probiotic management approach (e) Determine variation in native species microbiomes in space, by species, or by growth stage to allow some predictive patterns that may inform the timing of a manipulative strategy (f) Compare the endophytic communities of invasive *Phragmites* to that of native *Phragmites*
Benefits of microbes	(a) Test the plant response of *Phragmites* when inoculated by particular microbe or set of microbes (b) Determine endophytes that impact growth rate, biomass production, tolerance to stress, or other characteristics that may provide a competitive advantage (c) Assess the impacts of inoculants on *Phragmites'* competitive abilities (d) Determine the impact of *Phragmites*-associated pathogens on native plant communities (e) Identify particular microbes associated with *Phragmites* or with native plants that increase the relative competitiveness of native wetland species in the presence of *Phragmites* (f) Identify individual microbes or microbial consortia that impact plant developmental pathways (e.g., nitrogen-fixing bacteria)
Targeting relationships for control	(a) Test microbial sensitivities to inhibitors (e.g., fungicides or antibiotics) (b) Determine the selectivity of microbial inhibitors for particular groups microbes (c) Test endophyte sensitivity to treatments with limited environmental impact (d) Determine the competitive outcomes of *Phragmites* with native plants following the elimination or suppression of selected microbes (e) Determine competitive outcomes of *Phragmites* with native plants with the inoculation of mutualistic microbes or with the elimination or suppression of pathogens (f) Explore mechanisms that underly reductions in *Phragmites* competitiveness
Test control methods	(a) Analyze considerations for scaling up to landscape-level application of microbial-based control methods (b) Perform analysis for appropriate regulatory bodies and involve regulators in discussions and planning (c) Determine impacts of microbial manipulations on non-target species (d) Determine the direct environmental impacts of the method of manipulation (e.g., fungicide, boric acid) (e) Assess costs associated with microbiome manipulation management strategies (f) Explore optimal management efficacy at short- and long-term time scales

Phragmites is remarkably tolerant of and resilient to a variety of environmental and habitat disturbances (Hellings and Gallagher, 1992; Minchinton and Bertness, 2003; Silliman and Bertness, 2004; Li et al., 2010), but little is known about how endophytes may mediate such responses. Chen et al. (2012) surveyed endophytic bacteria associated with *Phragmites* and evaluated their capacities to degrade pesticides and other pollutants. They proposed that endophytic bacteria could enhance the capacity of *Phragmites* to detoxify polluted waters. The presence of such bacteria may also contribute to the tolerance of *Phragmites* to grow in contaminated sites, where this may contribute to its invasiveness (Meyerson et al., 2000), although endophytic microbial population shifts are observed along with environmental changes (Ravit et al., 2007; Ma et al., 2013). Overall, the presence of endophytes leads to an increase in plant-produced antioxidants and general up-regulation of other stress-defensive mechanisms that may enhance stress tolerance and increase invasive success (Waller et al., 2005; Hamilton et al., 2012; Torres et al., 2012).

PHRAGMITES MANAGEMENT VIA MICROBIOME MANIPULATION

The control of *Phragmites* in North America has become very resource-intensive and difficult to maintain. A recent study of land managers found that, between 2005 and 2009, about $4.6 million was spent annually on mechanical and chemical control of *Phragmites* on over 80,000 hectares in the United States, but there is no significant relationship between the resources invested and control success (Martin and Blossey, 2013). These findings indicate that there is a need for improved control methods using more effective and sustainable approaches.

Successful management of invasive *Phragmites*, like other invasive plant species, would benefit from an integrated management approach that engages multiple stakeholders and can attract substantial long-term funding. As a result of this need,

researchers at the U.S. Geological Survey partnered with the Great Lakes Commission to use principles of the collective impact approach (Kania and Kramer, 2011) to establish the Collaborative for Microbial Symbiosis and *Phragmites* Management (hereafter called the *Phragmites* Symbiosis Collaborative or PSC). The PSC was initiated in February 2013 to advance microbe-based research on the control of invasive *Phragmites*. This powerful collaborative approach is new because it brings together an international group of researchers from many disciplines and agencies to focus on the development of an innovative microbe-based control strategy for invasive *Phragmites*.

The objectives of the PSC are to establish the current state of the science, identify research gaps, and develop a science strategy (i.e., research agenda) to guide and support research on microbial symbiosis to maximize collective progress toward an integrated *Phragmites* control and habitat restoration strategy. The PSC agenda (**Table 1**) includes explicit steps that guide the scientific community in the development of new control methods based on microbiome manipulation. These mutually reinforcing steps target the competitive abilities of invasive *Phragmites*, as well as lay out principles and approaches that will serve as a foundation for application of microbiome manipulations to other invasive species. Using the five conditions of collective impact (a common agenda, a shared measurement system, mutually reinforcing activities, continuous communication, and a backbone support organization) to plan and support the initiative (Kania and Kramer, 2011), this collaboration of scientists is fostering progress toward a broader overall vision to maximize the collective impact of individual research efforts.

SUMMARY

Microbial communities affect plant health and productivity in many ways and likely contribute to the competitive success of invasive plant species. The symbiotic relationships between invasive plant species and their associated microbes offer a new target for development of control methods and management strategies. However, the spatial and temporal composition of microbial communities in invasive plants, as well as the roles they play in plant competition, are not well-characterized. Similarly, approaches for microbiome manipulation as a form of invasive species control are under development. Therefore, this paper reviewed the relevant science relating to plant-microbial interactions and identified a conceptual strategy for uncovering the microbial interactions that could influence invasion success. A case study on the invasive grass *Phragmites australis* showed how the collective impact approach can be applied to create a science agenda for development of microbe-based control strategies. The steps outlined in this case study will serve as both a foundation for similar microbe-based control efforts targeting other invasive species and a model of the collective impact approach that can be applied to other avenues of research and management.

AUTHOR CONTRIBUTIONS

The manuscript was drafted by KK, CB, WB, HB, KC, ML, MM, EN, MT, and JW with editorial remarks from DW.

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GLOSSARY

Arbuscular mycorrhizal fungi (AMF): Group of fungi in order Glomales that colonize plant roots and enhance plant growth by increasing absorption of minerals from soils.

Biotic resistance hypothesis: States that species-rich communities are more resistant to invasion because they are able to use the resources more efficiently than communities with low species richness.

Biotrophic: Describes an organism that can live and multiply only on another living organism, such as parasitic or symbiotic bacteria and fungi.

Class 1 Fungal endophytes: Fungi in the family Clavicipitaceae that have a narrow host range (grasses) and colonize shoot and rhizome tissues.

Class 2 Fungal endophytes: Non-clavicipitaceous fungi that have a broad host range and colonize shoot, root, and rhizome tissues.

Class 3 Fungal endophytes: Non-clavicipitaceous fungi that have a broad host range and colonize above-ground tissues.

Class 4 Fungal endophytes: Non-clavicipitaceous fungi that have a broad host range and colonize root tissues.

Collective impact: The commitment of a group of actors from different sectors to a common agenda for solving a complex problem; individual impacts are multiplied through collective effort.

Ecological resistance: Reduced invasion success in a native community associated with multiple biotic processes, including predation, competition, herbivory, or disease.

Endophyte: An organism, often a bacterium or fungus, that lives within the tissues of living plants; relationships with plant vary from symbiotic to nearly pathogenic.

Enemy release hypothesis: States that the success of exotic organisms is due to escape from natural enemies within their native range.

Epichloë: A genus of systemic and constitutive fungal symbionts of cool-season grasses.

Epiphytes: Microbes that grow and persist on plant surfaces.

Haplotype: A designation based on a group of genes within an organism that was inherited together from a single parent.

Horizontal transmission: Transmission of an infective agent (e.g., microbe) between individuals in a population.

Microbiome: All of the microorganisms that associate with another organism either externally or internally.

Mutualism: A relationship between two organisms in which both benefit from the association.

Mycorrhizal: Refers to fungi that associate with plant roots and facilitate the uptake of nutrients.

Oomycete: Eukaryotic microorganisms within the kingdom Chromista, characterized by biflagellate swimming zoospores and the formation of oospores.

Pathogen: A microbe capable of causing host damage.

Phyllosphere: Surface of plant leaves that may be colonized by microorganisms.

Phytosphere: Plant ecosystem including the exterior and interior of both aboveground and belowground portions of plants.

PSC: Collaborative for Microbial Symbiosis and *Phragmites* Management.

Rhizosphere: Area of soil surrounding plant roots where the abundance and activity of microorganisms is elevated due to root carbon deposition.

Symbiosis: Interaction between two different organisms living in close association, typically to the advantage of both (includes mutualism, commensalism, parasitism).

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ORIGINAL RESEARCH
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Crucial Roles of Abscisic Acid Biogenesis in Virulence of Rice Blast Fungus *Magnaporthe oryzae*

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Rice suffers dramatic yield losses due to blast pathogen *Magnaporthe oryzae*. *Pseudomonas chlororaphis* EA105, a bacterium that was isolated from the rice rhizosphere, inhibits *M. oryzae*. It was shown previously that pre-treatment of rice with EA105 reduced the size of blast lesions through jasmonic acid (JA)- and ethylene (ETH)-mediated ISR. Abscisic acid (ABA) acts antagonistically toward salicylic acid (SA), JA, and ETH signaling, to impede plant defense responses. EA105 may be reducing the virulence of *M. oryzae* by preventing the pathogen from up-regulating the key ABA biosynthetic gene *NCED3* in rice roots, as well as a β-glucosidase likely involved in activating conjugated inactive forms of ABA. However, changes in total ABA concentrations were not apparent, provoking the question of whether ABA concentration is an indicator of ABA signaling and response. In the rice-*M. oryzae* interaction, ABA plays a dual role in disease severity by increasing plant susceptibility and accelerating pathogenesis in the fungus itself. ABA is biosynthesized by *M. oryzae*. Further, exogenous ABA increased spore germination and appressoria formation, distinct from other plant growth regulators. EA105, which inhibits appressoria formation, counteracted the virulence-promoting effects of ABA on *M. oryzae*. The role of endogenous fungal ABA in blast disease was confirmed through the inability of a knockout mutant impaired in ABA biosynthesis to form lesions on rice. Therefore, it appears that EA105 is invoking multiple strategies in its protection of rice from blast including direct mechanisms as well as those mediated through plant signaling. ABA is a molecule that is likely implicated in both tactics.

Keywords: ***Magnaporthe oryzae*, rice blast, *Pseudomonas*, ABA, ISR**

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INTRODUCTION

Rice (*Oryza sativa*) is a staple food crop world-wide, providing about one fifth of the calories consumed by humans. One of the largest problems impacting rice production is crop loss due to blast disease, caused by the hemibiotrophic fungal pathogen, *Magnaporthe oryzae*. Previously, we isolated and characterized a natural rice rhizospheric bacterium, EA105, that shows a strong direct antagonism toward *M. oryzae* vegetative growth and pathogenesis. When EA105 is root-inoculated on rice plants, it also triggers Induced Systemic Resistance (ISR) resulting in smaller blast lesions (Spence et al., 2014a), facilitated through jasmonic acid (JA) and ethylene (ETH) but not salicylic

acid (SA) signaling (Spence et al., 2014b). Although SA, JA, and ETH are the three hormones that are most closely linked to plant defense, another important component is the stress responsive hormone, abscisic acid (ABA).

Abscisic acid is a small signaling molecule involved in multiple plant processes including seed dormancy, development, and response to abiotic and biotic stresses. Due to its involvement in numerous and overlapping processes, the activity of ABA is complex and tightly regulated at multiple steps. ABA is detected by a receptor complex, PYR/PYL/RCAR. The family of small soluble receptors termed PYR/PYL were identified using a synthetic ABA agonist, pyrabactin, and were named Pyrabactin Resistance 1 (PYR) and PYR1-Like (PYL). Within this family, some of the proteins were identified concurrently and also given the name Regulatory Components of ABA Receptors, or RCAR (Santiago et al., 2012). In the absence of ABA, PYR/PYL/RCAR receptors are found dimerized in the cytosol and nucleus. The binding of ABA results in dissociation of the dimers, and the monomer that is bound to ABA undergoes a conformational change forming a binding site for Phosphatase type 2 Cs (PP2Cs) (Santiago et al., 2012). The PP2Cs are inactivated when bound to the ABA-receptor complex. In the absence of ABA, PP2Cs inactivate SNF-1 Related Kinases (SnRK2s) which are positive regulators of ABA signaling (Szostkiewicz et al., 2010). When SnRK2s are no longer inhibited by PP2Cs, they enter the nucleus and phosphorylate targets such as *SLAC1, KAT1, AtRbohF*, thus activating, transcription factors which positively influence expression of stress/ABA-responsive genes (Kulik et al., 2011). These genes contain sequences within their promoters called ABA Responsive Elements (ABREs) that are recognized by transcription factors, referred to more specifically as ABRE-binding proteins (AREBs) or ABRE binding factors (ABFs) (Fujita et al., 2013).

The amount of ABA in a particular location within a plant tissue depends on biosynthesis, catabolism, transport, and compartmentalization (Ye et al., 2012). In plants, ABA is synthesized through the cleavage of carotenoids in a multi-step pathway (Schwartz et al., 1997, 2003). The key regulatory enzyme in ABA biosynthesis is coded for by Nine-*cis*-epoxycarotenoid dioxygenase 3 (*NCED3*) (Liotenberg et al., 1999; Qin and Zeevaart, 1999). The first committed step in ABA catabolism is hydroxylation of the methyl group at the eighth carbon position. ABA contains three methyl groups, that can be hydroxylated, with C8 hydroxylation being most closely linked to catabolism. Hydroxylation does not inactivate ABA, but it can flag ABA for conversion into phaseic acid (PA) and subsequently dihydrophaseic acid (Ye et al., 2012). In rice, there are three enzymes with differing expression patterns that hydroxylate ABA, *OsABA8ox1*, *2*, and *3* (Ye et al., 2012) and are homologs of *Arabidopsis thaliana CYP707A* (Nambara and Marion-Poll, 2005). *OsABA8ox1* is primarily responsible for ABA catabolism following drought stress, and is negatively regulated by ethylene (Saika et al., 2007). Additionally, ABA can be reversibly inactivated through conjugation, primarily to glucosyl esters forming ABA-GE that can be stored in vacuoles or apoplasts. The addition of glucosyl esters to ABA is catalyzed by an ABA glucosyl transferase (Xu et al., 2002) and the conjugate can be subsequently removed through hydrolysis by a β-glucosidase such as *AtBG1* (Lee et al., 2006). Glucosyl transferases and β-glucosidases involved in the inactivation and activation of ABA have not previously been characterized in rice.

While ABA is a crucial molecule for regulating plant growth, development, and stress response, some phytopathogens have evolved mechanisms to stimulate overproduction of ABA in plants, resulting in the suppression of Systemic Acquired Resistance (SAR), while also causing a reduction in growth, transpiration, and photosynthesis (Loake and Grant, 2007). SAR is typically mediated through SA signaling, and ABA acts antagonistically to SA signaling, blocking the SAR response (Jiang et al., 2010; Xu et al., 2013; Meguro and Sato, 2014). It has been shown multiple times that ABA suppresses not only SA-mediated defense signaling but also JA and ETH-mediated defense signaling (Anderson et al., 2004; Adie et al., 2007; Ton et al., 2009; Atkinson and Urwin, 2012; Nahar et al., 2012). Elevated ABA levels in rice plants are associated with increased disease severity of rice blast caused by *M. oryzae* (Koga et al., 2004; Jiang et al., 2010; Yazawa et al., 2012) as well as bacterial blight caused by *Xanthomonas oryzae* (Xu et al., 2013). The reverse highlighted the same point; knocking down ABA levels reduces susceptibility to blast by impairing the ability of *M. oryzae* to penetrate host cells, ultimately resulting in reduced disease symptoms (Yazawa et al., 2012).

In addition to increasing plant susceptibility to disease, we hypothesize that high levels of ABA in fungi may also directly promote virulence in *M. oryzae*. ABA biosynthesis and signaling is likely to be an ancient process found in early unicellular eukaryotes which has followed divergent evolution (Hauser et al., 2011). Several phytopathogens retain the ability to synthesize ABA, though the role of fungal-derived ABA is still unclear and there is evidence that most fungal-produced ABA is secreted (Hartung, 2010). The fungal ABA biosynthesis pathway is distinct from that of plants. The ABA biosynthesis gene cluster in fungi was first identified in *Botrytis cinerea* (Siewers et al., 2006) and has been named the "direct" or "mevalonate" pathway in contrast to plants that cannot use mevalonic acid as a precursor to ABA biosynthesis (Hirai et al., 2000). ABA perception and signaling mechanisms have also diverged, with differences arising even between monocots and dicots (Hauser et al., 2011). ABA production has been documented in *M. oryzae* races 007.0 and 102.0 (Jiang et al., 2010) though it has not previously been shown in the sequenced reference strain, 70-15. We have examined the role of ABA in a three-way communication between rice, the fungal pathogen *M. oryzae*, and a natural rice beneficial bacterial isolate with the goal of further elucidating the mechanisms by which this bacterium can directly antagonize *M. oryzae* and trigger ISR in rice to protect against blast.

MATERIALS AND METHODS

Fungal and Bacterial Strains and Growth Conditions

Wild type *M. oryzae* 70-15, the sequenced reference strain, was used throughout the experiments. For vegetative growth, the

fungi were placed on complete medium (CM) containing sucrose (10 g/L), casamino acids (6 g/L), yeast extract (6 g/L), and 1 mL of *Aspergillus nidulans* trace elements (Per 100 mL: 0.22 g $MnSO_4 \cdot H_20$, 0.05 g KI, 0.02 g $ZnSO_4 \cdot 7H_20$, 0.01 g H_3BO_4, 0.1 mL concentrated H_2SO_4, 0.008 g $NiCl_2 \cdot 6H_20$, 0.007 g $CoCl_2 \cdot 6H_20$). Oatmeal agar consisting of ground oats (50 g/L) and agar (15 g/L) were used for sporulation. Plates were kept at 25°C with constant fluorescent light. Bacterial strains EA105, EA106, and EA201 were isolated from rhizospheric soil surrounding the roots of rice cultivar M-104 grown in the field by Dr. Venkatesan Sundaresan's lab from the University of California (Davis). The bacteria were cultured in liquid or solid Luria Bertani (LB) medium at 28–30°C.

Plant Materials and Growth Conditions

Oryza sativa cultivar M-104 was donated by Dr. Venkatesan Sundaresan from the University of California (Davis). Hypersusceptible genotype Seraceltik was used for *M. oryzae* infections. Rice seeds were dehusked and sterilized using ethanol and bleach. Ten-day-old seedlings were transferred to sterile clear plastic boxes containing 50 mL of Hoagland's medium, pH 5.7 and placed on a shaker at 80 rpm. Treatments were done at 14 days.

Rice Treatments

Exponential phase bacterial cultures were washed in water and re-suspended to approximately 1×10^9 cells per mL. In each clear box containing rice seedlings, 50 μL of bacteria was added to the media, for a final concentration of 1×10^6 cells per mL. For 70-15 treatment, spores were grown on oatmeal, then scraped into sterile water and filtered through a sterile miracloth (EMD Millipore). Rice leaves were dipped in a spore suspension containing 0.2% gelatin and 1×10^5 spores per mL for 5 min. In the tri-trophic interaction, spores were added 24 h after bacterial treatment. For growth hormone treatments, each was added into the liquid media at the following concentrations: ABA (100 μM), IAA (20 μM), IBA (1 μM), Kinetin (100 μM), GA (50 μM). Kinetin stock was prepared in 1N NaOH, while ABA, IAA, IBA, and GA stocks were prepared in methanol. All were filter sterilized. Controls were treated with an equal amount (1 μl per mL) of methanol. Each treatment was done in biological triplicate, and each biological replicate contained five plants.

In Planta Infection Assays

Rice plants of cultivars M-104, Nipponbare, and Seraceltik were grown in soil for 3 weeks. To check the effect of bacterial priming, overnight cultures of bacteria were washed in water and re-suspended to 0.5 OD. For each plant, 2 mL of bacteria were dispensed onto the soil surface at the base of the plant. At 24 h post bacterial treatment, the second youngest leaf was cut and affixed to a large 15 cm diameter petri dish, on top of moistened paper towels and treated with 70-15 spores. Spores were grown on oatmeal agar for 10 days, and were subsequently scraped into sterile water with 0.2% gelatin. Spore concentration was adjusted to 10^5 spores/mL. On each leaf, a total of 4–30 μL droplets of spores were placed along the length. Plates were incubated in the dark for 24 h at 25°C, after which time the spore droplets were wicked away. Plates were then kept in cycles of 16 h light/8 h darkness for 5 days at 25°C. On the fifth day, the length and width of lesions were measured. A minimum of eight leaves with four droplets per leaf were included per replicate. Three biological replicates were completed.

Spore Germination and Appressoria Formation Assays

Plastic coverslips were sterilized with ethanol and UV, and used as a hydrophobic surface to encourage spore germination and appressoria formation. *M. oryzae* spores were grown on oatmeal agar for 10 days prior to being scraped into water and filtered through a miracloth. Each coverslip was inoculated with a 50 μL drop containing a final concentration of 10^5 spores/mL, and/or 10^5 bacterial cells/mL (also in water). The coverslips were placed in petri dishes with wet filter disks in the center to promote humidity. Plates were sealed and placed in the dark. Germination percentages were calculated after 2 h incubation, and appressoria formation was determined after 6 h. Coverslips were imaged using a Zeiss Axioscope2 light microscope. Three images were taken per coverslip, and five coverslips were used per treatment. Three biological replicates were examined.

ABA Quantification

Plants and spores were treated as described above. Roots, shoots, and fungal tissue were ground in liquid nitrogen. For plant samples and spore samples, approximately 100 mg of tissue was used. For fungal mycelial samples, approximately 250 mg of tissue was used. ABA was extracted from each sample in 1 mL of 90% methanol containing 10 mg of butylated hydroxytoluene and 20 mL of glacial acetic acid per liter. The extraction was done at 4°C for 24 h. The samples were then used with the Phytodetek Abscisic Acid ELISA-based kit, per the directions. Plant samples were run at the following dilutions (in TBS buffer): 1:10, 1:20, 1:40, 1:80, and 1:160. Fungal samples were run at the following dilutions: 1:10, 1:100, 1:200, and 1:400. ABA content was calculated per gram of tissue. Each biological replicate consisted of five plants or five fungal plates, and the experiment was done in biological triplicate.

Gene Expression

Tissue was ground in liquid nitrogen and RNA was extracted using the EZ-10 Total RNA Mini-prep kit (BioBasic). Samples were treated with DNaseI (Thermo Scientific) and cDNA was synthesized from 500 ng of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Standard Taq polymerase (New England Biolabs) was used for PCR, and products were run on a 1.4% agarose gel. Gene-specific primers are listed in Supplementary Table S2. Band intensities were quantified using ImageJ, and normalized to ubiquitin expression. Each biological replicate was pooled from 5 plants, and each treatment was done in biological triplicate.

Constructing Mutants in *M. oryzae* 70-15

Adaptamer-mediated PCR (Reid et al., 2002), a method based on homologous recombination, was used to create knock out mutants in strain 70-15. Briefly, genes of interest were replaced with a hygromycin resistance cassette. For each gene to be

knocked out, a 1.2 kb segment upstream of the 5′ UTR and another 1.2 kb segment downstream of the 3′ UTR was amplified. Adaptors were added to the 3′ end of the first segment and the 5′ end of the second segment. The hygromycin resistance cassette was amplified from plasmid pCB1003 using primers that had adaptors complementary to those used in amplifying the upstream and downstream segments. All three segments were combined in a PCR reaction to make the full-length constructs, approximately 3.3 kb. Creation of protoplasts and transformations were conducted following traditional methods (Sweigard et al., 1992). Gene disruption mutants were identified by PCR screening using primers outside the flanking regions and gene specific primers and further confirmed by Southern blot analysis. Primers are listed in Supplementary Table S3.

Southern Hybridization

Total DNA of *M. oryzae* 70-15 and mutants was isolated using Qiagen DNeasy plant mini kit (Qiagen, Inc.,) and digested with restriction enzymes, electrophoresed on 1.0% agarose gels in 1 × TAE buffer, and transferred onto Whatman Nytran SPC nylon membrane (Whatman, Inc.,) overnight by capillary action. DIG labeled DNA probes of HPH were generated using a DIG Probe Synthesis Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA), hybridized to blots overnight at 48°C, washed under high stringency conditions and exposed to Kodak Biomax light film. Procedures recommended by the manufacturer of the kits were used. Although we are aware of its utility, a complemented line for the ABA4 mutant was not created in this study due to time and resource limitations. However, our experiments with exogenously applied ABA provide strong proof that the phenotypes observed are related to the deletion of the ABA4 gene.

Sporulation Assay

A 5 mm plug of spores was plated in the center of an oatmeal agar plate. Plates were kept under fluorescent light with a cycle of 16 h light/ 8 h dark at room temperature. After 7 days, the surface of the plate was scraped to collect the spores, which were put into 1 mL of water. The spores were then counted using a haemocytometer. For each strain, there were three biological replicates, done in technical quadruplicate.

Statistical Analysis

Statistical analyses of the results were performed using the statistical software JMP 10. To compare across treatments, the Tukey's HSD test was used and results were considered to be statistically different when $p < 0.05$.

RESULTS

EA105 May Prevent 70-15 Spores from Increasing ABA Biosynthesis and Signaling in Rice

Previously, we showed that when *Pseudomonas chlororaphis* EA105 (hereafter EA105) was inoculated onto uninfected rice plants, there was approximately a 10-fold increase in the ETH responsive genes *EIL1* and *ERF1* as well an approximately threefold increase in the JA responsive genes *JAR1* and *WRKY30* at 24 h post treatment (Spence et al., 2014b). However, the SA responsive genes *PR1* and *WRKY77* were minimally affected (Spence et al., 2014b). From that data, it was concluded that isolate EA105 induces systemic resistance in rice against blast in an ETH and JA dependent manner (Spence et al., 2014b). Since elevated ABA levels are associated with increased susceptibility, we examined the expression of *NCED3*, the rate-limiting enzyme involved in ABA biosynthesis, in roots where *NCED3* is most active. 70-15 spores up-regulated *NCED3* while EA105 did not affect its expression. Interestingly, 70-15 spores were unable to induce *NCED3* expression in plants that were pre-treated with EA105 (**Figure 1A**). For comparison, two other isolates recovered from the same soil sample as EA105 were also tested (Spence et al., 2014a). Isolate EA201 inhibits fungal diameter *in vitro* but root treatment does not reduce lesions. Contrastingly, EA106 has no direct antifungal capabilities, but does induce resistance when treated at the root surface. EA105 is distinct from both, because it can directly inhibit fungi and also induce resistance against *M. oryzae* (Spence et al., 2014a). Pretreatment with rice isolates EA106 and EA201 did not prevent 70-15 from up-regulating *NCED3* (**Figures 1B,C**). To see if *NCED3* up-regulation coincided with higher ABA levels, total ABA concentrations in roots and shoots treated with bacteria, fungus, or both were examined. However, there were no significant differences in the ABA content (**Figure 2A**). In all treatments, there were approximately 2000–2500 picomoles of ABA per gram of plant tissue. ABA content was checked at the same time-point used for expression analysis as well as 24 h later, and still no differences were apparent (**Figure 2B**). At the second time-point, there was actually a slight increase in ABA levels in plants that were treated with both EA105 and spores (**Figure 2B**). ABA concentrations were also determined in 70-15 spores and mycelia. We found that ABA is produced by 70-15, with mycelia producing around 200 picomoles per gram and spores producing more than 400 picomoles per gram (**Figure 2C**).

Since the ABA concentrations did not match what was seen with *NCED3* expression patterns, it was necessary to examine other steps at which ABA signaling is regulated. We designed primers for multiple genes related to ABA including those involved in catabolism, perception, signaling, and response, to gain a better understanding of how EA105 and 70-15 spores are affecting ABA signals in rice. The expression of *OsABA8ox1*, involved in ABA catabolism in rice, was largely unaffected by any of the treatments; however, there was slightly less of an induction of *OsABA8ox1* in plants treated with spores or exogenous ABA (Supplementary Figures S1 and S2). Similarly, there were no significant expression changes in ABA receptor *RCAR5* (Supplementary Figures S1 and S2).

Abscisic acid activity can also be modulated through inactivation and activation by adding or removing, respectively, glucosyl esters. In rice, the genes involved in this process had not previously been characterized. A BLAST search of the protein sequence for *AtBG1*, a gene implicated in the activation of ABA in *A. thaliana*, revealed a putative rice β-glucosidase. This gene was

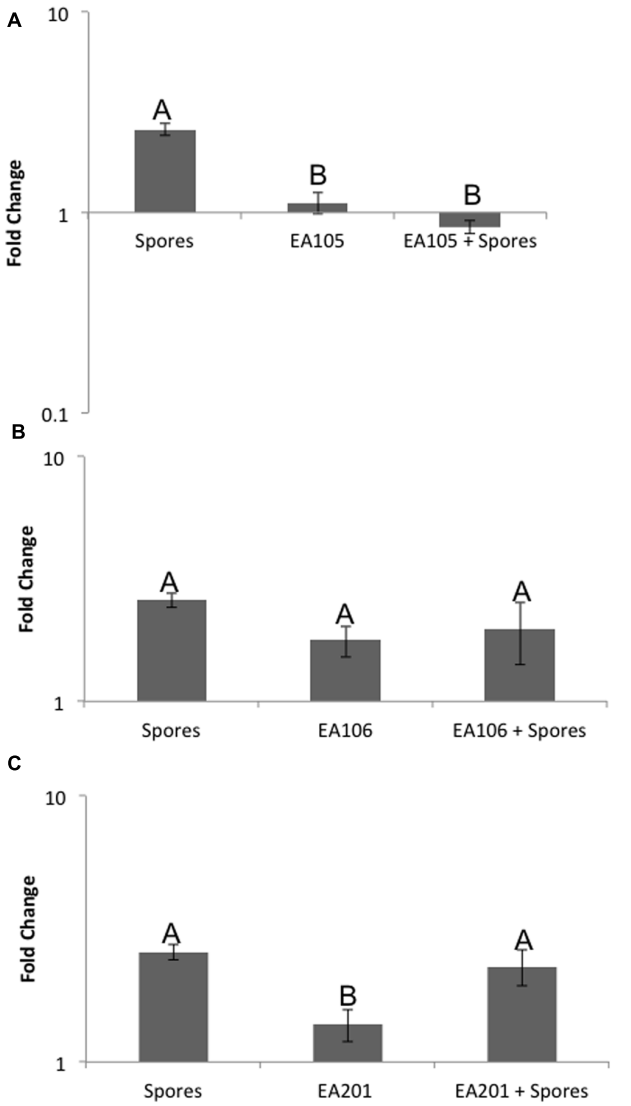

FIGURE 1 | Expression of ABA biosynthesis gene, *NCED3*. Roots were inoculated with either **(A)** EA105, **(B)** EA106, or **(C)** EA201, 24 h prior to being exposed to spores. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test, $p < 0.05$.

expressed more strongly in shoots than roots, and its expression in shoots was induced by ABA (Supplementary Figure S2). When plants were treated with EA105 and ABA together, there was no longer up-regulation of this gene. Although the expression of this gene was weaker overall in roots, there was nearly 24-fold up-regulation in plants infected with 70-15 spores (Supplementary Figure S3). Similar to what was seen with *NCED3*, the presence of EA105 prevents spores from up-regulating this ABA-activating gene in roots. In *A. thaliana*, the inactivation of ABA is typically catalyzed by *UGT71B6*, a UDP glucosyl transferase. Using a protein BLAST of this gene, a putative rice glucosyl transferase was found. Expression of this gene in roots was very low compared to shoots. Interestingly, shoot expression patterns mimicked those of the β-glucosidase, with ABA inducing this gene more than 10-fold while EA105 + ABA eliminated the induction of this gene (Supplementary Figure S2).

Moving further through the ABA signaling pathway, the expression of Mitogen-activated protein (MAP)-kinase *OsMPK1* was also examined, but showed minimal expression changes (Supplementary Figure S1). When ABA signaling is able to proceed, it culminates in the expression of genes containing ABREs in their promoter regions. One such gene of many is *Rab25*, although its expression was not affected by the treatments (Supplementary Figure S2).

Exogenous ABA Promotes Virulence in *M. oryzae*

Pathogenesis of *M. oryzae* begins with spore germination. The crucial step in virulence is the formation of the appressorium, a specialized infection structure that accumulates high turgor pressure and forms a penetration peg that can enter the rice cuticle. To test whether ABA could enhance pathogenicity in *M. oryzae*, 70-15 spores were treated with ABA ranging from 10 to 100 μM. By 3 h almost all spores germinated in the untreated controls, and by 24 h most formed appressoria. To see if ABA was accelerating these processes, germination was examined at 2 h and the initiation of appressoria formation was examined at 6 h. Spores that had been exposed to 50 or 100 μM ABA had a higher percent germination than those that were not exposed to ABA at 2 h post treatment (**Figure 3**). Also, the percent of germinated spores that were forming appressoria at 6 h post treatment was higher in the presence of 50 or 100 μM ABA (**Figure 3**). In addition to ABA, other plant growth regulators were tested including gibberelic acid (GA), the natural auxin indole-3-acetic acid (IAA), a synthetic auxin indole-3-butryic acid (IBA), and the cytokinin, kinetin. Aside from ABA, only GA was able to increase percent germination at 2 h, but did not increase appressoria formation at 6 h (Supplementary Figure S4). Kinetin had no effect on germination, but increased appressoria formation at 6 h. The natural and synthetic auxins had no effect on *M. oryzae* 70-15 spore germination or appressoria formation (Supplementary Figure S4). The germination and appressoria formation was also quantified in spores treated with EA105 and ABA together. Previously, we have shown that EA105 had a minimal effect on spore germination at 3 h, but almost completely abolished appressoria formation in *M. oryzae* at 24 h (Spence et al., 2014a). At the earlier time points, EA105 behaves similarly. The percent germination is not statistically different from the control, while appressoria formation is greatly reduced (**Figure 4**). When EA105 and ABA are co-treated on spores, the percent germination is about half way between what was seen with ABA or EA105 treatment alone. The percent of spores that formed appressoria decreased from about 84% with ABA treatment alone to about 23% when treated with both EA105 and ABA together (**Figure 4**).

Impairing ABA Biosynthesis in *M. oryzae* Dramatically Reduces Virulence

Although the genes responsible for ABA biosynthesis in *M. oryzae* have not been identified or characterized, we found

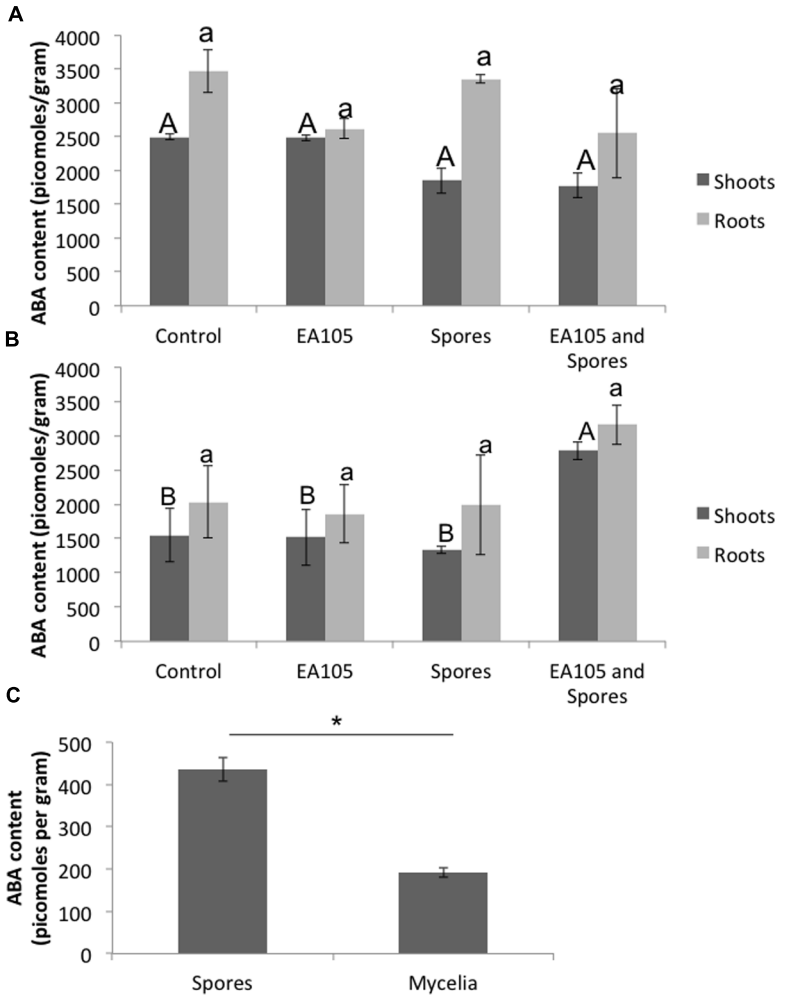

FIGURE 2 | Abscisic acid (ABA) concentrations in rice roots and shoots, as well as 70-15 spores and mycelia, using an ELISA-based kit. Root and shoot tissue was collected separately at **(A)** 24 h post 70-15 treatment and **(B)** 48 h post 70-15 treatment for ABA extraction. **(C)** ABA content was quantified in untreated 70-15 spores and mycelia. Error bars indicate standard error based on three biological replicates, each pooled from 5 plants, or 3 plates of fungi. Each replicate was quantified in quadruplicate. Different letters represent a statistically significant difference based on the Tukey-Kramer test when $p < 0.05$. For spores and mycelia, mean ABA concentration is statistically significant, $p = 0.0036$, based on a *t*-test.

hypothetical genes in 70-15 that were orthologous to those responsible for ABA biosynthesis in the fungal pathogen *Botrytis cinerea*. In *B. cinerea*, there are four biosynthesis genes (Siewers et al., 2006). Orthologs were found for all the genes except ABA3 (Supplementary Table S1). Based on the annotated *M. oryzae* 70-15 genome, we also found an ABA G-protein coupled receptor (GPCR) gene. All four genes are located on chromosome 3 in 70-15 (Supplementary Table S1; Supplementary Figure S5). To determine if these genes were functional, their expression was examined in 70-15 spores and mycelia. For all the genes, expression in spores was higher than in mycelia and ABA2 did not appear to be expressed at all in mycelia (Supplementary Figure S6). Expression was also examined in spores and mycelia that were treated with EA105, but only very subtle down-regulation of the genes occurred (Supplementary Figure S6). There were no significant changes in ABA content with EA105 treatment, though there was a slight but non-significant decrease in ABA content of mycelia treated with EA105 compared to untreated mycelia (Supplementary Figure S6).

An attempt was made to knock out ABA1, 2, and 4 in 70-15, however, potential ABA1 and ABA2 mutants were extremely slow growing and small, and we were unable to confirm that these were mutants. ABA4 was successfully knocked out, as well as the ABA GPCR (Supplementary Figure S5). The GPCR mutant largely resembled the parental 70-15, but the ABA4 mutant had distinct phenotypic differences in multiple stages of its life cycle. Vegetatively, the 70-15ΔABA4 mutant grew slower than 70-15 and never reached the same size (**Figure 5A**). Additionally, the mycelia became darkly pigmented beginning around 7 days after re-culturing and striking difference were apparent by 14 days (**Figure 5B**). Sporulation was also impaired in 70-15ΔABA4, while 70-15ΔGPCR produced more conidia

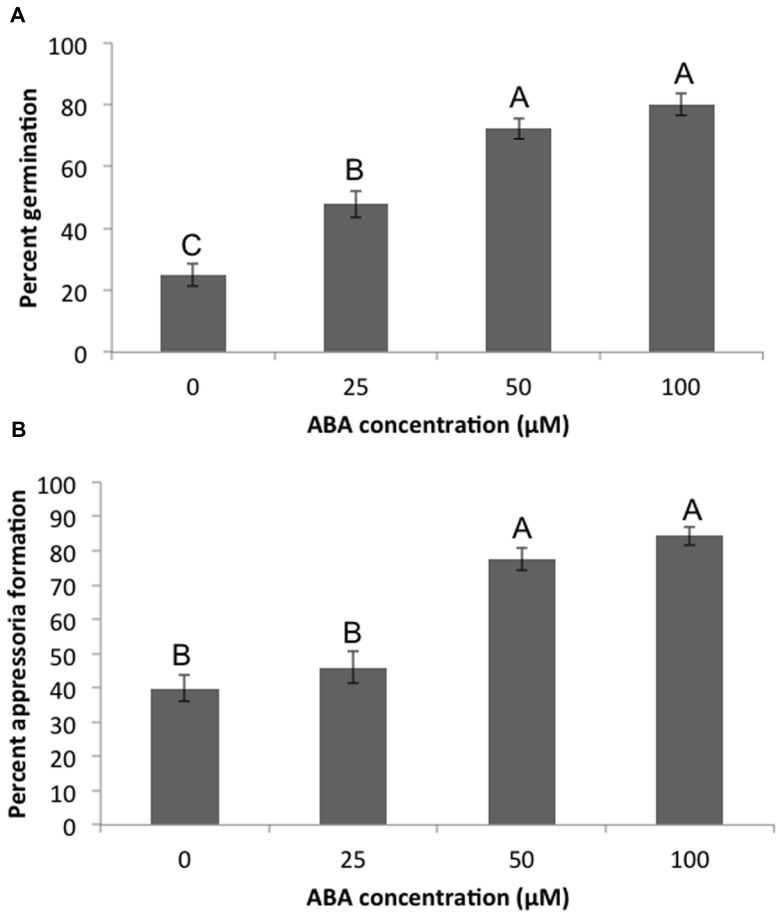

FIGURE 3 | ***Magnaporthe oryzae*** **70-15 spores treated with different concentrations of ABA.** Images were taken at 2 and 6 h post-treatment and the percentage of spores **(A)** germinating or **(B)** forming appressoria were quantified. This experiment was repeated three times with five coverslips per treatment and three images per coverslip. Different letters represent statistical significance based on the Tukey-Kramer test ($p < 0.05$).

than 70-15 (Supplementary Figure S7). The appearance of the 70-15ΔABA4 spores was also different than that of the wild-type, with unusual white patches visible on the spore plates (Supplementary Figure S7). Additionally, a very dark pigment was secreted into the media, and was left behind after spores were removed from the plates. There were not any significant differences in germination, although germination of both mutants was inhibited by EA105 treatment, which was not the case for 70-15 (**Figure 6A**). The 70-15ΔABA4 mutant was severely impaired in appressoria formation as compared to the other two strains (**Figure 6B**) and there were distinct phenotypic differences that could be seen microscopically. The ABA4 mutants showed hyper-branching of the germ tubes, as well as unusual bulges along the hyphae with less melainized appressoria (**Figure 6C**).

Abscisic acid was quantified in the mycelia and spores of both mutants and compared with parental 70-15. In mycelia, both mutants produced roughly half the ABA of 70-15. The total amount of ABA produced by spores was higher than that in mycelia. The ABA4 mutant spores produced slightly less than half the amount of ABA produced by wild type spores. Similarly, the GPCR mutant spores also produced approximately half the amount of ABA that was produced by wild-type (**Figure 5D**).

Interestingly, adding exogenous ABA to the media did not complement 70-15ΔABA4's vegetative growth defect, nor did it effect growth of 70-15 or 70-15ΔGPCR (**Figure 5C**). However, adding exogenous ABA to spores enabled the ABA4 mutant to form as many appressoria as the parental strain (**Figure 6B**) and partially complemented the aberrant phenotype of hyperbranching and bulging.

The mutants were also tested for their ability to form lesions on rice leaves. Strikingly, the 70-15ΔABA4 mutant was unable to properly infect. In many cases, the mutant did not have any effect on the leaf, or left a tiny black speck (**Figure 7**). The small spots that formed were much smaller than the lesions caused by 70-15 or 70-15ΔGPCR (**Figure 7**). When the experiment was extended for several more days, all of the leaves began drying and curling, and the lesions from 70-15 and 70-15ΔGPCR kept spreading, but the 70-15ΔABA4 spots did not grow. The mutants were also tested on plants that had been root-primed with EA105 for 24 h. EA105 retained the ability to reduce lesion size caused by 70-15 and 70-15ΔGPCR, but since 70-15ΔABA4 did not form lesions,

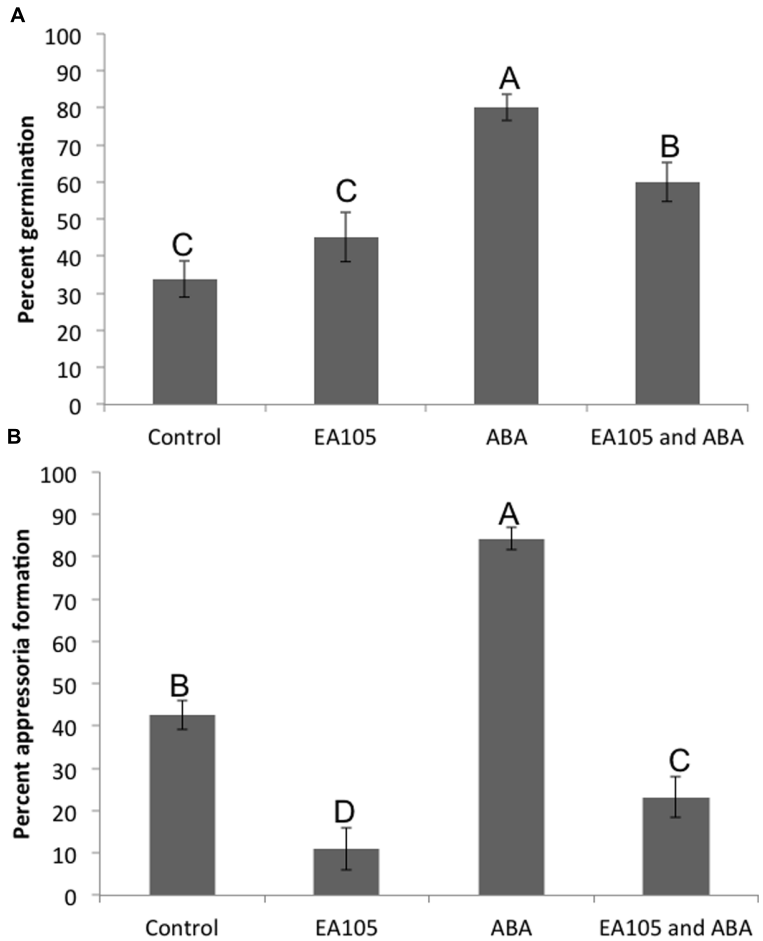

FIGURE 4 | ***Magnaporthe oryzae*** **70-15 spores treated with EA105 and/or exogenous ABA. (A)** Percent germination was quantified at 2 h post treatment and **(B)** percent appressoria formation was quantified at 6 h post treatment. The experiment was repeated three times with five coverslips per treatment and three images per coverslip. Different letters represent statistical significance based on the Tukey-Kramer test ($p < 0.05$).

there was no effect from EA105 priming. The same trend was apparent in rice cultivars M-104 and Seraceltik (Supplementary Figures S7–S9).

DISCUSSION

With the global population expected to exceed 9 billion by 2050, developing an effective and sustainable method to reduce crop loss from rice blast could have a significant impact on food security. We have identified natural rice rhizospheric isolate EA105, which has the ability to reduce the symptoms of blast in rice. However, another important aspect in the development of a biocontrol solution is understanding normal disease progression and how it is interrupted. The small signaling molecules SA, JA, and ETH have been studied for their involvement in SAR and ISR but they do not act independently within plants. Crosstalk from multiple additional signals can promote or antagonize the effects of these signaling molecules on plant resistance. It is becoming apparent that ABA is a critical factor involved in modulating plant defenses that may be manipulated in different ways by pathogens and beneficial microbes. The study of ABA as it relates to biotic stress is relatively new, and there is very little known about how biocontrol bacteria affect this important hormone. However, a crucial role for ABA was recently discovered in the growth promotion of tomato by *Bacillus megaterium* (Porcel et al., 2014). Using the interaction between EA105, rice, and *M. oryzae* as a model system, we sought to investigate how the beneficial bacteria and the pathogenic fungus may be manipulating ABA to influence the susceptibility of rice to blast.

Since elevated ABA levels have been shown to increase rice susceptibility to *M. oryzae* and *X. oryzae* (Koga et al., 2004; Jiang et al., 2010; Yazawa et al., 2012; Xu et al., 2013), the effect of EA105 and 70-15 on the expression of key ABA biosynthesis gene *NCED3* was examined. As expected, 70-15 treatment up-regulated *NCED3* in rice, while EA105 had very little effect. Interestingly, when rice plants were treated with EA105 prior to infection with spores, there was no longer an up-regulation of *NCED3*, potentially indicating that EA105 is preventing spores

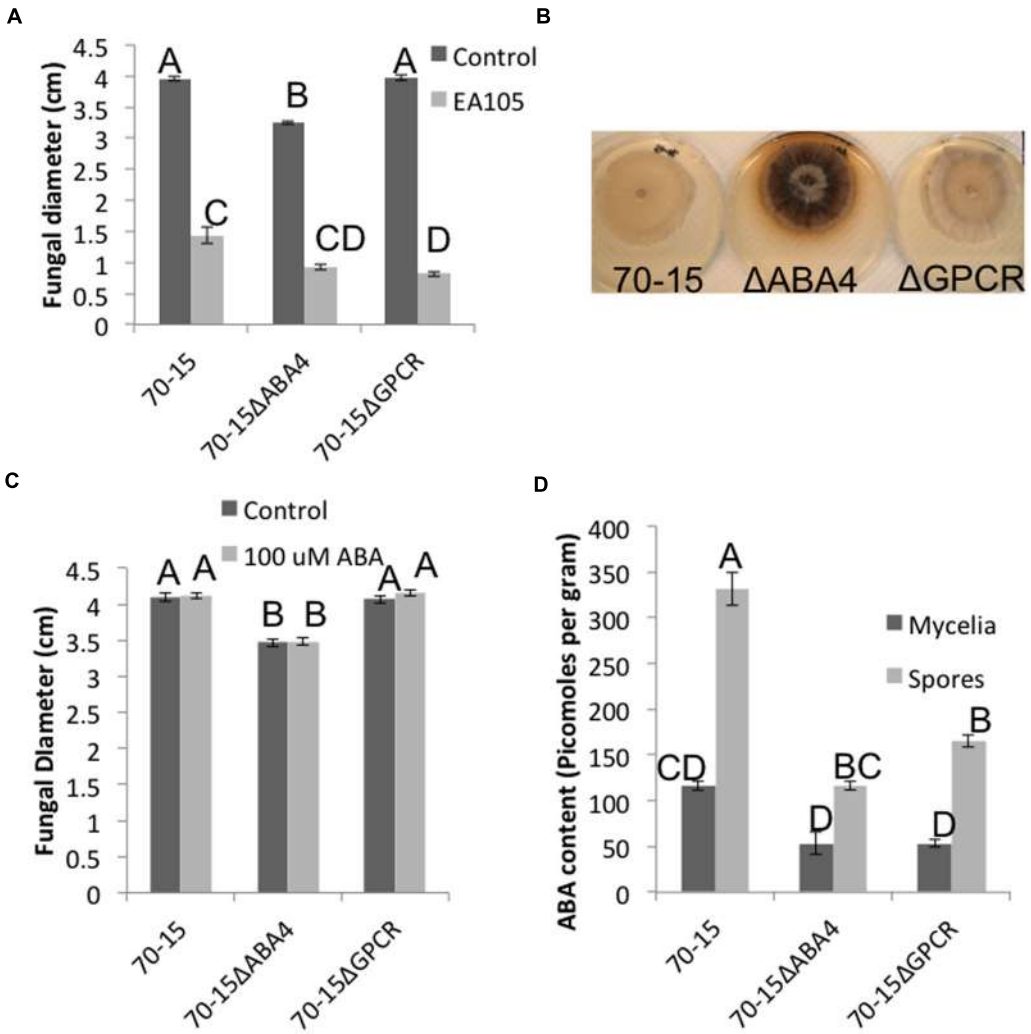

FIGURE 5 | Vegetative growth characteristics of ABA4 and ABA GPCR knockout mutants. (A) Fungal diameter of 70-15, 70-15ΔABA4, and 70-15ΔGPCR at 7 days after culturing, the in presence and absence of EA105. **(B)** At 14 days post culturing, 70-15ΔABA4 looks strikingly different than its parental strain due to pigmentation. **(C)** Fungi were grown on CM amended with 100 μM ABA and fungal diameters were measured after 7 days. **(D)** ABA content was quantified in spores and mycelia of 70-15 and the two mutants. Different letters represent statistical significance based on the Tukey-Kramer test ($p < 0.05$).

from increasing ABA biosynthesis in rice. When plants were treated with ABA, which also induces *NCED3* expression, and EA105, the up-regulation of *NCED3* persisted. EA105 was able to prevent spores but not exogenous ABA from up-regulating *NCED3* in rice, indicating that spores are affecting plants in ways that differ from ABA alone. In this aspect, EA105 was not able to counter the effects of ABA, yet it did attenuate the effects of spores.

In contrast to the expression of ABA biosynthesis gene *NCED3,* actual ABA concentrations were examined in plants with the same treatments and no differences were found. While it has been shown that elevated levels of ABA increase susceptibility to *M. oryzae* during very early stages of infection (Yazawa et al., 2012), it is possible that ABA once again becomes important in later stages of disease progression. For instance, in sugar beets the highest levels of ABA were not seen until 15 days post-infection, when the fungal pathogen switched to the necrotic phase of infection (Schmidt et al., 2008). *M. oryzae* is also a hemi-biotrophic fungus, and it is possible that ABA levels in rice only reach their maxima in later stages of necrosis.

The discrepancy between *NCED3* expression and ABA concentration has been shown previously (Priest et al., 2006; Ye et al., 2012). Beyond biosynthesis, ABA concentrations are also influenced by catabolism, activation and inactivation, transport, compartmentalization, and inhibition of signal transduction. In rice, primers were designed based on putative genes and both were highly responsive to exogenous ABA treatment. Similarly to what was seen with *NCED3*, spores increased expression of the putative β-glucosidase, potentially involved in activating ABA, in rice roots while the presence of EA105 prevented this.

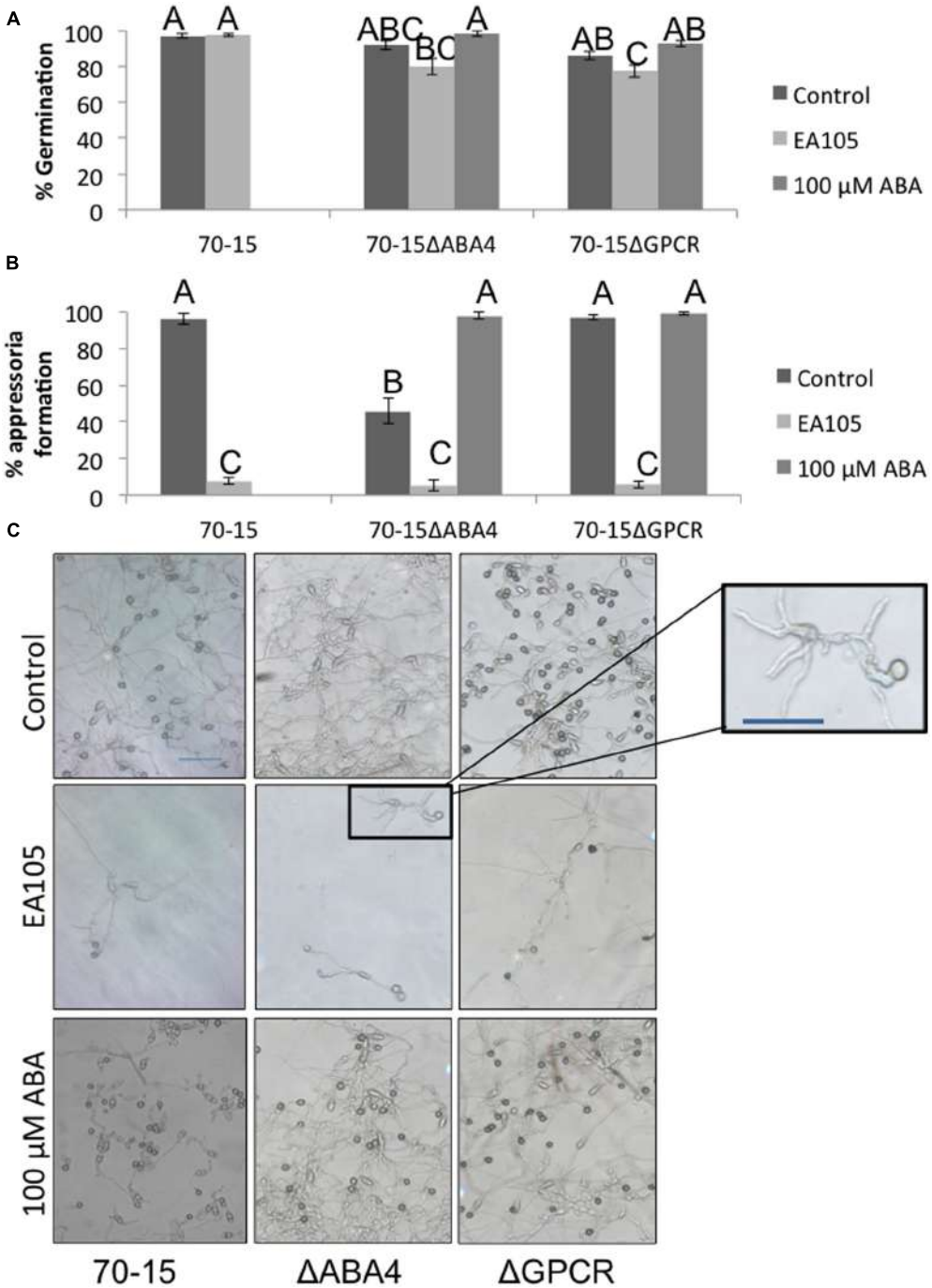

FIGURE 6 | Spore germination and appressoria formation in 70-15 and ABA mutants. (A) Spore germination was measured 3 h after resuspension and incubation on a hydrophobic plastic coverslip. **(B)** Appressoria formation was measured at 24 h. **(C)** Light microscope images were taken in areas which were dense with spores (top row) as well as in sparse areas where individual spores could be discerned. An enlarged imaged of the ABA4 mutant is displayed to show the hyperbranching phenotype. Scale bars are 50 μM. Supplementation of ABA (100 μM) reverts the phenotype in ABA4 mutant. Different letters represent statistical significance based on the Tukey-Kramer test ($p < 0.05$).

Yet another factor to consider is the degree by which actual ABA concentrations contribute to ABA-mediated responses. ABA signaling pathways are intersected by multiple pathways including GA, SA, JA, and ET (Robert-Seilaniantz et al., 2011; Denance et al., 2013; Yang et al., 2013; Meguro and Sato, 2014), all of which can affect ABA-mediated responses without directly altering ABA concentrations. Regulation within ABA signaling, such as inactivation and binding of PP2Cs to the SnRK2s, also

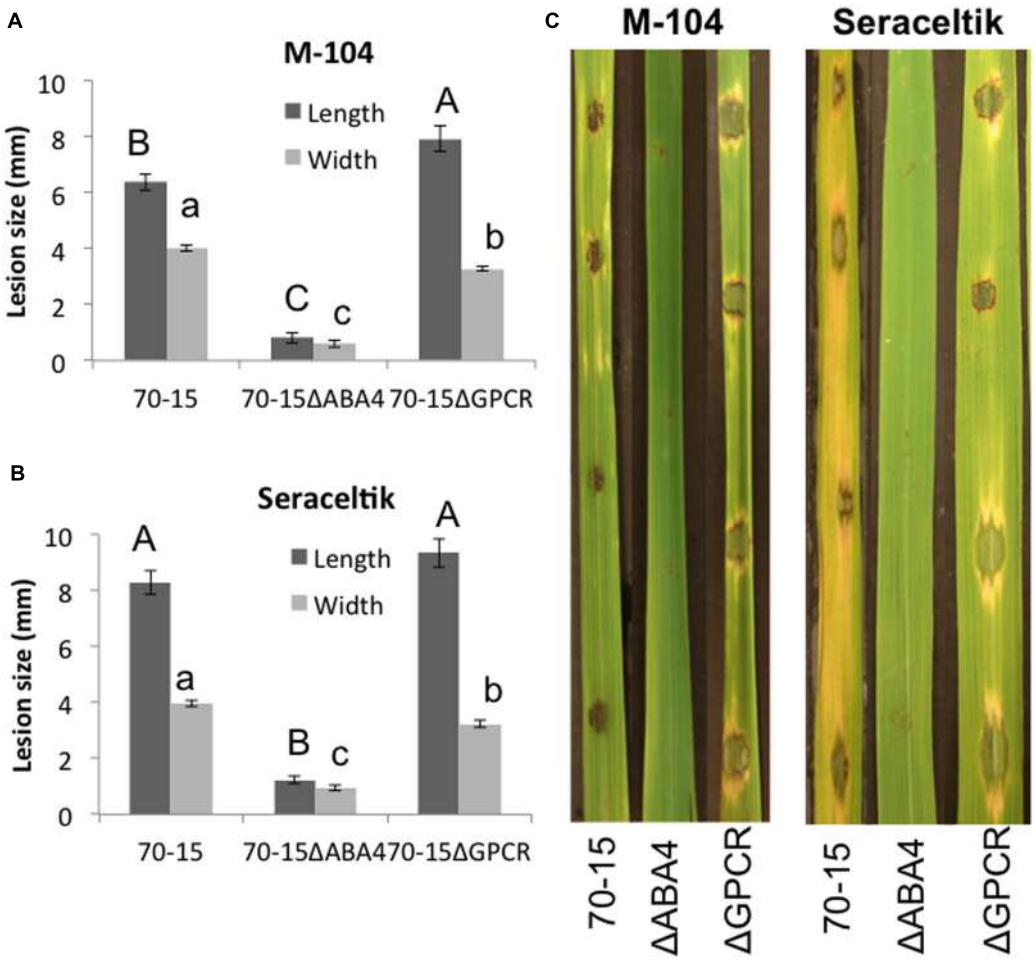

FIGURE 7 | Ability of ABA mutants to infect rice. M-104 and Seraceltik rice plants were infected with 70-15, 70-15ΔABA4, or 70-15ΔGPCR and lesion length and width were measured. Photographs were taken 5 days after infection. Different letters represent statistical significance based on the Tukey-Kramer test ($p < 0.05$).

effect ABA-mediated responses, as does the availability of ABA receptors. Yazawa et al. (2012) noticed that in the mutant *OsABI* line, impaired in ABA signaling, there were actually higher levels of ABA though they still saw fewer lesions (Yazawa et al., 2012). It is an important observation that lesions were reduced in plants which had elevated levels of ABA, but were impaired in a later step in ABA signaling, indicating that the actual ABA concentrations may not be as crucial as ABA perception and signaling in ABA-mediated resistance. Corroborating this, we saw that EA105 affected ABA gene expression in rice and *M. oryzae* without altering ABA concentration.

A hormone such as ABA that is involved in a multitude of normal developmental and stress-induced responses in plants must be tightly regulated. Each process for regulating the amount of active ABA, such as inactivation or catabolism, has multiple genes that can be triggered by different stimuli. ABA responses and ABA signal transduction can differ based on the type of stress, with particular differences having been found between abiotic and biotic stress (Kim, 2012; Ye et al., 2012). ABA was originally studied for its role in abiotic stress response, and most of what is known has been determined through experimenting with drought, temperature, and salt stress (Lim et al., 2012) while there is comparatively much less known about the role of ABA in biotic stress responses. There were only minimal expression changes to ABA-catabolism gene *OsABA8ox1*, ABA receptor *RCAR5*, *OsMPK1*, or *Rab25*. It is possible that these genes are not involved in this particular biotic stress situation, while other ABA-related genes that were not tested may be playing a role.

Based on expression of *NCED3* and the putative rice β-glucosidase, it is possible that modulating ABA signaling in plants may be a contributing factor to how EA105 reduces blast lesions. It has been established previously that ABA plays a role in plant susceptibility to pathogens. However, ABA may be playing a dual role by not only increasing susceptibility in plants, but also by directly promoting virulence in *M. oryzae*. We have shown that 70-15 produced ABA during vegetative growth and during spore formation. Exogenous ABA accelerated both spore germination and appressoria formation in 70-15, both of which are necessary

for virulence. *M. oryzae* may be producing its own ABA as well as utilizing the increased ABA produced in plants to enhance pathogenicity. ABA is a growth regulator in plants, and may also regulate growth in fungi. To determine if the effect of ABA on 70-15 germination and appressoria formation was specific, other growth regulators were also tested including auxins, cytokinin, and GA. Only ABA was able to accelerate both germination and appressoria formation. Previously, EA105 has been shown to almost completely prevent the formation of appressoria (Spence et al., 2014a,b). When EA105 and ABA were added together on spores, there was a large reduction in the number of spores forming appressoria when compared to ABA treatment alone. Not only is EA105 interfering with ABA's ability to accelerate appressoria formation, but this also shows that high levels of ABA (100 μM) are still unable to prevent EA105 from reducing appressoria formation.

While ABA's role in drought tolerance in plants has driven an extensive body of research on plant ABA biosynthesis and signaling, very little is known about fungal ABA biosynthesis, signaling, or even the functional significance of ABA in fungi. Fungal ABA biosynthesis follows an entirely different pathway, utilizing a different set of enzymes. Using characterized ABA biosynthesis gene sequences from *B. cinerea*, we were able to identify a gene in *M. oryzae*, orthologous to ABA4, which appears to significantly reduce the concentration of ABA synthesized by *M. oryzae* spores and mycelia. Not surprisingly, this mutant suffers growth defects and phenotypic abnormalities. However, the most striking characteristic of this ABA4 knockout mutant is its inability to properly form lesions on rice, supporting our hypothesis that not only plant ABA, but also endogenous fungal ABA may be required for infection. Further, adding an un-biologically high level of exogenous ABA (100 μM) only partially restored the wild-type phenotype, indicating that internal fungal ABA biosynthesis and intermediate signaling events, rather than absolute ABA content, may be a crucial part of the infection process. When examining germination and appressoria formation in an isolated, *in vitro* assay, exogenous ABA fully restored the defects of the ABA4 mutant. The process of plant infection is a far more complex and integrated system than germination or appressoria formation alone, which may explain why exogenous ABA can fully rectify deficiencies in those two isolated processes but not in plant infection as a whole.

Another interesting and unexpected set of data has stemmed from the deletion of an *M. oryzae* GPCR involved in ABA signaling. The GPCR deletion mutant sporulated faster and formed bigger lesions than the parental strain, but contained levels of ABA similar to the ABA4 mutant, which was about half the level of the parental strain. Thus, the two mutants behave oppositely yet both have reduced levels of ABA. Again, this points toward a conclusion that absolute ABA levels may not directly determine outcomes, but rather ABA signaling, possibly during the intermediate steps of ABA biosynthesis, might have a larger impact. The complexity and multifaceted nature of ABA in plants has led to elaborate checks and balances to regulate ABA-mediated effects. Fungi are likely to have similar systems in place to control whether ABA is in an active or inactive state, as well as when it is perceived, and when and how the signals are transduced. However, this is an unexplored area and should be the focus of future efforts, as it could shed light on the pathogenesis of economically important phytopathogens.

AUTHOR CONTRIBUTIONS

HB conceived the research. CS conducted the experiments and drafted the manuscript. HB provided suggestions and improvements on the manuscript. VL performed Southern blot analysis and contributed to the manuscript. ND provided ideas and guidance in addition to improving the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01082

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Expression of *Agrobacterium* Homolog Genes Encoding T-complex Recruiting Protein under Virulence Induction Conditions

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The proteins encoded by three Agrobacterial genes, *atu5117*, *atu4860,* and *atu4856*, are highly homologous to each other in amino acid sequence. All three proteins can bind to VirD2 and are named VBP1, VBP2, and VBP3 (VirD2-binding protein), respectively. VBP is involved in T-DNA transfer by recruiting the T-complex from the cytosol to the polar transport apparatus T4SS (type IV secretion system) and is defined as the "T-complex recruiting protein." However, it remains unknown how these three homologous genes co-exist in a relatively small prokaryotic genome. To understand whether these three homologous genes are expressed differentially under virulence induction conditions, we examined the effects of virulence induction conditions, including various pH values, temperatures and acetosyringone (AS, an effective virulence inducer to *Agrobacterium tumefaciens*) concentrations, on the expression of the three VBP-encoding genes. Our data showed that *vbp*1 (*atu5117*) and *vbp3* (*atu4856*) maintained constant expression under the tested induction conditions, whereas the expression of *vbp2* (*atu4860*) was affected by the conditions. Culture conditions favorable to the expression of *vbp2* differed from the reported induction conditions for other virulence proteins. In particular, the pH value was a crucial factor for the expression of *vbp2*. In addition, the deletion of *vbp1* affected the expression of *vbp2*. Taken together, these results suggest that the mechanisms regulating the expression of these three homologous genes are different from the virulence induction mechanism and that VBP homologs are presumably involved in other biological processes in addition to T-complex recruitment.

Keywords: ***Agrobacterium,*** **virD2-binding protein (VBP), virulence induction, gene expression, T-complex recruiting protein**

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INTRODUCTION

Agrobacterium tumefaciens is a well-known phytopathogen that causes crown gall tumor disease in various dicotyledonous plants. Pathogenicity is achieved by the transfer of a T-DNA fragment from the bacterial tumor-inducing (Ti) plasmid into host cells, genetically transforming the host. *Agrobacterium* uses the VirB/D4 T4SS to transfer the T-DNA in the form of a VirD2-T-DNA

Abbreviations: AS, acetosyringone; HEPN domain, higher eukaryotes and prokaryotes nucleotide-binding domain; IB medium, inducing broth medium; T4SS, type IV secretion system; Ti plasmid, tumor-inducing plasmid; VBP, VirD2-binding protein; Vir proteins, virulence proteins.

nucleoprotein complex (called T-complex; Guo et al., 2011; Pacurar et al., 2011; Chandran, 2013; Kado, 2014). The T-DNA transfer process has been largely described, and accumulating data have revealed that many Vir proteins are involved in T-DNA transfer. VirD2, one of the Vir proteins, is a relaxase that can cleave the bottom strand of the T-DNA and covalently attach to the 5′ end of the single-stranded T-DNA, thereby forming the VirD2-T-DNA nucleoprotein complex. In 2007, we used VirD2 as a pull-down bait to identify a VBP; (Guo et al., 2007a). An *Agrobacterial* genome-wide search demonstrated that in addition to the identified VBP-encoding gene *atu5117*, two other *A. tumefaciens* C58 genes, *atu4860* and *atu4856*, can encode two VBP homologs. All three VBP homologs were confirmed to be able to bind VirD2 and thus designated VBP1 (encoded by *atu5117*), VBP2 (encoded by *atu4860*), and VBP3 (encoded by *atu4856*; Guo et al., 2007a,b). Our further investigation showed that VBPs are able to recruit the T-complex from the cytosol to the polar VirB/D4 transport apparatus T4SS (Guo et al., 2007a). Thus, VBPs were also defined as being T-complex recruiting proteins.

Bioinformatics investigations have demonstrated that three VBPs are highly conversed with regard to their functional domains, and all of the VBPs contain a putative nucleotidyltransferase domain near the N-terminus and a putative higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domain near the C-terminus (Guo et al., 2007b; Gao et al., 2013). Structural studies have shown that VBP is a dimer and that the C-terminal HEPN domain is the dimerization domain of VBP. Associated functional studies of the HEPN domain of VBP have demonstrated that the dimerization of VBP is essential for the induction of tumors in plants (Padavannil et al., 2014). Our recent experimental data confirmed that VBP1 (encoded by *atu5117*) is an NTPase that might energize the recruitment of T-complex to the transport site (Gao et al., 2013).

As a ubiquitous soil-born bacterium, *Agrobacterium* has two lifestyles: independent free-living or acting as a phytopathogen. Its pathogenicity is not indispensable for its life cycle (Baek and Shapleigh, 2005; Gao and Lynn, 2005). However, it is currently not understood how a relatively small prokaryotic genome maintains three homologs for a non-essential biological function despite the ability of *Agrobacterium* tumorigenesis to be attenuated only by inactivating all three *vbp* genes. In addition, all three VBPs can complement each other in recruiting the T-complex (Guo et al., 2007a). True genetic redundancy is evolutionarily unstable (Brookfield, 1992; Nowak et al., 1997; Kafri et al., 2008), and bacterial genes that are redundant and not under efficient selection could be rapidly lost (Mira et al., 2001). Intuitively, VBPs may potentially be involved in other biological processes as well as in T-complex recruitment. To explore this possibility, many questions remain unanswered, but one important question is how the expression of three *vbp* genes respond to the virulence induction conditions.

Because VBPs are involved in *Agrobacterium* tumorigenesis, we investigated whether the growth conditions that induce tumorigenesis could affect the expression of the three *vbp* genes. Phenolic compounds and sugar compounds released by wounded plant tissue can be sensed and recognized by *A. tumefaciens*, and induce *Agrobacterium* to express *vir* genes. The induced *Agrobacterium* cells can then transfer the T-DNA to host plant cells, resulting in the formation of a tumor (Pitzschke and Hirt, 2010). Of these compounds, AS is the most effective inducing agent to *Agrobacterium* tumorigenesis. Although the effects of AS concentration, pH and temperature during the inducing process on the transformation efficiency vary in different reports (Baron et al., 2001; Gelvin, 2006; McCullen and Binns, 2006; Sudarshana et al., 2006), it has been proposed that acidic pH (4.8–5.5), moderate temperature (25°C), and a relatively high AS concentration (200 μg/ml) could induce most tumor tissues (Holford et al., 1992; McCullen and Binns, 2006). Thus, we investigated the effects of tumor-inducing conditions, including AS concentration, pH and temperature, on the expression of the three *vbp* genes using western blot analyses. Our data showed that the expression levels of *vbp1* and *vbp3* were nearly unchangeable, independent of the variable induction conditions. However, the expression of *vbp2* was controlled by the culture conditions. Both the temperature and pH optimal for *vbp2* expression were higher than those for virulence induction. The most effective virulence inducer, AS, appears to inhibit the expression of *vbp2*. Among these three tested factors, pH plays an important role in regulating the expression of *vbp2*. The expression of *vbp2* was also affected by *vbp1*-deletion. These results indicate that the expression of three *vbp* genes may be regulated by a novel unknown pathway, which is contradictory from the reported virulence induction pathway. This unknown *vbp*-regulating pathway may be involved in the regulation of *Agrobacterium* tumorigenesis. Taken together, these results also provide additional details for further elucidating the potential versatile functions of VBP homologs.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The strains and plasmids used in the present study are listed in Supplementary Table S1. *Escherichia coli* strains were cultured in Luria broth at 37°C. *A. tumefaciens* strains were cultured in YEP medium, AB-sucrose medium or IB medium at different culture steps, as previously described (please refer to the text; Gelvin, 2006; Guo et al., 2007a). Corresponding antibiotics were added to the culture medium according to Supplementary Table S1.

DNA Manipulations

The *vbp1* gene fragment was amplified using primers, vbp1-F and vbp1-R (Supplementary Table S1), and cloned into the sites of *Xho* I and *Hind* III endonucleases of the expression vector pRSET-A (The corresponding restriction sites of the *vbp1* gene fragment are underlined in the primers). The resulting plasmid pR-vbp1 was transformed into *E. coli* BL21 (DE3) using the heat shock method (Sambrook et al., 1989). Plasmids expressing *vbp2* and *vbp3* genes were previously constructed (Guo et al., 2007b).

Heterogeneous Production of Three VBPs

Escherichia coli BL21 harboring the corresponding plasmid with the His-tag fused *vbp* gene was grown to an OD_{600} of approximately 0.5 and was subsequently induced by IPTG with a final concentration of 0.3 mM for 1-h to express the *vbp* gene. The induced *E. coli* BL21 cells were harvested by centrifugation (13,000 × *g*, 4°C, 10 min) and washed twice with phosphate-buffered solution (PBS, 10 mM, pH 7.2). Cell pellets from 10 ml of culture were lysed with 2 ml of sodium dodecyl sulfate (SDS)-loading buffer (0.1 M Tris-Cl, pH 6.8, 4% SDS, 0.1% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) and stored at −20°C until further analysis.

Expression of Three *vbp* Genes in *A. tumefaciens*

To test the responses of *vbp* genes to the virulence induction condition, the preparation of medium for culturing *A. tumefaciens* and the induction culture for different *A. tumefaciens* strains was performed according to ref. 16. For normal virulence induction, cells of different *A. tumefaciens* strains were first inoculated into YEP liquid medium containing appropriate antibiotics and grown overnight at 28°C (while shaking at 250 rpm) and diluted (in the ratio of 1/100, v/v) into AB-sucrose medium containing appropriate antibiotics. After *A. tumefaciens* in AB-sucrose medium grew to OD_{600} approaching 0.8, the cells were harvested by centrifugation (∼3,800 × *g*, 10 min) and washed two times with IB medium. The cell pellets were resuspended in IB medium (pH 5.5) containing 100 μg/ml AS (The cell concentration in IB medium was adjusted to $OD_{600} = 0.4$) and induced at 25°C for 15–16 h, while shaking at 50 rpm. The induced *Agrobacterium* cells were harvested by centrifugation as described above. *Agrobacterium* cells from 10 ml of IB medium culture were lysed by 1 ml of SDS-loading buffer and stored at −20°C until further protein analysis. Meanwhile, another sample of *Agrobacterium* cells from equal volume of IB medium culture was prepared for the determination of protein concentration in the *Agrobacterium* sample, so that each *Agrobacterium* sample can be adjusted to the same protein concentration for running SDS-polyacrylamide gel electrophoresis (PAGE).

To test the effect of pH on the expression of three *vbp* genes, the pH in IB medium was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5, respectively, and the AS concentration in IB medium was 100 μg/ml. *Agrobacterium* cells were induced in IB medium with different pH and 100 μg/ml AS at 25°C for 15–16 h. To test the effect of temperature on the expression of three *vbp* genes, the pH of the IB medium was adjusted to 5.5; the AS concentration of the IB medium was 100 μg/ml. *Agrobacterium* cells were induced in IB medium at the following five different temperatures: 19, 25, 28, 32, and 37°C. To test the effect of AS concentration on the expression of three *vbp* genes, four different AS concentrations, 0, 50, 100, and 200 μg/ml, were chosen to induce the *Agrobacterium* cells; the pH of the IB medium was adjusted to 5.5. *Agrobacterium* cells were induced in IB medium at different AS concentrations at 25°C for 15–16 h.

Generation of Antibodies Against Three VBPs

Polyclonal antibodies against three VBPs were supplied by Jinsirui Bio (GenScript-China, Nanjing, China). To obtain the polyclonal antibodies that can discriminate three highly homologous VBPs, peptide fragments from the variable sequences of three VBPs (Supplementary Figure S1) were artificially synthesized and used as antigens to generate polyclonal antibodies against VBPs in rabbits.

Protein Analysis

Proteins were analyzed using SDS-PAGE and Western blotting analyses. Each protein sample was adjusted to the same concentration. An equal volume of each protein sample was mixed with an equal volume of 2x loading buffer (0.1 M Tris-Cl [pH 6.8], 4% SDS, 0.1% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) and incubated at 100°C for 5 min before loading. After electrophoresis, the gels were stained with Coomassie blue R-250 solution to visualize the protein bands, so that we could further confirm that every sample was loaded equal amount of protein and the protein band pattern of each sample was comparable. For Western blotting, the proteins in gels, which were run in parallel with the Coomassie blue-stained gels, were electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) and detected using the BCIP/NBT alkaline phosphatase color development kit (Beyotime Corp., China) according to the procedure recommended by the manufacturer. Polyclonal antibodies against the variable sequence regions of three VBPs were used as the primary antibodies for detection.

RESULTS

Effective Discrimination of Three VBPs

Western blotting is a routine method to assess protein expression. However, the high homology of amino acid sequences of three VBPs (Supplementary Figure S1) suggests that the polyclonal antibody generated by any full-length VBP would most likely cause cross-reaction with each of the three VBPs, resulting in non-specific results. Consequently, the peptide fragments from the variable sequence regions of the three VBPs (283–313 amino acid residues of VBP1, 163–191 amino acid residues of VBP2, and 161–189 amino acid residues of VBP3; Supplementary Figure S1) were artificially synthesized and used as antigens to generate antibodies that could differentiate between the three VBPs. As shown in **Figure 1**, three VBPs heterogeneously produced by *E. coli* could be specifically identified by their corresponding antibodies.

Expression of Three *vbp* Genes in Wild-type Strain *A. tumefaciens* C58 Differ in Response to *vir* Gene Induction Conditions

Agrobacterium tumefaciens C58 is a wild-type strain isolated from a cherry tree tumor; it was completely sequenced in 2001

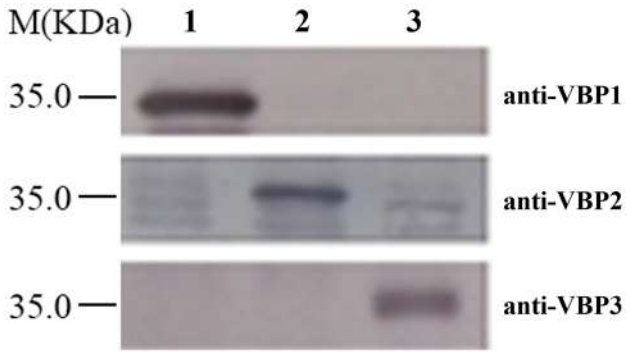

FIGURE 1 | Discrimination of three virD2-binding proteins (VBPs) by Western blot assay. Crude extracts from *Escherichia coli* cells expressing different *vbp* genes were separated using SDS-PAGE in the indicated lanes and then analyzed by Western blotting using antibodies against the variable sequence regions of the three VBPs. M: Molecular weight marker. The anti bodies used for each assay are listed on the right. Lane 1: crude extracts from *E. coli* cells expressing *vbp1*. Lane 2: crude extracts from *E. coli* cells expressing *vbp2*. Lane 3: crude extracts from *E. coli* cells expressing *vbp3*.

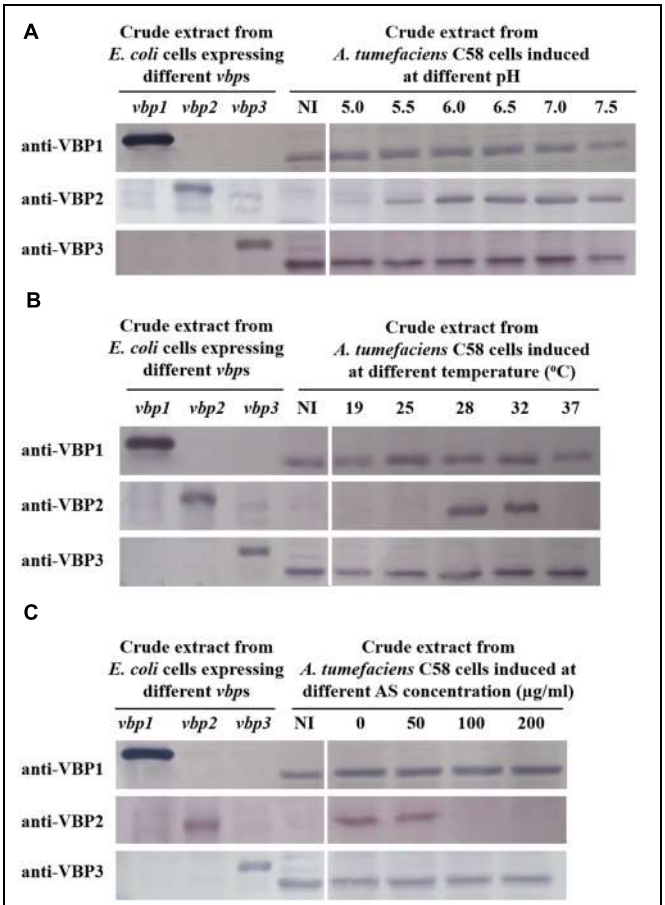

FIGURE 2 | Expression of three *vbp* genes in *Agrobacterium tumefaciens* strain C58 at different pHs (A), temperatures (B) and AS concentrations (C). *A. tumefaciens* strain C58 cells were induced under the indicated pH, temperature or AS concentration. Crude extracts from the differentially induced C58 cells were separated using SDS-PAGE and then analyzed by Western blotting. Crude extracts from *E. coli* cells expressing different *vbp* genes with His-tag were used as positive controls. The anti bodies used in this study are listed on the left. NI: crude extracts from C58 cells not induced in IB medium. **(A)** Effect of pH on the expression of *vbp*s in *A. tumefaciens* C58. The temperature is 25°C and AS concen tration is 100 μg/ml. **(B)** Effect of temperature on the expression of *vbp*s in *A. tumefaciens* C58. The pH is 5.5 and AS concentration is 100 μg/ml. **(C)** Effect of AS concentration on the expression of *vbp*s in *A. tumefaciens* C58. The pH is 5.5 and temperature is 25°C. Some differences in protein size of *E. coli* recombinant VBP2 protein (the positive control band in panel C immigrated faster than that in other panels) are likely attributable to prolonged storage.

(Goodner et al., 2001; Wood et al., 2001). The *A. tumefaciens* C58 genome contains a circular chromosome, a linear chromosome, a Ti plasmid pTiC58 and a cryptic megaplasmid pAtC58. In the genome of *A. tumefaciens* C58, *vbp1* is located on the plasmid pAtC58, whereas *vbp2* and *vbp3* are located on the linear chromosome (Guo et al., 2007b). The expression of homologous genes located at different loci may be regulated by different mechanisms. This encourages the exploration of the molecular mechanisms that control the expression of three *vbp* genes.

To test the responses of the three *vbp* genes to *vir* gene induction conditions, *A. tumefaciens* C58 strain was induced by different pHs (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5), AS concentrations (0, 50, 100, and 200 μg/ml) or temperatures (19, 25, 28, 32, and 37°C), and *vbp* gene expression in the differentially induced *Agrobacterium* cells were examined using Western blotting (**Figure 2**). The results showed that the responses to *vir* gene induction conditions differed among the three *vbp* genes. Both *vbp1* and *vbp3* were expressed at any of the tested pH, temperature or AS concentration conditions, whereas the *vbp2* gene was expressed only under some specific induction conditions. Strikingly, *Agrobacterium* cells that were not induced by IB medium did not express *vbp2*, and the optimal pH for *vbp2* expression was within the range of 6.0–7.5, which was significantly higher than that for *vir* gene expression. Furthermore, *vir* gene induction is maximal in the pH range of 5.2–6.0, whereas this pH range was not optimal for the expression of *vbp2* (**Figure 2A**). The results in **Figure 2B** show that *vbp2* was expressed only in the temperature range of 28–32°C, which was higher than the optimal temperature for virulence gene expression. The most effective virulence inducer AS also affected the expression of *vbp2*, but the effect of AS on *vbp2* expression inhibited the expression of *vbp2*. When the concentration of AS was higher than 100 μg/ml, the expression of *vbp2* gene was fully inhibited. Combining all of the results in **Figure 2**, we conclude that the culture conditions optimal for virulence gene induction were not very favorable to the expression of *vbp2*. When comparing the results in **Figure 2**, we found little inconsistency in the expression of *vbp2*. The results in **Figure 2A** show that *vbp2* was weakly expressed under the culture condition of pH 5.5, temperature 25°C and AS concentration 100 μg/ml, but the results shown in both **Figures 2B,C** show that *vbp2* was not expressed under the same culture condition. This slight difference for *vbp2* expression suggested that one of the three factors (pH, temperature, and AS concentration) is critical to the expression of *vbp2* and that any slight fluctuation of the key

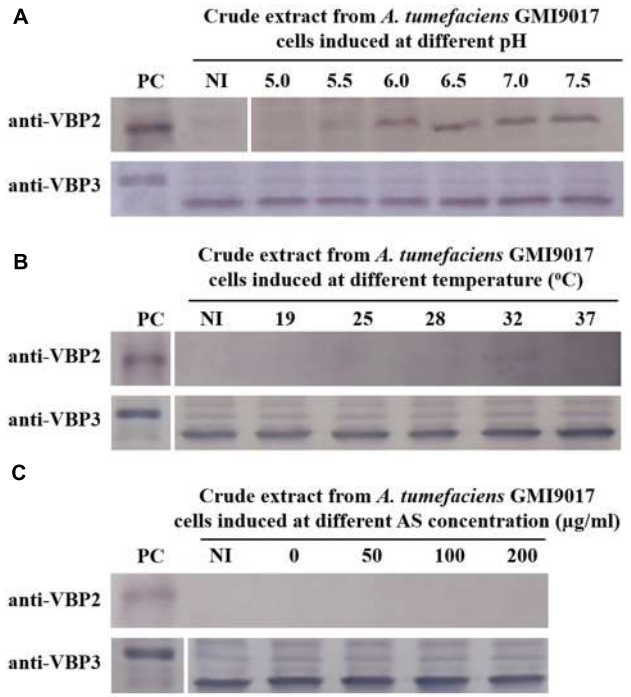

FIGURE 3 | Expression of *vbp2* and *vbp3* in *A. tumefaciens* strain GMI9017 at different pHs (A), temperatures (B), and AS concentrations (C). *A. tumefaciens* strain GMI9017 cells were induced under the indicated pH, temperature or AS concentration. Crude extracts from the differentially induced GMI9017 cells were separated using SDS-PAGE and then analyzed by Western blotting. PC, positive control, crude extracts from *E. coli* cells expressing *vbp2* (up) or *vbp3* (down) with His-tag. The antibodies used in this study are listed on the left. NI: crude extracts from GMI9017 cells not induced in IB medium. **(A)** Effect of pH on the expression of *vbp2* and *vbp3* in *A. tumefaciens* GMI9017. The temperature is 25°C and AS concentration is 100 μg/ml. **(B)** Effect of temperature on the expression of *vbp2* and *vbp3* in *A. tumefaciens* GMI9017. The pH is 5.5 and AS concentration is 100 μg/ml. **(C)** Effect of AS concentration on the expression of *vbp2* and *vbp3* in *A. tumefaciens* GMI9017. The pH is 5.5 and temperature is 25°C.

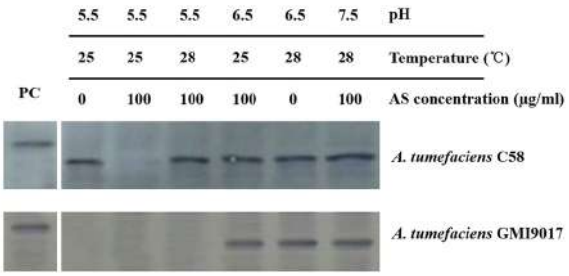

FIGURE 4 | Comparison of the expression of *vbp2* in *A. tumefaciens* C58 and *A. tumefaciens* GMI9017 at different pHs, temperatures and AS concentrations. Both the *A. tumefaciens* strain C58 cells and GMI9017 cells were induced in parallel under the indicated pH, temperature or AS concentration. Crude extracts from the differentially induced *Agrobacterium* cells were separated using SDS-PAGE and then analyzed by Western blotting using antibody against VBP2. PC, positive control, crude extract from *E. coli* cells expressing *vbp2* with His-tag.

factor under this culture condition could result in the differential expression of *vbp2*. Data (**Figure 2B**) also showed that the expression of the *vbp2* gene was inhibited at a temperature of 37°C, which was higher than the optimal temperature (28–32°C) for the vegetative growth of *Agrobacterium*. In conclusion, these data indicated that the expression of three *vbp* genes were different from each other under the above-tested induction conditions. Both *vbp1* and *vbp3* genes were expressed constantly, whereas the expression of *vbp2* gene was selective and only occurred near a neutral pH, temperature of 28–32°C and AS concentration lower than 100 μg/ml.

Deletion of the *vbp1* Gene Affects the Regulation of *vbp2* Expression by *vir* Gene Induction Conditions

As the expression of the three *vbp* genes differed under *vir* gene induction conditions, we investigated whether some regulatory relationships exist among these three homologous genes. To examine the effect of *vbp1* deletion on the expression of *vbp2* and *vbp3*, *Agrobacterium* strain GMI9017, in which the *vbp1* gene was deleted, was grown under the same induction conditions as the *A. tumefaciens* C58 strain.

The data showed that deletion of *vbp1* did not affect the expression of *vbp3*. As shown in **Figure 3**, *vbp3* in GMI9017 was expressed under all of the tested induction conditions, similar to C58. However, the expression of *vbp*2 displayed a greater difference in *A. tumefaciens* GMI9017 compared with *A. tumefaciens* C58. The *vbp1*-deleted GMI9017 strain was able to express the *vbp2* gene in the pH range of 6.0–7.5, which was similar to the C58 strain, but it did not express *vbp2* at the acidic pH 5.5, regardless of the temperature and AS concentration (**Figures 3B,C**). Unlike the GMI9017 strain, wild-type strain C58 was able to express *vbp2* at pH 5.5 when grown in a temperature range of 28–32°C or with an AS concentration of less than 50 μg/ml (**Figures 2B,C**). The effects of pH, temperature or AS concentration on the expression of *vbp2* in GMI9017 were different from those in the wild-type strain C58, indicating that deletion of *vbp1* affects the responses of *vbp2* expression to pH, temperature and AS concentration. In other words, *vbp1* is involved in the regulation of *vbp2* expression by pH, temperature and AS concentration, though the mechanism underlying this phenomenon is unclear. When grown at pH 5.5, which is not the pH optimum for *vbp2* expression, GMI9017 did not express *vbp2* at any tested temperature and AS concentration, suggesting that pH may be a crucial external factor in regulating the expression of *vbp2*. Combined with the analysis on the inconsistency of *vbp2* expression at pH 5.5 in C58 (**Figure 2**), we speculated that pH 5.5 might be the pH critical point of *vbp2* expression.

To eliminate any uncontrollable error that is potentially caused by bacterial culture and induction, the wild-type strain C58 and *vbp1*-deleted strain GMI9017 were induced under different pHs (5.5, 6.5, and 7.5), temperatures (25 and 28°C) or AS concentrations (0 and 100 μg/ml) in parallel. As expected, the results shown in **Figure 4** were consistent with the results

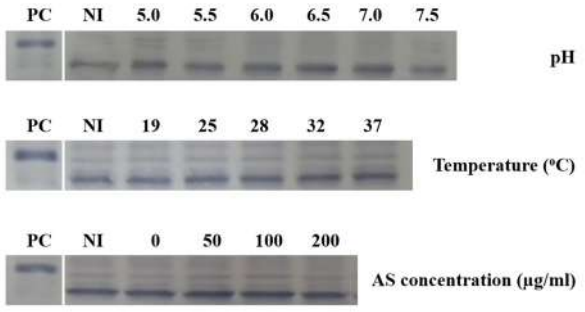

FIGURE 5 | Expression of *vbp3* in the double-mutant (*vbp1* and *vbp2*) strain GMV12 at different pHs, temperatures and AS concentrations. *A. tumefaciens* strain GMV12 cells were induced under the indicated pH, temperature or AS concentration. Crude extracts from the differentially induced GMV12 cells were separated using SDS-PAGE and then analyzed by Western blotting with antibody against VBP3. PC, positive control, crude extract from *E. coli* cells expressing *vbp3* with His-tag. NI: crude extracts from GMV12 cells not induced in IB medium.

in **Figures 2** and **3**. This further confirmed that *vbp1* deletion affected the responses of *vbp2* expression to *vir* gene induction conditions (pH, temperature and AS concentration).

The Expression of *vbp3* Gene is Not Affected by the Double Deletion of *vbp1* and *vbp2*

Although the expression of *vbp3* is not affected by the *vir* gene induction conditions and deletion of *vbp1*, we determined whether a double deletion of *vbp1* and *vbp2* affects the expression of *vbp3*. Thus, we examined the expression of *vbp3* in a double-mutant strain of *vbp1* and *vbp2*, GMV12 under different pHs, temperatures or AS concentrations. No significant change was observed in the expression level of the *vbp3* gene of the GMV12 strain under all of the tested *vir* gene-inducing conditions (**Figure 5**), indicating that in addition to the virulence induction conditions, the homologous genes *vbp1* and *vbp2* do not regulate the expression of *vbp3*.

DISCUSSION

The responses of three VBP-encoding genes to pH, temperature, and AS concentration are very different from the responses of other reported virulence genes to these three virulence induction factors. According to our data, both *vbp1* and *vbp3* could be expressed ubiquitously, despite varying induction conditions, indicating that neither of these genes are affected by well-known virulence induction conditions. However, *vbp2* showed selective expression under diverse induction conditions. Importantly, pH is a crucial factor for the expression of *vbp2* and is favorable near 7.0. The *vbp2* promoter region was subcloned in front of a promoterless *egfp* gene, and the expression of the reporting gene *egfp* further confirmed the response of the *vbp2* promoter to pH (unpublished data). However, the pH optimal for the expression of virulence genes is approximately 5.5. In *A. tumefaciens*, the reported virulence genes that were induced by acidic conditions (pH 5.5) include all virulence genes located on the Ti plasmid, chromosome virulence genes (*chvG* and *chvI*), and some other genes involved in tumorigenesis, such as *katA* and *aopB* (Xu and Pan, 2000; Jia et al., 2002; Li et al., 2002; Yuan et al., 2008). The induction of *vir* genes by acidic conditions is regulated by a two-component system VirA-VirG. VirA perceives acidic, phenolic or monosaccharide signals, processes all these signals and finally phosphorylates VirG. Subsequently, phosphorylated VirG binds to a conserved 12-bp AT-rich sequence (*vir* box) in the promoter regions of all *vir* genes and initiates their transcription (Pazour and Das, 1990; Yuan et al., 2008). DNA sequence analysis revealed no "*vir* box" in the promoter regions of *vbp* genes, indicating that the *vbp* promoters cannot bind phosphorylated VirG. These findings suggest that *vbp* transcription is not regulated by VirA-VirG-controlling acidic induction. This silica prediction is consistent with our experimental data. In addition, a recent study on the transcriptome of *A. tumefaciens* in response to acidic conditions did not demonstrate that *vbp* transcription was significantly affected by acidic conditions and a deep sequence analysis on the promoters of all four replicons of *A. tumefaciens* did not identify any small RNA targeting sequence in *vbp* gene promoters (Yuan et al., 2008; Wilms et al., 2012). Thus, we conclude that the expression of *vbp1* and *vbp3* is not regulated by *vir*-inducing conditions and that the expression of *vbp2* is regulated by a mechanism different from the reported pH-regulating mechanism. The molecular mechanism regulating the expression of *vbp2* is requires further study.

VBP proteins are able to recruit the VirD2-T-DNA complex to the T4SS apparatus and are thus important for the pathogenicity of *A. tumefaciens* (Guo et al., 2007a). The similarity of three VBPs in the amino acid sequence is over 70% and three VBPs are redundant for the function of T-complex recruitment. However, from an evolutionary perspective, functional overlaps of homologous proteins are inherently unstable. If a protein's function can be fully compensated for by a redundant homolog, then the mutations in the protein-encoding gene would have no effect on the phenotype. Consequently, such mutations would not remain, and the redundancy would be gradually eliminated (Lynch and Conery, 2000). Homologous genes can be obtained from duplications and lateral acquisitions. A recent study declared that the secondary chromosome (linear chromosome) of *A. tumefaciens* C58 originated from an intragenomic transfer from the primary chromosome (circular chromosome) to an ancestral plasmid (Slater et al., 2009). Both *vbp2* and *vbp3* genes are located on the linear chromosome, indicating that these two homologous genes were likely to originate from duplication, whereas, the *vbp1* gene is located on the cryptic megaplasmid pAtC58, indicating that *vbp1* was potentially obtained from lateral acquisition. Several homologs inhabit the same genome stably, indicating that either their functions or their *cis*-regulatory motifs have diversified (Gu et al., 2004). The difference between the expression of *vbp2* and *vbp3* genes demonstrated that the *cis*-regulatory motifs of these two homologs have diversified. However, the expression of *vbp1* is very similar to the expression of *vbp3*, implying

that both promoter regions of *vbp1* and *vbp3* may have similar *cis*-regulatory motifs. We used several databases[1] to analyze the promoter regions of *vbp1* and *vbp3*. The analysis showed that both promoter regions of *vbp1* and *vbp3* have the same transcription factor-binding motifs (data not show). The co-inhabitancy of *vbp1* with its homologs in the same genome indicates that the function of *vbp1* has diversified from its homologs. Therefore, these three *vbp* homologs may provide a good example for the research of homologous gene evolution. Recent bioinformatics studies have shown that redundant protein partners are significantly more frequently associated with the essential core proteins of protein-interaction networks (Kafri et al., 2008; Plata and Vitkup, 2014). T-DNA transfer is not necessary for the life cycle of *A. tumefaciens*. Nevertheless, the *A. tumefaciens* mutant of the *vbp* triple-deletion was difficult to construct (Guo et al., 2007b), demonstrating that VBP may be involved in some essential biological process as well as T-complex recruitment. Evidence that the expression of three *vbp* genes is not involved in the expression of other virulence genes suggests that the major function of VBP homologs is not T-complex recruitment. Taken together, these data suggest that redundant VBPs are versatile proteins and are involved in biological processes other than T-complex recruitment. It is possible that VBP1 has diversified to specify T-complex recruitment. However, the biological processes involving VBP require further investigation.

[1] http://linux1.softberry.com/; http://www.cbil.upenn.edu; http://pepper.molgenrug.nl

AUTHOR CONTRIBUTIONS

MG planned the experiments. JY, MW, XZ, and ZH prepared and performed the experiments. JY, MG, and MW analyzed the data. JY and MG wrote the paper.

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SUPPLEMENTARY MATERIAL

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Induced systemic resistance against *Botrytis cinerea* by *Micromonospora* strains isolated from root nodules

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Micromonospora is a Gram positive bacterium that can be isolated from nitrogen fixing nodules from healthy leguminous plants, where they could be beneficial to the plant. Their plant growth promoting activity in legume and non-legume plants has been previously demonstrated. The present study explores the ability of *Micromonospora* strains to control fungal pathogens and to stimulate plant immunity. *Micromonospora* strains isolated from surface sterilized nodules of alfalfa showed *in vitro* antifungal activity against several pathogenic fungi. Moreover, root inoculation of tomato plants with these *Micromonospora* strains effectively reduced leaf infection by the fungal pathogen *Botrytis cinerea,* despite spatial separation between both microorganisms. This induced systemic resistance, confirmed in different tomato cultivars, is long lasting. Gene expression analyses evidenced that *Micromonospora* stimulates the plant capacity to activate defense mechanisms upon pathogen attack. The defensive response of tomato plants inoculated with *Micromonospora* spp. differs from that of non-inoculated plants, showing a stronger induction of jasmonate-regulated defenses when the plant is challenged with a pathogen. The hypothesis of jasmonates playing a key role in this defense priming effect was confirmed using defense-impaired tomato mutants, since the JA-deficient line *def1* was unable to display a long term induced resistance upon *Micromonospora* spp. inoculation. In conclusion, nodule isolated *Micromonospora* strains should be considered excellent candidates as biocontrol agents as they combine both direct antifungal activity against plant pathogens and the ability to prime plant immunity.

Keywords: biocontrol, defense priming, induced systemic resistance, jasmonic acid, *Micromonospora*, PGPR, tomato

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Introduction

Most actinobacteria were considered to inhabit environments such as soil, rhizosphere, or lake sediments. However, it was later discovered that actinobacteria are closely associated with plants and they have been isolated from a great number of different plant genera, colonizing different parts of the plant (Coombs and Franco, 2003; Hasegawa et al., 2006; Qin et al., 2010; Velázquez et al., 2013). Actinobacteria have been described to promote plant growth and their beneficial effect has been reported previously in various plant species (El-Tarabily and Sivasithamparam, 2006; Franco et al., 2007).

Most legumes are engaged in a symbiotic relationship with nitrogen-fixing rhizobia hosted in root nodules. A common misconception was that nodules from leguminous plants were thought to be inhabited by only one type of microorganism, but it is now clear that they conform an ecosystem of their own: besides nodule forming bacteria, several other endophytes with PGP activities are found (Velázquez et al., 2013). Among other actinobacteria, *Micromonospora* strains have been found in a wide selection of leguminous plants, including *Medicago sativa*, the plant of choice for the isolation of *Micromonospora* spp. in our study. Previous studies (Martínez-Hidalgo et al., 2014b) showed that nodule isolated *Micromonospora* strains excel at plant growth promotion when inoculated in alfalfa. This effect was not due to biological nitrogen fixation although the nitrogen content was significantly higher than in control plants (Martínez-Hidalgo et al., 2014a).

A number of actinobacteria have been also described to reduce the negative effects of pathogens in plants, inhibiting pathogen growth via production of antibiotics, lytic enzymes or siderophores or inducing the plant defense mechanisms (Conn et al., 2008; Hirsch and Valdés, 2010; Verma et al., 2011) but very few have proven ability to promote plant growth, inhibit the growth of pathogens and also boost plant defensive capacity in plants of agronomic relevance.

Plants have developed mechanisms to detect potential aggressors and to coordinate the appropriate defense responses, including the production of toxic substances and lytic enzymes. Regulation of these responses is under phytohormone control, being salicylic acid (SA), jasmonic acid (JA) and ethylene among the major regulators (Pieterse et al., 2009). In general terms, JA coordinates responses effective against necrotrophic pathogens and chewing insects, while SA targets mainly biotrophic pathogens such as viruses, but intensive cross-talk among both pathways (generally antagonistic) allows the plant to shape the final immune response triggered against specific invaders (Pieterse et al., 2009). Upon appropriate stimulation plants can develop a state of enhanced defensive capacity, known as induced systemic resistance (ISR). Different soil beneficial microorganisms have been shown to trigger ISR in plants, usually relying on JA signaling (van Loon et al., 2006; Pozo and Azcón-Aguilar, 2007; Van Wees et al., 2008; Pieterse et al., 2014).

Induced resistance may result in direct activation of defense mechanisms – including increased basal levels of defense-related compounds, or the priming of the plant defensive capacity. In the latter, no major changes appear upon induction in the absence of a challenge, but a more efficient activation of defense mechanisms occurs upon attack. Thus, priming is a cost-effective way of increasing plant resistance (Conrath et al., 2006; Pastor et al., 2013).

The fungus *Botrytis cinerea* is a plant necrotrophic pathogen that colonizes senescent or dead plant tissues and causes gray mold in vegetables and softening in fruits. Its hyphae can penetrate plant tissues through wounds or natural openings and spread from previously colonized dead tissues into healthy ones. It has a broad host range of food crops, including tomato (*Solanum lycopersicum*), with the gray mold disease being responsible for substantial economical crop losses every year (Dean et al., 2012). Tomato is one of the most important crops in the world and it is considered an important model plant because, besides its economic importance, it display interesting features, a known genome and a considerable number of mutants and genomic tools available (Kimura and Sinha, 2008). Remarkably, *B. cinerea* is able to manipulate tomato defense regulatory pathways to promote fungal colonization and disease progression (El Oirdi et al., 2011; Rahman et al., 2012). *B. cinerea* control is usually achieved by cultural measures and application of broad spectrum fungicides.

The increasingly strict regulation on chemical pesticides and environmental and safety concerns, have evidenced the need of sustainable and safe solutions for crop protection. Thus, formulating bioinoculants with both growth and defense promotion for plants is a major goal in modern agriculture. *Micromonospora* strains have been isolated from leguminous root nodules and found to improve crop performance in alfalfa (Martínez-Hidalgo et al., 2014b). They are sporulating bacteria, a highly valued trait relevant for its use as bioinoculants, as it allows cultures to be stored for long periods of time without a significant loss in survival.

The aim of this study was to test the potential of *Micromonospora* strains isolated from alfalfa nodules as biocontrol agents exploring their antifungal properties and their ability to boost plant defense mechanisms using the agronomically relevant pathosystem tomato-*B. cinerea*. The potential of these strains for improving disease control in horticulture is discussed.

Materials and Methods

Bacterial and Fungal Cultures

All endophytic *Micromonospora* cultures were maintained in SA1 medium (Trujillo et al., 2005), while all pathogenic fungi were cultured in PDA at 24°C. *B. cinerea* to be used for plant bioassays was cultured similarly but on PDA supplemented with tomato leaves at 40 mg ml^{-1} (Vicedo et al., 2009). *Micromonospora* strains used in this study were isolated from *M. sativa* root nodules (**Table 1**).

In vitro Antagonistic Bioassays for Inhibition of Fungal Growth

Pathogens were selected based on their importance as plant pathogens in Spain. The species chosen were *Fusarium circinatum, Sclerotinia sclerotiorum, Rhizoctonia solani,* and *B. cinerea*.

Pathogens routinely grown in PDA cultures, were grown in petri dishes with SA1 medium (Trujillo et al., 2005) in order to confirm they could grow normally under these conditions. For the trial, the 13 selected *Micromonospora* strains (**Table 1**) were streaked in a thick line in the center of the plate and let grow for 7 days at 28°C. After this time, 1 cm plugs of PDA media containing actively growing fungi of two different species (*F. circinatum* and *R. solani* or *S. sclerotiorum* and *B. cinerea*) were located equidistant at both sides of the streak of *Micromonospora* sp. (Supplementary Figure S1) and incubated

TABLE 1 | Strains of *Micromonospora* used in this study and comparison of gene *rrs* with already described species.

Strain (accession)	*rrs* identification	%	Source
AL4 (KF876221)	*Micromonospora viridifaciens* DSM 43909T (X92623)	99,52	Martínez-Hidalgo et al. (2014b)
AL16 (KF876222)	*Micromonospora saelicesensis* Lupac 09 (AJ783993)	99,65	Martínez-Hidalgo et al. (2014b)
AL20 (KF876223)	*Micromonospora chokoriensis* 2-19/6 (AB241454)	99,79	Martínez-Hidalgo et al. (2014b)
ALF1 (KF876224)	*Micromonospora humi* P0402 (GU459068)	99,51	Martínez-Hidalgo et al. (2014b)
ALF2 (KJ187181)	*Micromonospora narathiwatensis* BTG4-1 (AB193559)	98,76	This work
ALF4 (KF876225)	*Micromonospora coxensis* 2-30-b/28 (AB241455)	99,31	Martínez-Hidalgo et al. (2014b)
ALF7 (KF876233)	*Micromonospora saelicesensis* Lupac 09 (AJ783993)	99,86	Martínez-Hidalgo et al. (2014b)
ALFb5 (KF876226)	*Micromonospora saelicesensis* Lupac 09 (AJ783993)	99,86	Martínez-Hidalgo et al. (2014a)
ALFb7 (KF876227)	*Micromonospora saelicesensis* Lupac 09 (AJ783993)	99,58	Martínez-Hidalgo et al. (2014b)
ALFb1 (KF876228)	*Micromonospora echinospora* ATCC 15837 (U58532)	97,78	Martínez-Hidalgo et al. (2014b)
ALFpr18c (KF876230)	*Micromonospora tulbaghiae* TVU1 (EU196562)	99,93	Martínez-Hidalgo et al. (2014a)
ALFpr19a (KF876231)	*Micromonospora lupini* Lupac 14N (AJ783996)	99,31	Martínez-Hidalgo et al. (2014b)
ALFr4 (KF876234)	*Micromonospora cremea* CR30 (FN658654)	98,62	Martínez-Hidalgo et al. (2014b)

for 2 days at 24°C. Three replicates were performed for each fungus.

Plant Material and *Micromonospora* Inoculation Procedures

Five tomato (*S. lycopersicum* L.) genotypes were used in our studies including the three cultivars 'Roma,' 'Castlemart' and 'Moneymaker,' and the following lines altered in defense signaling: the JA-deficient mutant *def1* (Howe et al., 1996; in background 'Castlemart,' gently provided by G. Howe, Michigan State University) and SA-impaired transgenic line *nahG* (Brading et al., 2000; in background 'Moneymaker' gently provided by J. Jones, John Innes Centre).

To test the capacity of *Micromonospora* spp. to ISR in plants against *B. cinerea*, tomato seeds were sterilized with sodium hypochlorite for 20 min and rinsed three times in sterile distilled water. Seeds were placed on sterile vermiculite, grown until the first true leaf appeared and then transplanted to pots with commercial substrate (Projar Seed Pro 5050, Spain). Plants were randomly distributed and grown in a greenhouse at 24/16°C with a 16/8 h photoperiod and 60% humidity, and watered three times a week with Long Ashton nutrient solution (Hewitt, 1966).

After transplantation, the tomato plants were used in two sets of experiments: to analyze long-term effect of *Micromonospora* spp. inoculation the plants were inoculated immediately after transplantation with each microbial strain. To analyze short-term effect of *Micromonospora* spp. inoculation, the plants were inoculated 24 h before the infection with the pathogen. The experiments were repeated at least twice, and for each experiment, five replicates per treatment were used.

Inoculation of tomato plants with the selected *Micromonospora* strains was performed with 1 ml of bacterial suspensions (10^9 cells per ml) of each microbial strain grown on solid medium. The bacterial suspensions were carefully strewn in the soil near the roots of the seedling using a micropipette.

Pathogen Bioassays

One month after transplantation, *B. cinerea* was inoculated either by adding plugs of the fungal culture to each leaflet or by spray on the leaflets of tomato plants with 10^6 *B. cinerea* conidia (in whole plants or in detached leaves; Zhai et al., 2013). The extension of the disease was measured 48 and 72 h after the challenge.

B. cinerea Bioassays in Intact Plants

Solanum lycopersicum L. 'Roma' were grown in the greenhouse in pots of 1 L capacity, filled with commercial substrate (Compo Sana® Universal potting soil, Compo Iberia S.L.) and inoculated with *Micromonospora* strains ALFb5 and ALFpr18c a month before inoculation with *B. cinerea*. Plugs of agar containing *B. cinerea* micellium were attached to seven leaflets in each plant and located in a humidity chamber kept at 20–23°C. 48 h after challenge with *B. cinerea*, the diameter of the necrotic lesions formed by the fungal hyphae in the leaflets was measured with the aid of a Vernier caliper (Martínez-Medina et al., 2013). Two measurements were taken for each lesion considering the biggest and the smallest diameter and the average between the two was calculated.

B. cinerea Bioassays on Detached Leaves

Tomato plants were grown as described above, and the inoculation with *Micromonospora* spp. was performed 30 days or 24 h before pathogen challenge.

In these experiments *B. cinerea* was applied to detached leaves. Two leaves of each plant were detached with a sharp blade, placed in plastic trays on wet paper and challenged with *B. cinerea* by applying 1 cm diameter plugs of PDA media containing actively growing mycelia of *B. cinerea* obtained from 3 weeks-old culture plates. The leaves were then placed in a humidity chamber and kept at 20–23°C with constant light. Disease damage level was scored after 72 h and three levels of damage were established, according to the severity of the symptoms: mild for necrosis extending 1–2 mm from the plug, moderate for necrosis ranging from 3 to 5 mm from the plug and severe for lesions that extended for more than one third of the leaflet (Supplementary Figure S2).

Gene Expression Analysis

Quantitative analysis of defense related gene expression. To evaluate the effects of *Micromonospora* spp. inoculation on defense gene expression upon pathogen attack, *B. cinerea*

was applied to detached leaves of tomato plants that had been inoculated with *Micromonospora* spp. at transplanting, 30 days before the challenge with the fungus. Two leaves of each plant were detached, placed in a humidity chamber as described above and challenged by spraying a spore suspension of *B. cinerea* on the leaf surface. This inoculation method ensures homogeneous and simultaneous contact of the leaf tissue with the pathogen, allowing a more precise quantification of changes in gene expression levels. Spores collected from 15 days-old cultures were incubated in Gambor's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 10 mM sucrose and 10 mM KH_2PO_4 for 2 h in the dark without shaking (Vicedo et al., 2009). The suspension was adjusted to 5×10^6 spores ml^{-1}. Controls were mock inoculated in a similar way. All leaves were kept under high humidity and harvested at 72 h.

Total RNA was extracted from tomato leaves taken from four different treatments: uninoculated and unchallenged control plants (Control), plants treated only with *Micromonospora* sp. (pr18c), plants challenged only with *B. cinerea* (*Botrytis*) and plants inoculated with *Micromonospora* sp. and then challenged with *B. cinerea* (pr18c+*Botrytis*).

The RNA was extracted following the Tri-Reagent (Sigma) protocol following the manufacturer's procedure. Contaminating DNA was removed with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey- Nagel). cDNA was synthesized with 3 μg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. The conditions of RT-qPCR experiments and the relative quantification of specific mRNA levels was performed according to López-Ráez et al. (2010), using tomato gene specific primers previously described, coding for Pathogenesis related protein PR1a (ID M69247, *PR1a*; Martínez-Medina et al., 2013), Proteinase inhibitor II (ID K03291, *Pin II*; Uppalapati et al., 2005), Lipoxygenase A (ID U09026, *LoxA*; López-Ráez et al., 2010) and elongation factor 1α (ID X14449, *SlEF*; Rotenberg et al., 2006).

Expression values were normalized using the housekeeping gene *SlEF*, which encodes for the tomato elongation factor-1α. At least three independent biological replicates were analyzed per treatment, and each qPCR reaction was performed in duplicate.

Detection of *Micromonospora* sp. in Plant Tissues

Presence of *Micromonospora* in plant roots and shoots was assessed by amplification of *Micromonospora* DNA in the samples by PCR. Primers to amplify the *gyrB* gene, which encodes for the subunit B of DNA gyrase, were designed in our laboratory on the basis of available sequences of this gene in the *Micromonospora* genus. The primer sequences are: F: TCGACGGCAAGGCGTACG and R: CGCAGCTTCTCSATGTCG. Genomic DNA of root or leaf samples was extracted using NucleoSpin® Plant II columns (Macherey Nagel, Duren, Germany). DNA quality and PCR performance were confirmed in all samples by amplification of the tomato *SlEF* gene. Bacterial DNA obtained from a pure *Micromonospora* culture was used as a positive control for *gyrB* amplification. *gyrB* amplification conditions were as follows: 9 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 62°C and 2 min at 72°C, followed by 7 min final extension at 72°C. PCR products were electrophoresed in 1% agarose gels containing ethidium bromide (0.5 μg/ml) using modified Tris-Acetate EDTA buffer (Millipore, Cork, Ireland).

Statistical Analysis

The statistical analysis of data was performed using SPSS software, version 21 (SPSS, Inc., Chicago, IL, USA). The data on lesion diameter in tomato leaves was processed with one-way analysis of variance (ANOVA). The statistical significance of the results was determined using Fisher's LSD test ($P < 0.05$).

Association between severity of leaf fungal damage and inoculation treatments was examined using Chi-square tests, followed by *z*-tests to compare the inoculation groups at each damage level. A criterion of $P < 0.05$ for statistical significance was used for all analyses and *P*-values were corrected for multiple tests using the Bonferroni correction.

Results

Micromonospora Strains Inhibit the Growth of Fungal Plant Pathogens *In Vitro*

The strains under study were isolated from alfalfa root nodules and have been found to have great genetic diversity according to the *rrs* gene sequences (**Table 1**).

Selected *Micromonospora* isolates were tested for their ability to inhibit the growth of the damaging fungal pathogens *F. circinatum, S. sclerotiorum, R. solani,* and *B. cinerea* (Supplementary Figure S1).

All of the 13 isolates assayed inhibited the growth of one or more of the selected pathogenic fungi (**Table 2**). Only two strains of *Micromonospora* were capable of inhibiting growth of *F. circinatum* (ALF4 and ALFb7), five strains (AL4, AL16, AL20, ALFpr18c y ALFr4) inhibited *S. sclerotinum*, seven strains were inhibitory to *R. solani* (AL20, ALF1, ALF2, ALFb5, ALFb7, ALFpr18c y ALFpr19a) and 10 strains inhibited *B. cinerea* (AL4, AL20, ALF1, ALF2, ALF7, ALFb1, ALFb5, ALFpr18c, ALFpr19a y ALFpr4). The results suggest the potential of these strains to control fungal diseases through direct effects on the pathogen.

Micromonospora strains ALFb5 and ALFpr18c, were selected for *in planta* studies as they inhibited a large number of fungal pathogens (three for ALFpr18c and two for ALFb5) and were previously shown to efficiently promote plant growth (Martínez-Hidalgo et al., 2014b).

Micromonospora sp. Induce Systemic Resistance against *B. cinerea* in Tomato

The two selected *Micromonospora* strains (ALFpr18c and ALFb5) were tested for its efficiency to increase tomato resistance against *B. cinerea*.

S. lycopersicum L. 'Roma' plants were root inoculated with *Micromonospora* strains at transplanting or 24 h prior challenge

TABLE 2 | Antimicrobial activity of selected strains of *Micromonospora* against four selected plant pathogenic fungi.

Strains	AL4	AL16	AL20	ALF1	ALF2	ALF4	ALF7	ALFb1	ALFb5	ALFb7	ALFpr18c	ALFpr19a	ALFr4
Fusarium circinatum	−	−	−	−	−	+	−	−	−	+	−	−	−
Sclerotinia sclerotiorum	+	+	+	−	−	−	−	−	−	−	+	−	+
Rhizoctonia solani	−	−	+	+	+	−	−	−	+	+	+	+	−
Botrytis cinerea	+	−	+	+	+	−	+	+	+	−	+	+	+

with *B. cinerea*, as described in materials and methods. Plants treated with any of the two *Micromonospora* strains looked healthier than control plants, even though only plants treated with *Micromonospora* sp. ALFpr18c showed less disease symptoms at the long term (**Figure 1**).

Remarkably, this significant reduction on the severity of symptoms caused by the pathogen was also evidenced even when pathogen inoculation was performed on detached leaves in a different experiment. As *Micromonospora* sp. ALFpr18c was consistently the most efficient strain in reducing disease severity, it was selected for the follow up studies.

We extended our analysis to other tomato cultivars to find out if the protection by *Micromonospora* sp. ALFpr18c was a consistent effect for tomato and not a cultivar-specific response, choosing two well-characterized cultivars with defense impaired mutants available: 'Castlemart' and 'Moneymaker.' The sensitivity to *B. cinerea* of the two tomato cultivars differ significantly, being 'Castlemart' less severely affected than 'Moneymaker' ($X^2 = 18{,}871$, $P = 0.001$; **Figure 2**). Remarkably, inoculation with *Micromonospora* spp. resulted in a significant reduction of the disease symptoms in both cultivars. The ISR by ALFpr18c against *B. cinerea* was effective regardless the timing of the inoculation of the bacteria, 30 days or 24 h before the challenge with the pathogen (**Figure 2**). *Micromonospora* sp. protected plants challenged with *B. cinerea* by reducing the severity of damage caused by the pathogen, as it was shown by a significant decrease in the number of leaflets with the highest level of damage (**Figure 2**). Indeed, there was a statistically significant

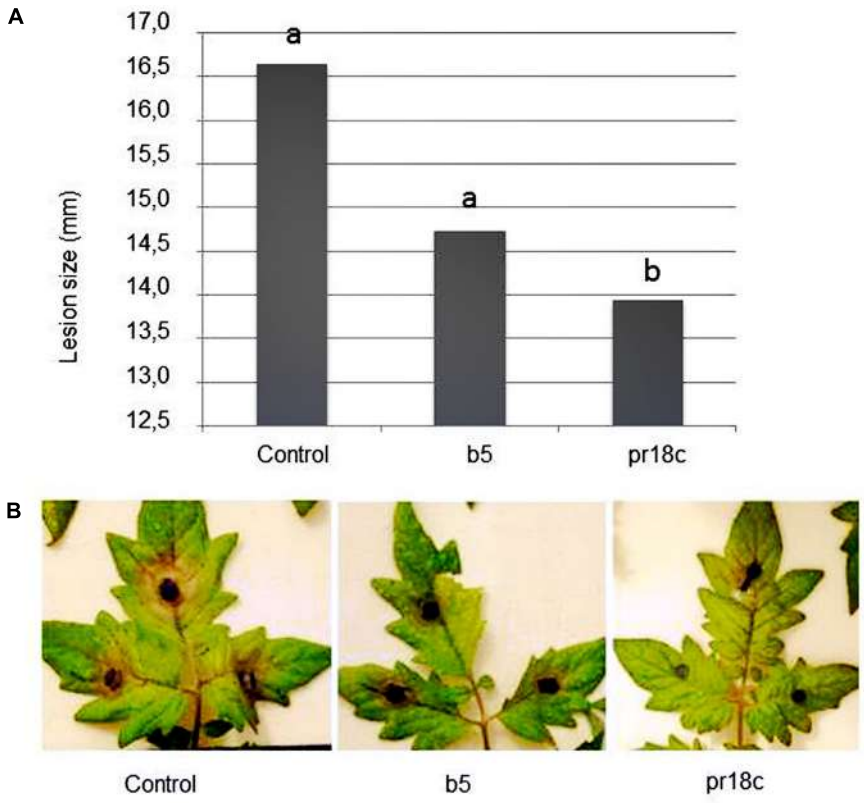

FIGURE 1 | Effect of root inoculation with selected *Micromonospora* strains (ALFb5 and ALFpr18c) on tomato resistance against *Botrytis cinerea*. **(A)** Symptom severity in leaves of tomato plants upon challenge with *B. cinerea* was determined by measuring the diameter of the necrotic lesions 42 h post-pathogen inoculation. Plants were grown in the absence of *Micromonospora* (Control) or inoculated at transplanting with *Micromonospora* strain ALFpr18c (pr18c) or strain ALF b5 (b5). Data not sharing a common letter are significantly different (Fisher's protected LSD test at $P \leq 0.05$). **(B)** Examples of *B. cinerea* symptom development in leaves of *Micromonospora* spp. inoculated (b5 or PR18c) or non-inoculated (control) plants.

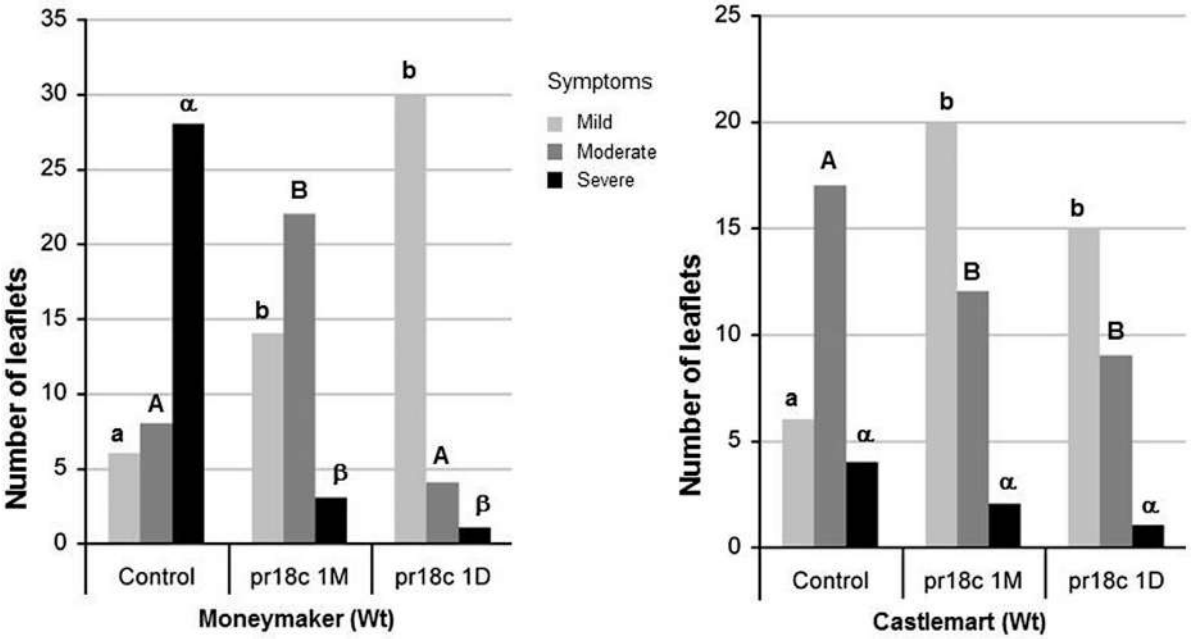

FIGURE 2 | Effect of *Micromonospora* ALFpr18c root inoculation on *B. cinerea* disease development in different tomato cultivars (*Solanum lycopersicom* L. 'Moneymaker' and 'Castlemart'). Necrosis severity caused by *Botrytis* was scored using a three levels disease scale: mild, moderate and severe, as shown in Supplementary Figure S2. The number of leaflets within each category is shown. For each tomato cultivar, disease damage level was compared between the control and the bacterial treatments. Control, plants not inoculated with *Micromonospora*; pr18c 1M and pr18c 1D, plants inoculated with *Micromonospora* sp. pr18c 1 month or 1 day before challenge with *B. cinerea,* respectively. Bars not sharing a common letter (lowercase for mild, uppercase for moderate and greek for severe symptoms) are significantly different using Bonferroni corrected Chi-square tests, followed by *z*-tests ($P \leq 0.05$).

association between levels of leaf fungal damage and inoculation treatments for 'Castlemart' ($X^2 = 8{,}374$, $P = 0.015$) and for 'Moneymaker' ($X^2 = 29{,}824$, $P = 0.001$).

The spatial separation between both microorganisms (*Micromonospora* was inoculated in the soil, close to the roots, and the pathogen in the leaves) suggest that the protection is related to the activation of plant defense mechanisms. However, to rule out a possible direct antifungal activity of the bacteria through an eventual colonization of the leaf tissue in the long term treatments, we evaluated the presence of the bacteria in roots and leaves of inoculated and non-inoculated tomato plants using *Micromonospora* gene *gyrB* specific primers. *GyrB* amplification, and therefore, the presence of *Micromonospora* sp. ALFpr18c was evidenced in the roots of the plants inoculated at transplanting, 30 days before harvesting, confirming effective root colonization by *Micromonospora*. However, no bacterial gene amplification was detected in the leaves of the same plants, confirming the bacteria localization in the roots and the physical separation of the resistance inducer and the pathogen (Supplementary Figure S3).

Micromonospora sp. Induced Resistance is Related to Priming of Jasmonate-Regulated Responses

To understand the mechanism behind this long term induced resistance, and to determine what defense signaling pathways were involved, we compared the response against *B. cinerea* in plants inoculated or not with the strain *Micromonospora* sp. ALFpr18c 30 days before the challenge with the pathogen. Plant defense responses to *Botrytis* are known to depend on the interplay between SA and JA dependent responses, and those regulated by JA have been proposed as the major players in resistance (El Oirdi et al., 2011). Accordingly, we monitored the expression levels of well-characterized marker genes for both signaling pathways in leaves from the different treatments. Non-challenged plants showed no differences in the expression levels of any of the marker genes analyzed regardless of the presence of *Micromonospora* sp. (**Figure 3**). In contrast, important differences were found in the transcription levels of marker genes from both pathways between non-inoculated plants and plants preinoculated with *Micromonospora* spp. upon challenge with *B. cinerea* (**Figure 3**).

The *PR1* gene, encoding for an acidic form of the Pathogenesis Related Protein 1, is well-recognized as a marker of SA dependent responses. The expression of *PR1* was strongly induced in control plants upon challenge with the pathogen (sevenfold), but the increase upon *B. cinerea* challenge was much lower in plants previously inoculated with *Micromonospora* sp.

Regarding the JA regulated signaling pathway, the expression of the marker genes *PinII*, encoding for Proteinase inhibitor II, known to be involved in the plant resistance to *B. cinerea*, and *LoxA*, coding for a JA-inducible lipoxygenase involved in the biosynthesis of oxylipins was monitored. Transcript levels

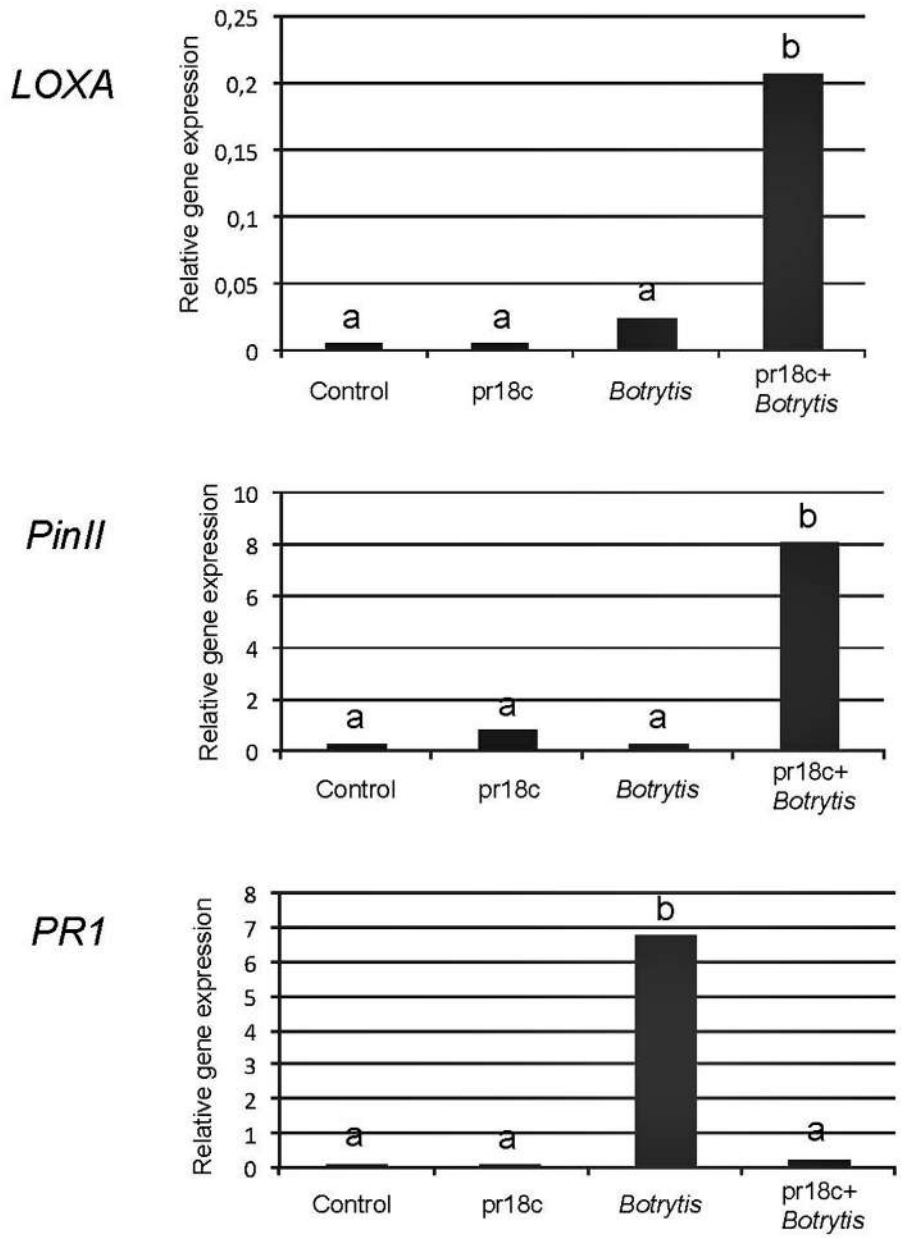

FIGURE 3 | Expression levels of the defense related genes *PR1*, *LoxA,* and *PinII* in leaves of tomato plants. Relative expression levels for *PR1*, *LoxA,* and *PinII* were determined by qPCR and normalized against the constitutive *SlEF* (tomato elongation factor α). Control: uninoculated, unchallenged plants; pr18c: plants inoculated with *Micromonospora* strain ALFpr18c; *Botrytis*: uninoculated plants challenged with *B. cinerea*; pr18c+*Botrytis*: plants previously inoculated with *Micromonospora* strain ALFpr18c and challenged with *B. cinerea*. *Micromonospora* inoculation was performed at transplanting, a month before infection with *Botrytis*. Data not sharing a common letter are significantly different according to Fisher's protected LSD test at $P \geq 0.05$.

of both *PinII* and *LoxA* genes were significantly higher in plants inoculated with *Micromonospora* sp. and then challenged with *B. cinerea* than in the rest of treatments (**Figure 3**). Remarkably, control plants without *Micromonospora* sp. but challenged with *B. cinerea* showed very low levels of expression of both JA-marker genes, indicating a poor activation of the efficient (JA regulated) defense mechanisms in the absence of *Micromonospora* ALFpr18c.

Micromonospora sp. Induced Resistance against *B. cinerea* is Compromised in Defense-Deficient Tomato Mutants

The ability of *Micromonospora* sp. ALFpr18c to induce resistance against *B. cinerea* was analyzed in tomato plants impaired in JA or SA signaling (**Figure 4**). Tomato plants were challenged with *B. cinerea* 30 days after root inoculation with *Micromonospora* sp., as described in Section "Materials and Methods."

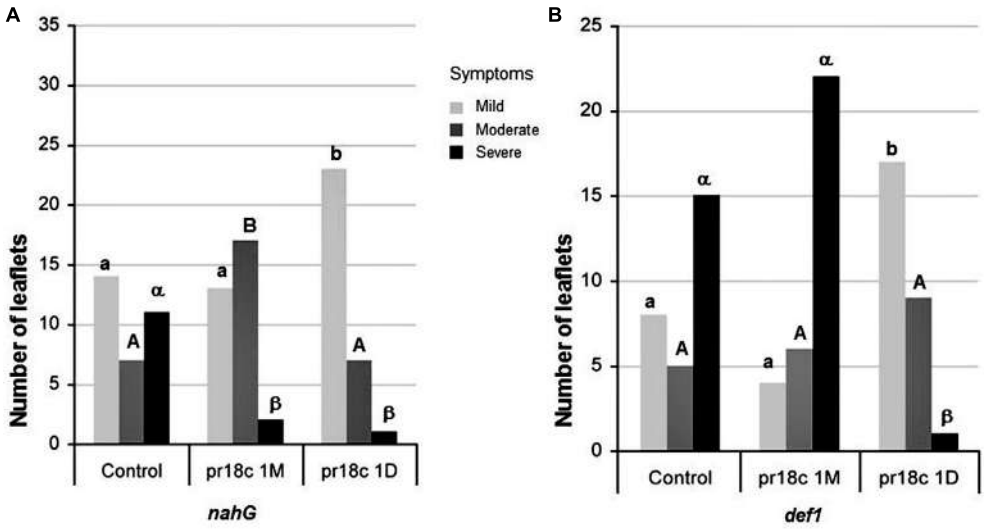

FIGURE 4 | Effect of *Micromonospora* ALFpr18c root inoculation on disease development caused by *B. cinerea* in different defense deficient tomato lines. (A) SA-deficient *nahG* and **(B)** JA-deficient *def 1* plants. Necrosis severity caused by *B. cinerea* was scored using a three levels disease scale: mild, moderate and severe, as shown in Supplementary Figure S2. Control, plants not inoculated with *Micromonospora*; pr18c 1M and pr18c 1D, plants inoculated with *Micromonospora* sp. ALFpr18c 1 month or 1 day before challenge with *B. cinerea*, respectively. Within each genotype, bars not sharing a common letter (lowercase for mild, uppercase for moderate and greek for severe symptoms) are significantly different using Bonferroni corrected Chi-square tests, followed by *z*-tests ($P \leq 0.05$).

To address the possible role of the SA regulated defense pathway in the *Micromonospora* ISR, *nahG* plants and their corresponding wild type ('Moneymaker') were used. *nahG* plants, unable to accumulate SA, were significantly less susceptible to *B. cinerea* than their corresponding wild type ($X^2 = 9{,}499$, $P = 0.009$). Thus, SA appears to have a negative role in disease resistance, in agreement with recent reports showing that *B. cinerea* manipulate SA signaling to promote infection and disease progression (El Oirdi et al., 2011).

When studying the effect of ALFpr18c on *nahG* mutant, there is a statistically significant association between levels of leaf fungal damage and inoculation treatments: ($X^2 = 10{,}434$, $P = 0.005$); *Micromonospora* sp. inoculated *nahG* plants showed a significant reduction on the severity of symptoms caused by the pathogen, as evidenced by the decrease in the percentage of leaves with higher severity symptoms (**Figure 4**). No significant differences were found ($X^2 = 0{,}190$, $P = 0.909$) when comparing ALFpr18c-inoculated *nahG* or wild type (Moneymaker) plants, confirming that *Micromonospora* induced resistance was not impaired in the SA deficient mutant.

To analize the relevance of JA signaling on *Micromonospora* ISR, *def1* mutants, deficient in JA regulated responses and their corresponding wild type cultivar ('Castlemart') were used. Differences in the basal resistance of *def1* and its parental line ('Castlemart') were clear, being *def1* plants significantly more susceptible to *B. cinerea* than the corresponding wild type cultivar ($X^2 = 13{,}186$, $P = 0.001$; **Figure 4**), supporting the key role of JA in resistance to *B. cinerea*. When analyzing the effect of ALFpr18c on *def1* mutant, there is not a statistically significant association between levels of leaf fungal damage and inoculation treatments: ($X^2 = 2{,}493$, $P = 0.288$). Accordingly, plant protection against the pathogen by the strain ALFpr18c is lost in the JA deficient mutant.

Taken all together, our results show that JA has a positive role in tomato resistance against *B. cinerea*, and these responses are effectively primed by *Micromonospora* sp.

We also tested the efficacy of the short term induction of resistance, comparing disease severity when *Micromonospora* ALFpr18c inoculation was performed 24 h before the challenge. Plants treated with ALFpr18c showed less severe lesions than control plants regardless the tomato line tested, even in the case of the JA compromised mutant *def1*, unable to maintain long term ISR (**Figure 4**).

Discussion

In the context of the new agricultural sustainability directives that have been outlined by the European Union, the search for effective bioinoculants is a major goal in agronomic research. The *Micromonospora* strains presented here, isolated from alfalfa root nodules showed very good plant growth promoting activity (Martínez-Hidalgo et al., 2014a). Plant growth promotion is often achieved by both improved plant nutrition (Vessey, 2003; Velázquez et al., 2013) and enhanced stress tolerance, for example, reducing disease damages. In this study we evaluated the potential of *Micromonospora* strains for biological control of pathogens. We evaluate the two different ways by which *Micromonospora* spp. could act as biocontrol agents, by testing their direct inhibitory activity against plant pathogens, and their ability to boost plant defense mechanisms.

Most of the *Micromonospora* strains tested showed a clear inhibitory effect on multiple plant pathogenic fungi in *in vitro* antagonistic assays (**Table 2**). Production of secondary metabolites is common in *Actinobacteria*, many of them with antibiotic effect (El-Tarabily et al., 1997; Gadelhak et al., 2005; Hirsch and Valdés, 2010). Production of antimicrobial substances by biocontrol agents is associated to plant protection by selective inhibition of fitopathogenic fungi, thus avoiding crop losses linked to diseases. Root diseases caused by soil pathogens are a major problem in agriculture and antagonism in the rhizosphere is an effective tool to decrease their incidence and damage. Inoculation with *Micromonospora* spp. may contribute to this antagonistic effect. The metabolic versatility of *Micromonospora* spp. is very high, they are able to produce multiple metabolites, such as antibiotics (Weinstein et al., 1966), antitumorals (Igarashi et al., 2007) or lytic enzymes, like chitinases, or proteases that could inhibit germination of *B. cinerea* spores or suppress fungal growth (Frankowski et al., 2001; Kamensky et al., 2003). Further studies will be needed to discover the metabolites or enzymes responsible for the observed inhibitory effects of our selected strains.

Besides a direct antimicrobial effect, that can contribute to reduce pathogens propagation in the soil, *Micromonospora* sp. ALFpr18c is able to stimulate plant defenses and ISR against foliar pathogens. Here we show that root inoculation with *Micromonospora* sp. enhanced disease resistance to the foliar pathogen *B. cinerea*. For every tomato cultivar tested in the different trials ('Roma,' 'Moneymaker' and 'Castlemart'), the extent and severity of the symptoms caused by *B. cinerea* in plants pretreated with *Micromonospora* sp. was significantly lower than in untreated controls.

Micromonospora sp. inoculation was performed in the soil, near the root system, while *B. cinerea* was directly applied to leaves, so that there was no contact between both microorganisms. Absence of bacteria on the leaves was confirmed by DNA amplification analysis. Thus, the direct antagonism of the bacteria on the fungus in our experimental conditions is very unlikely. The protection observed was, therefore, likely related to an effect on the plant defense mechanisms, further confirmed by the lack of *Micromonospora* triggered ISR in the *def1* defense deficient mutant lines.

The two *Micromonospora* strains tested in plant bioassays, ALFpr18c and ALFb5, reduced disease progression when inoculated 24 h before challenging the plants with *B. cinerea*. However, only ALFpr18c was able to induce long term systemic resistance, since inoculation at transplanting -30 days before challenge- with these bacteria, but not with ALFb5, did reduce disease severity significantly. For this reason, ALFpr18c was selected as an effective inducer of durable resistance and used to uncover the mechanisms underlying such effect.

Plant defense responses to *B. cinerea* are coordinated by the interplay of the JA and SA regulated signaling pathways (El Oirdi et al., 2011), the two major branches of defense related signaling. Quantitative analysis of transcript levels of marker genes for both pathways and the use of tomato lines defective in the signaling pathways allow to determine which pathways are activated in the different treatments, as previously described for other microbial inducers of plant resistance (Martínez-Medina et al., 2013). In the absence of pathogen attack, the bacteria do not have a clear impact on these major defense signaling pathways in the leaves (**Figure 3**). In contrast, the pathogen *B. cinerea* triggers a strong induction of the SA pathway, as previously described (El Oirdi et al., 2011). It has been proven that *B. cinerea* is sensitive to JA regulated responses, but not to those regulated by SA. Since SA and JA pathways are mutually antagonistic, the pathogen manipulate SA signaling to downregulate JA dependent responses to promote disease and necrosis spreading (El Oirdi et al., 2011; Rahman et al., 2012). The negative role of SA in tomato resistance against *B. cinerea* is here confirmed by the fact that the SA impaired line *nahG* was significantly less susceptible to *B. cinerea* than its corresponding wild type background (**Figure 4**), so SA pathway impairment renders the plant less susceptible to the fungus. Remarkably, the plants previously treated with the bacteria showed a minimal induction of the SA pathway upon challenge, thus the bacteria prevented the SA response triggered by the pathogen. In contrast, JA regulated responses were strongly upregulated in the preinoculated plants (**Figure 3**), particularly, *Pin II* that encodes for a proteinase inhibitor with strong inhibitory effects on *B. cinerea* (El Oirdi et al., 2011). As *Micromonospora* sp. itself does not induce a response from the plant, but this increase in defense was only evident upon challenge, the results support that *Micromonospora* sp. ISR against *B. cinerea* through priming of JA regulated defense responses. Priming is a cost efficient way of inducing resistance since defenses are only boosted upon pathogen attack, but they remain in basal levels in the absence of pathogen pressure (Conrath et al., 2006). Other beneficial microorganisms prime JA dependent responses, appearing as a common mechanism for efficient resource management in beneficial plant-microbe interactions (Van Wees et al., 2008; Selosse et al., 2014). It has been postulated that during initial stages of the interaction, beneficial soil microorganisms are perceived as potential aggressors by the plants, thus triggering some general defense responses. Later on, the beneficial bacteria deal with the plant immune system modulating plant defenses to achieve successful colonization, and as result, defenses may remain under "alert," or primed (Zamioudis and Pieterse, 2012).

The analysis of the induction of resistance in tomato lines altered in the JA and SA signaling pathways further support this notion. The different lines showed a decrease in the severity of *B. cinerea* symptoms when inoculated with *Micromonospora* sp. Plants that were inoculated 24 h before the challenge with the pathogen show a reduction in disease severity in all tomato lines, including the JA and SA signaling deficient lines *nahG* and *def1*. Thus, this protection is probably due to an early general defense response to the bacteria that affect *B. cinerea,* but differs from the long term protective effects. Differences in the mechanisms underlying early and late responses associated to priming have been described, with early responses associated to the accumulation of reactive oxygen species and cell wall reinforcements, and late responses being under phytohormone control (Pastor et al., 2013), and a similar distinction may be applying here. In fact, the early unspecific defense response

from the plant upon colonization by beneficial bacteria seems frequent. Even *Rhizobium,* known for establishing a very well-coordinated symbiosis with legumes, triggers a peak of defense pathway activation during the early stages of the interaction (Santos et al., 2001; Soto et al., 2006).

When *Micromonospora* sp. was inoculated at transplanting, 30 days before the challenge, disease severity was significantly reduced in the different tomato cultivars tested lines. However, the induction of resistance was completely lost in the JA deficient mutant *def1*, that was even more severely affected when inoculated with the bacteria.

In summary, the transcriptional analysis and the genetic approach with tomato deficient lines evidenced that the durable systemic resistance induced by *Micromonospora* sp. ALFpr18c is based on priming of JA regulated defense responses. JA dependent defenses, although mainly effective against necrotrophs, may also affect hemibiotrophs and even biotrophs (Pozo et al., 2005), so the spectrum of efficiency of *Micromonospora* sp. induced resistance deserves further exploration.

In this article, we provided evidences of the potential of *Micromonospora* strains as biocontrol agents for long lasting crop protection against phytopathogenic fungi. The priming activity of *Micromonospora* spp. is sustained in time -more than a month in our experiments- without significantly reducing its effectiveness, therefore inoculation with these bacteria could be performed at the time of sowing without a reduction in the effectiveness of the protection over time. These results, together with the direct antifungal potential evidenced for these strains, their proven role as plant probiotic bacteria and their sporulation capacity makes bacteria from the genus *Micromonospora* a very promising source of multifunctional bioinoculants.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00922

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The importance of chorismate mutase in the biocontrol potential of *Trichoderma parareesei*

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Species of *Trichoderma* exert direct biocontrol activity against soil-borne plant pathogens due to their ability to compete for nutrients and to inhibit or kill their targets through the production of antibiotics and/or hydrolytic enzymes. In addition to these abilities, *Trichoderma* spp. have beneficial effects for plants, including the stimulation of defenses and the promotion of growth. Here we study the role in biocontrol of the *T. parareesei Tparo7* gene, encoding a chorismate mutase (CM), a shikimate pathway branch point leading to the production of aromatic amino acids, which are not only essential components of protein synthesis but also the precursors of a wide range of secondary metabolites. We isolated *T. parareesei* transformants with the *Tparo7* gene silenced. Compared with the wild-type, decreased levels of *Tparo7* expression in the silenced transformants were accompanied by reduced CM activity, lower growth rates on different culture media, and reduced mycoparasitic behavior against the phytopathogenic fungi *Rhizoctonia solani*, *Fusarium oxysporum* and *Botrytis cinerea* in dual cultures. By contrast, higher amounts of the aromatic metabolites tyrosol, 2-phenylethanol and salicylic acid were detected in supernatants from the silenced transformants, which were able to inhibit the growth of *F. oxysporum* and *B. cinerea*. In *in vitro* plant assays, *Tparo7*-silenced transformants also showed a reduced capacity to colonize tomato roots. The effect of *Tparo7*-silencing on tomato plant responses was examined in greenhouse assays. The growth of plants colonized by the silenced transformants was reduced and the plants exhibited an increased susceptibility to *B. cinerea* in comparison with the responses observed for control plants. In addition, the plants turned yellowish and were defective in jasmonic acid- and ethylene-regulated signaling pathways which was seen by expression analysis of lipoxygenase 1 (*LOX1*), ethylene-insensitive protein 2 (*EIN2*) and pathogenesis-related protein 1 (*PR-1*) genes.

Keywords: shikimate pathway, *Tparo7* gene, silencing, antifungal, tyrosol, 2-phenylethanol, salicylic acid

INTRODUCTION

The use of biopesticides is an alternative for sustaining high production with low ecological impact in different agricultural production systems (Harman et al., 2010), *Trichoderma*-based products being the biofungicides available on the market most widely used (Harman et al., 2004; Lorito et al., 2010). Species of *Trichoderma* protect plants against pathogens owing to the plasticity of their genomes regarding the expression of their abilities to compete for nutrients, the inhibition or killing of plant pathogens through the production of antibiotics and/or cell wall (CW)-degrading enzymes (Druzhinina et al., 2011), the promotion of plant growth, and the induction of defenses against biotic and abiotic damage (Hermosa et al., 2012; Brotman et al., 2013).

Trichoderma parareesei is an efficient cellulase-producing species isolated from soil that has recently been described as a new species (Druzhinina et al., 2010). This species resembles the ancestor that originally gave rise to *T. reesei* and it exhibits the properties of an environmental opportunist (Atanasova et al., 2010). *T. parareesei* shows fast growth and produces abundant numbers of propagules on a variety of carbon sources, and is adapted to various lighting conditions. In contrast to *T. reesei*, strains of *T. parareesei* have shown biocontrol potential against fungal and oomycete plant pathogens (Atanasova et al., 2010; Rubio et al., 2014), as well as beneficial effects for plants, in terms of seedling lateral root development, and in adult plants improved defense against *Botrytis cinerea* and growth promotion under salt stress (Rubio et al., 2014).

The sequencing of *Trichoderma* genomes has revealed numerous secondary metabolite (SM) gene clusters (Mukherjee et al., 2013). The production of pyrones, peptaibols, terpenes, and polyketides is relevant in this genus (Degenkolb et al., 2006; Reino et al., 2008). Several *Trichoderma* SMs belonging to these structural families not only exhibit antagonistic activity against plant pathogens (Schirmböck et al., 1994; Rubio et al., 2009), but also have proven beneficial effects in plants (Viterbo et al., 2007; Vinale et al., 2008; Malmierca et al., 2015).

The shikimate pathway (**Figure 1**) is not present in animals but it is essential in other organisms such as bacteria, fungi, or plants for the synthesis of aromatic compounds, including aromatic amino acids (AAA) tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp) (Helmstaedt et al., 2001). Thus, it is a promising target for antimicrobial or antifungal agents and herbicides (Sasso et al., 2004). The shikimate biosynthetic route is connected to central carbon metabolism since it begins with phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E-4P) (Tzin and Galili, 2010). This first step is catalyzed by the enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (DAHPS), which is considered to be a critical regulatory checkpoint (Bentley, 1990; Light and Anderson, 2013). The shikimate pathway includes the production of chorismate, the precursor of Tyr and Phe, through the chorismate mutase (CM) enzyme (Romero et al., 1995; Sträter et al., 1997), which also generate a wide range of SMs (Vogt, 2010). CMs can be monofunctional, where they convert chorismate to prephenate through a Claissen rearrangement, or bifunctional, where they subsequently display prephenate dehydrogenase, prephenate dehydrate, or DAHPS activities (Tohge et al., 2013). CM is an important point of regulation for maintaining the correct balance of AAA in the cell. A protein-folding structural classification separates the CMs into three classes: the AroQ prokaryotic type, the AroQ eukaryotic type, and AroH, the latter two being present in monofunctional CMs. It is known that the CM of plants and eukaryotic microorganisms such as *Saccharomyces cerevisiae* belong to the monofunctional AroQ class and that they exhibit allosteric inhibition through Tyr and/or Phe and allosteric activation by Trp (Gu et al., 1997; Krappmann et al., 1999; Tzin and Galili, 2010). It has been described that *Ustilago maydis* also secretes a monofunctional aroQ CM lacking allosteric regulation that acts as a metabolic effector in favor of this pathogen during the colonization of maize plants (Djamei et al., 2011).

In this study we describe the characterization of the *Tparo7* gene that encodes a CM in *T. parareesei*. *Tparo7*-silenced mutants were used to study the role of this primary and secondary metabolic node in *T. parareesei* biocontrol activity and its interaction with tomato plants in terms of root colonization and the induction of systemic defenses.

MATERIALS AND METHODS

Microorganisms and Plants

Escherichia coli DH5α was used as a host for plasmid construction and propagation. This bacterial strain was grown in Luria-Bertani (LB) broth or on LB plates, supplemented with ampicillin (100 μg/ml), X-gal (40 μg/ml), and IPTG (10 μg/ml), when required.

T. parareesei (formerly *T. reesei*) IMI 113135 (CABI Bioscience, Egham, UK), referred to here as the T6 strain, was used as the wild-type (WT) throughout this study. T6 was used as a source of DNA to clone the *Tparo7* gene and also as a host in the transformation experiments to silence the *Tparo7* gene. All strains were propagated on potato dextrose agar (PDA, Difco Laboratories, Detroit).

Fusarium oxysporum CECT 2866 (Spanish Type Culture Collection, Valencia, Spain) (FO), *Rhizoctonia solani* strain19 (RS), belonging to the anastomosis group AG-2-2 IIIB, and *Botrytis cinerea* B05.10 (BC) were used as plant pathogenic microorganisms in antagonism assays. BC was used as pathogen in *in vivo* assays. These strains were stored at -80°C in 30% glycerol (FO, BC) and at 4°C in PDA plugs suspended in sterile water (RS).

Tomato seeds (*Solanum lycopersicum* "Marmande") were sterilized in 2% sodium hypochlorite for 20 min and washed thoroughly in sterile distilled water before use.

Culture Conditions

For gene expression and CM activity studies, *T. parareesei* mycelia were obtained following a two-step liquid culture procedure (Cardoza et al., 2006). First, the strains were grown in potato dextrose broth (PDB, Difco Laboratories) at 28°C and 200 rpm for 48 h. The fungal biomass was harvested, washed and transferred to minimal medium (MM) with 2% glucose as the

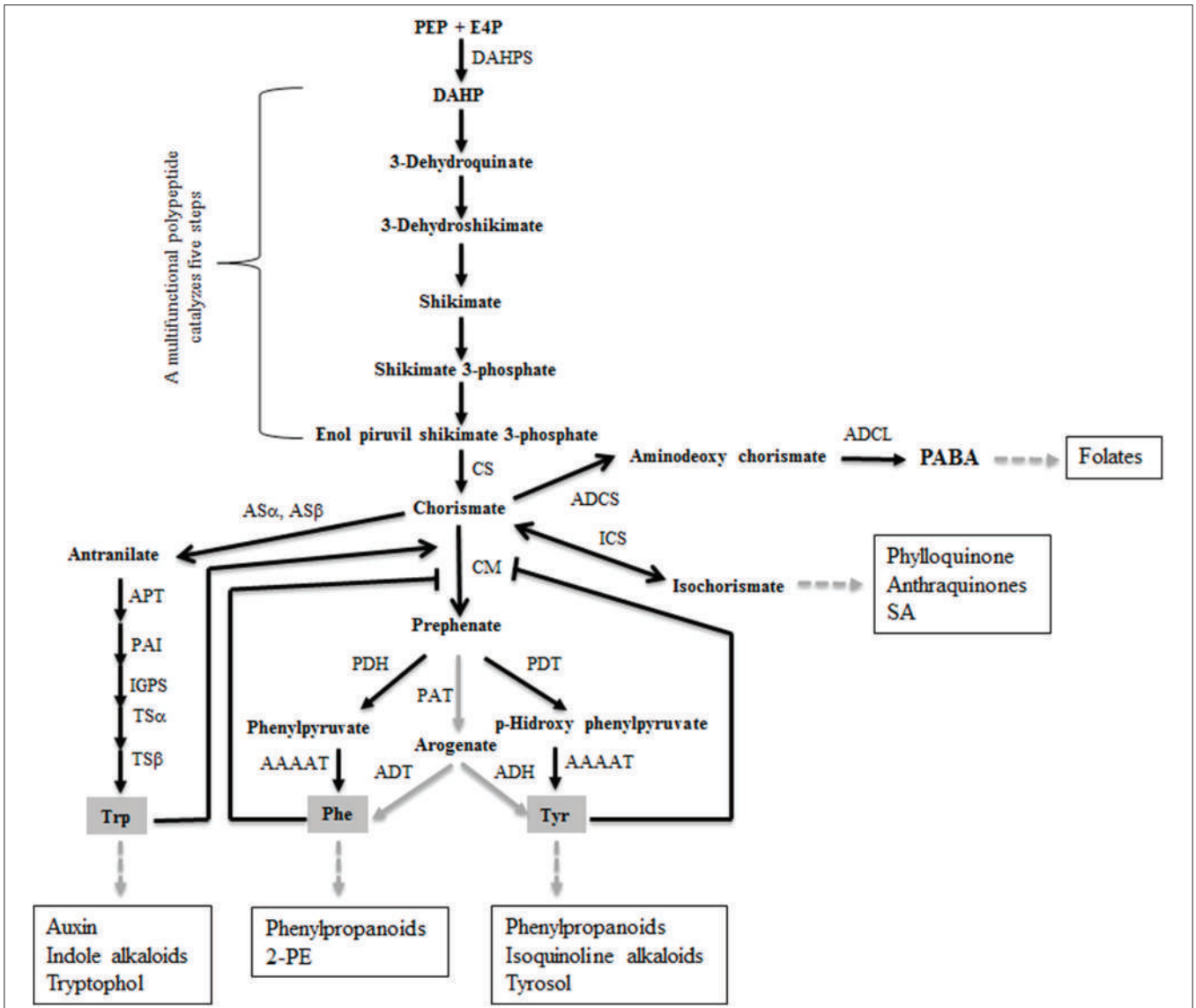

FIGURE 1 | Enzymes and metabolites comprising the shikimate pathway in *Trichoderma*. Black and gray arrows indicate the existence and absence, respectively, of genetic evidence (genes were identified in the available *T. reesei* genome and/or previously reported steps in yeast) for a given reaction. For simplicity, the AROM protein catalyzing the steps two to six is not shown. Abbreviations: PEP, phosphoenolpyruvic acid; E4P, erytrose 4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; DAHPS, DAHP synthase; CS, chorismate synthase; CM, chorismate mutase; PDH, prephenate dehydrogenase; PDT, prephenate dehydratase; AAAAT, aromatic amino acid amidotransferase; ASα, antranilate synthase alpha subunit; ASβ, antranilate synthase beta subunit; PAT, prephenate aminotransferase; ADT, arogenate dehydratase; ADH, arogenate dehydrogenase; APT, antranilate phosphoribosyl transferase; PAI, phosphoribosylantranilate isomerase; IGPS, indole-3-glycerol phosphate synthase; TSα, tryptophan synthase alpha subunit; TSβ, tryptophan synthase beta subunit; ICS, isochorismate synthase; ADCS, 4-amino-4-deoxychorismate synthase; ADCL, adenylsuccinate lyase; 2-PE, 2-phenylethanol; SA, salicylic acid; PABA, p-amino-benzoic acid. Major metabolites derived from the shikimate pathway are marked in squared boxes.

carbon source (Penttilä et al., 1987), and used as the control condition, or MM supplemented with 5 mM Tyr, Phe, Trp, or chorismic acid barium salt, when indicated, or PDB. After 24 h of incubation at 28°C and 200 rpm, mycelia were collected by filtration, thoroughly washed with sterile water, lyophilized, and kept at −80°C until RNA extraction.

For the determination of growth rates, fungi were inoculated at the center of MM or PDA plates and colony diameters were recorded every 24 h. To test the influence of different carbon and nitrogen sources on fungal growth, MM (with 2% glucose) or MM in which 2% glucose was replaced by 2% glycerol containing or not 38 mM ammonium sulfate or 5 mM Tyr, 5 mM Phe, and 2 mM Trp, were also used. For the determination of fungal biomass after growth in liquid culture, mycelium was collected by filtration, lyophilized and the dry weight was measured.

For the analysis of hydrolytic activities, intracellular proteins were extracted from mycelia of *T. parareesei* strains obtained from PDB containing 0.5% *R. solani* cell walls (RS-CWs) grown at 28°C and 200 rpm for 48 h. RS-CWs were obtained as previously described (Fleet and Phaff, 1974), using fungal mycelium from a PDB culture incubated at 25°C and 200 rpm for 4 days.

For metabolite quantification, *T. parareesei* supernatants were obtained from MM or PDB cultures incubated at 28°C and 200 rpm for 48 h.

To perform plant rhizosphere colonization tests, eight tomato seeds were grown in a Phytatray II box (Sigma-Aldrich) containing 100 ml of liquid MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose for 2 weeks. Each box was inoculated with 10^5 conidial germlings ml^{-1} of *T. parareesei* strain or not (control). The collection of germlings and the maintenance of plant-fungal co-cultures were carried out as previously described (Rubio et al., 2014). After 48 h of plant-fungal interaction, the roots from eight plants per treatment were collected, washed with distilled water, frozen, lyophilized, and kept at −20°C until total DNA extraction. Three independent tomato-*T. parareesei* co-culture experiments were used for each fungal strain.

DNA and RNA Procedures

Total DNAs from fungi were extracted following the method of Raeder and Broda (Raeder and Broda, 1985), using mycelium collected from a PDB culture incubated at 28°C and 200 rpm for 2 days. DNA isolation from tomato roots was performed with the cetyltrimethylammonium bromide (CTAB) extraction method (Dellaporta et al., 1983). Total RNA was extracted using TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad), following the instructions of the manufacturer.

For Southern analyses, 10 μg of genomic DNA was *Sac*I-digested, electrophoresed on a 0.7% agarose gel and transferred to a Hybond-N^+ membrane (Amersham Biosciences AB, Uppsala, Sweden). The *phleomycin* gene was labeled using the DIG High Prime kit (Roche, Penzberg, Germany), following the manufacturer's instructions, and used as probe. Hybridization, washes and detection were carried out as previously described (Tijerino et al., 2011).

Construction of the Gene-silencing Vector and Transformation

Plasmid pSIL (Cardoza et al., 2006) was linearized with *Spe*I-*Bam*HI and then ligated to a 500-bp *Spe*I-*Bam*HI fragment of the *Tparo7* gene. This fragment was in "sense" relative to the orientation of the *ta* gene promoter from *T. harzianum*. The construct was then linearized with *Hin*dIII-*Xho*I and ligated to the same 500-bp *Tparo7* fragment but in an "antisense" orientation relative to the *ta* gene promoter. A 159-bp intron was introduced between both the sense and antisense fragments of the *Tparo7* gene. The resulting plasmid also contained the terminator region of the *cbh*2 gene from *T. reesei* and was designated pSIL-ARO7. In order to transform this cassette in *T. parareesei* T6, pSIL-ARO7 was digested with *Sac*I to isolate the SIL-*Tparo7* fragment, which was then ligated to the same restriction site of plasmid pJL43b1 (Gutiérrez et al., 1997), giving rise to the final construct, pJL43b1-ARO7 (7.6 kb). This latter vector was used to transform *T. parareesei* T6 by a protoplast-based method described previously (Cardoza et al., 2006). In parallel, strain T6 was also transformed with pJL43b1 to obtain empty vector transformants; one of them, Tp-TC, was included in the assays as a transformation control. Transformants were selected for phleomycin resistance.

Real-time PCR Analysis

Gene expression was analyzed by quantitative real-time PCR. cDNA was synthesized from 2 μg of RNA, which was treated with DNase RQ1 (Promega Biotech Ibérica, Alcobendas, Spain) and then used for reverse transcription with an oligo(dT) primer with the Transcriptor First Strand cDNA Synthesis kit (Takara Inc., Tokyo, Japan), following the manufacturer's protocol. Real-time PCR reactions were performed with a StepOnePlus thermocycler (Applied Biosystems, Applied Foster City) in a total volume of 10 μl, using SYBR FAST KAPA qPCR (Biosystems, Buenos Aires, Argentina) and a final primer concentration of 100 nM each. Reactions were performed with cDNA from four pooled biological replicates for each condition, with the exception of five pooled biological replicates used to analyze plant defense gene expression from *in vivo* cultures. All reactions were performed in triplicate under the following conditions: an initial denaturation step (10 min at 95°C) followed by 40 cycles of denaturation (30 s at 95°C), annealing (1 min 60°C), and extension (1 min 72°C). CT values were calculated using the Applied Biosystems software, and transcript abundance was calculated with Microsoft Excel from Ct (cycle threshold) values normalized to the *actin* gene signal. The slopes and efficiency for each primer pair, Qaro7-C/Qaro7-D, and Act-F/Act-R, were measured for dilution series of pooled cDNA samples and calculated using the Applied Biosystems software (Table S1). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

DNA quantification of wild-type, Tp-TC and *Tparo7*-silenced transformants from colonized tomato roots was performed by real-time PCR as previously described (Morán-Diez et al., 2009), using the primer couples Act-tomF/Act-tomR and Act-F/Act-R (Table S1) to amplify a fragment of the *actin* gene from tomato and *Trichoderma*, respectively. Mixture and real-time PCR conditions were as indicated above. Ct values were calculated and the amount of fungal DNA was estimated using standard curves. Values were normalized to the amount of tomato DNA in the samples. Each sample was tested in triplicate.

Activity Assays

Mycelia collected from liquid cultures, grown as described above, were lyophilized and homogenized in 100 mM Tris-HCl (pH 7.5) plus 10% sodium dodecyl sulfate (w/v) buffer at 800 rpm for 1 h at 4°C in a Thermomixer (Eppendorf). Protein extracts were recovered by centrifugation at 12000 × g at 4°C for 20 min and stored at −20°C. Quantitative protein determination was performed with the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as standard protein.

CM activity was determined spectrophotometrically based on the formation of phenylpyruvate by treatment with

HCl (Davidson and Hudson, 1987). The reaction mixture contained 1 mM chorismate, 0.1 mg/ml BSA, and 10 mM 2-mercaptoethanol in 50 mM Tris-HCl buffer with 1 mM EDTA, pH 8.0. For the CM activity assay, 0.4 ml of this solution was preheated for 5 min at 37°C in a water bath. After the addition of 50 µl of enzyme solution, the reaction was incubated at 37°C for 5 min. For the conversion of chorismate to phenylpyruvate, 0.4 ml of 1 M HCl was added and the mixture was further incubated at 37°C for 10 min. The samples were rendered alkaline with 1 ml of 2.5 M NaOH, and absorbance was measured at 320 nm against a blank sample without enzyme. Protease and chitinase activities were determined in colorimetric assays as previously described (Montero-Barrientos et al., 2011), measuring the hydrolysis of azocasein at 366 nm (Holwerda and Rogers, 1992), and the release of N-acetylglucosamine during the hydrolysis of chitin at 585 nm (Reissig et al., 1955), respectively. All activity measurements were performed in triplicate. Activities are expressed as µmol min^{-1} per mg of protein.

Quantification of Metabolites

One hundred ml of culture supernatant was collected by filtration through sterile filter paper and lyophilized. Then, the resulting powder was extracted with 1 ml of a H_2O:methanol solution (9:1) and filtered through a 0.22-µm membrane. Tyrosol and 2-PE quantifications were carried out at the Mass Spectrometry Service of NUCLEUS (University of Salamanca) by capillary gas chromatography-mass spectrometry using an Agilent GC7890A gas chromatograph with a MS220 detector. A VF5ms capillary column was used (30 m, 0.25 mm 0.25 micron), with a starting temperature of 50°C kept for 5 min, followed by a ramp at 5°C per min up to 270°C, this temperature being held for 5 min.

Salicylic acid (SA) production was determined in 4 ml of culture supernatants as previously described (Leeman et al., 1996; Mercado-Blanco et al., 2001).

In Vitro Antifungal Assays

Confrontation assays (dual cultures) between *T. parareesei* strains and RS, FO, or BC plant pathogens were carried out in triplicate as previously described (Rubio et al., 2009). The dual cultures were photographed after 4 days at 28°C. BC dual cultures were also photographed after 18, 48, and 72 h at 28°C.

Growth assays on cellophane sheets were carried out in triplicate, as previously described (Rubio et al., 2009). Growth diameters were measured after 72 h for RS, 96 h for BC and after 120 h for FO. The results are expressed as the percentage of growth inhibition of each pathogen by each *T. parareesei* strain with respect to the mean colony diameters of each pathogen in control cultures.

The antifungal activity of *T. parareesei* supernatants against FO and BC was tested. A conidial suspension (2000 conidia in 5 µl) was added to the wells of sterile 96-well flat-bottomed microtiter plates along with 50 µl of water (control) or filter-sterilized (0.22-µm syringe filter; Millipore) supernatant from a 48 h-PDB culture, previously boiled for 10 min to avoid interference of enzymatic activities, or unboiled. PDB medium was added to each well up to a final volume of 150 µl. The plates were shaken briefly and placed in the dark at 28°C for 72 h. FO and BC growth were determined at 0, 24, 48, and 72 h by measuring optical density at 595 nm using a Sunrise microtiter plate reader (Tecan Ibérica, Barcelone, Spain). Each test was performed in sixtuplicate.

In Vivo Assays

Tomato seeds were coated with a conidial suspension containing 2×10^8 conidia/ml of *T. parareesei* T6, Tp-TC, or *Tparo7*-silenced transformants Tparo7-S3 and Tparo7-S4, or not (control) and were left in open Petri dishes to air-dry overnight in a laminar flow hood. One ml was used to coat 40 seeds. Plants were maintained under greenhouse conditions with a photoperiod of 16 h light: 8 h dark, a temperature between 18 and 30°C, and humidity maintained at 75% for 4 weeks. At this time, measurements of stem height and stem diameter were manually taken. Aboveground and root tissues were separated and dried until constant weight. Chlorophyll values were obtained with a Chlorophyll meter SPAD-502 Plus (Konica Minolta, Japan). The sensitivity of 4-week-old tomato plants to BC was evaluated. One leaf from each plant was inoculated on three leaflets, using 10 µl of a germination solution (20 mM glucose and 20 mM potassium phosphate) containing 2500 conidia per point. Necrotic leaf area was evaluated after three days using ImageJ free software. Ten plants were considered for each condition.

The aerial part of each tomato plant was sampled before being infected with BC and used for RNA extraction. Marker genes representative of the SA [pathogenesis-related protein 1 (*PR-1*)], jasmonic acid (JA) [lipoxygenase 1 (*LOX1*)] and ethylene (ET) [ethylene-insensitive protein 2 (*EIN2*)] signaling pathways were analyzed using real-time PCR, as described above. The primer pairs used are shown in Table S1.

Sequence Analyses

Sequences were analyzed using the DNAstar package (Lasergene, Madison). Protein sequences were aligned using the CLUSTAL X algorithm (Thompson et al., 1994). The nucleotide sequence of *Tparo7* was deposited in the GenBank database with Accession No. KT240045.

Statistical Analyses

Analysis of variance (ANOVA) was conducted with SPSS v. 19 software (SPSS Inc., Chicago) and Tukey's test was used at the 95% significance level.

RESULTS

Isolation and Expression of the *Tparo7* Gene

A gene encoding a putative CM was identified in the available *T. reesei* genome (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Based on its genomic sequence, two oligonucleotides were designed, Tparo7-F and Tparo7-R, and used to amplify a 974-bp fragment from *T. parareesei* T6 genomic DNA by PCR. This 974-bp fragment had an open reading frame (ORF) of 801 bp, two introns of 82 and 91 bp, and it was designated *Tparo7*. The ORF

of *Tparo7* encodes a protein of 266 amino acids with a theoretical molecular mass of 23.94 kDa and an isoelectric point of 5.44. An analysis of the primary structure of TpARO7 revealed the conserved monofunctional aroQ eukaryotic-type CM domain from position 14–262, related to its catalytic mechanism. There was also similarity of the deduced TpARO7 sequence to cytosolic CMs from other filamentous (AROC of *Aspergillus nidulans,* 60% identity) or yeast (ARO7 of *S. cerevisiae*, 46% identity) ascomycetes, basidiomycetes (ARO7 of *U. maydis*, 40% identity), plants (*Arabidopsis thaliana*, 37–41%) or bacteria (*Chitinovibrio alkaliphilus*, 37% identity). Sequence similarity was lower with the secreted Cmu1 of *U. maydis* (12%). A secondary structure analysis of TpARO7 predicted the prevalence of the alpha-helix conformation.

The allosteric regulation effect by AAA was explored in intracellular proteins, obtained from T6 mycelia, measuring CM activity after incubation in the presence of 100 μM Tyr, Phe, or Trp, or a mixture of these three AAA at a concentration of 100 μM each. In comparison with the control condition, a 38% reduction in CM activity was recorded when 100 μM Tyr was added to the reaction mixture, whereas an activity increase of 20% was detected with 100 μM Trp. No significant changes in CM activity were observed when Phe or the AAA mixture was added.

Tparo7 expression was investigated using real-time PCR after growing T6 for 24 h in the presence of 5 mM chorismic acid or each AAA—Tyr, Phe, or Trp. All these compounds upregulated *Tparo7* expression as compared with the transcript levels detected in 24-h MM-grown mycelia (control condition) (**Figure S1**). However, compared with the CM activity detected in MM, lower values were recorded from T6 cultures supplemented separately with these four compounds (**Table 1**).

TABLE 1 | Chorismate mutase activity of *T. parareesei* T6 intracellular protein extracts.

	Mean CM activity (μmol·min^{-1}/mg protein) ± *SD*
MM-glucose	1.20 ± 0.09^{a}
MM-Tyr	1.02 ± 0.02^{ab}
MM-Phe	0.90 ± 0.10^{b}
MM-Trp	1.12 ± 0.09^{ab}
MM-chorismic acid	0.85 ± 0.02^{b}

Values are means of three replicates with the corresponding standard deviations. Values followed by different superscript letters are significantly different ($P < 0.05$). Activity was measured in mycelia from 24 h MM supplemented with 2% glucose or 5 mM Tyr, Phe, Trp, or chorismic acid barium salt cultures.

Isolation of *Tparo7*-silenced Transformants

In order to characterize the *Tparo7* gene functionally, plasmid p43b1-ARO7 was constructed and transformed in *T. parareesei* T6. Eight out of fourteen transformants showing phleomycin resistance were checked by PCR using the primers TADIR2 and Intro-R. The presence of a 1200-bp amplification fragment was observed for seven of them. Integration of the transformation cassette was analyzed by Southern blot using *phleomycin* cDNA as a probe (**Figure S2**). Strains Tparo7-S2, Tparo7-S3, and Tparo7-S4, representing the three different integration patterns found among these seven transformants, and the transformation control strain Tp-TC, were chosen for further studies.

We analyzed both the expression of the *Tparo7* gene and CM activity in the selected transformants under two different culture conditions, using the expression level in strain T6 as a reference condition. The Tparo7-S3 and Tparo7-S4 transformants showed significantly lower *Tparo7* transcript levels linked to lower CM activity than those detected in T6 or Tp-TC after 24 h growth in MM and PDB (**Figure 2**, **Table 2**). Taking into account that Tparo7-S2 showed higher *Tparo7* transcript levels and CM activity than T6 or Tp-TC in PDB, the Tparo-S3 and TparoS4 strains were selected as *Tparo7*-silenced transformants for inclusion in ensuing assays. Since lower *Tparo7* expression levels were accompanied by lower CM activity, this gene must encode a CM enzyme in *T. parareesei*.

CM constitutes a central node of primary metabolism. Thus, the silenced transformants displayed lower growth rates than the

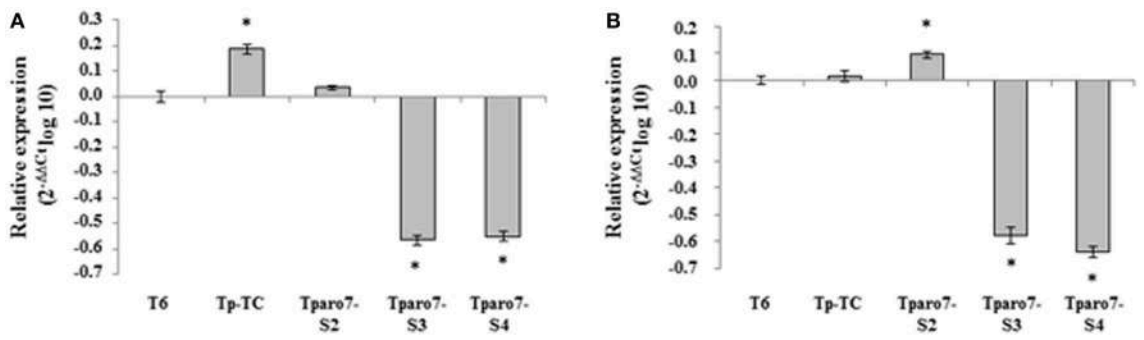

FIGURE 2 | Quantification of *Tparo7* transcripts in a control transformant (Tp-TC) and three silenced transformants (Tparo7-S2, Tparo7-S3 and Tparo7-S4) by real-time PCR. Values correspond to relative measurements against the *Tparo7* transcript in the wild-type *T. parareesei* T6 ($2^{-\Delta\Delta Ct} = 1$). The experiment was carried out with mycelia grown for 48 h on PDB and transferred to MM containing 2% glucose **(A)** or PDB **(B)** for 24 h. *T. parareesei* T6 actin was used as an internal reference gene. Bars represent the standard deviations of the mean of three replicates. Asterisk (*) represents statistically significant differences ($P < 0.05$).

T6 and Tp-TC control strains in all the different culture media tested (**Table 3**). In addition, *Tparo7*-silenced transformants showed lower sporulation but a higher production of diffusible pigments on PDA. All strains displayed the highest colony diameters at 37°C, the optimal growth temperature for *T. parareesei*, and significantly lower sizes were observed in the silenced mutants in comparison with those from T6 and Tp-TC (data not shown).

TABLE 2 | Chorismate mutase activity of *T. parareesei* T6, transformation control Tp-TC, or silenced-transformant intracellular protein extracts.

	Mean CM activity (μmol min^{-1}/mg protein) ± *SD*	
	MM	**PDB**
T6	1.87 ± 0.09^{b}	0.41 ± 0.01^{b}
Tp-TC	2.50 ± 0.68^{a}	0.33 ± 0.02^{c}
Tparo7-S2	1.78 ± 0.06^{b}	0.49 ± 0.03^{a}
Tparo7-S3	0.70 ± 0.03^{c}	0.23 ± 0.02^{d}
Tparo7-S4	0.08 ± 0.05^{d}	0.08 ± 0.02^{e}

Values are means of three replicates with the corresponding standard deviations. For each medium, values followed by different superscript letters are significantly different ($P < 0.05$). Activity was measured in mycelia from 24 h MM supplemented with 2% glucose or PDB cultures.

TABLE 3 | Growth rates of *T. parareesei* strains on different culture media.

	PDA	MM-glucose	MM-glycerol	MM-glycerol-AAA*
T6	7.0 ± 0.1^{a}	4.2 ± 0.0^{a}	3.8 ± 0.1^{a}	3.0 ± 0.1^{a}
Tp-TC	7.0 ± 0.1^{a}	4.1 ± 0.1^{a}	3.9 ± 0.1^{a}	3.2 ± 0.2^{a}
Tparo7-S3	4.2 ± 0.0^{c}	1.9 ± 0.1^{b}	1.5 ± 0.2^{c}	0.7 ± 0.2^{b}
Tparo7-S4	5.0 ± 0.3^{b}	2.0 ± 0.1^{b}	1.9 ± 0.0^{b}	0.9 ± 0.1^{b}

**The ammonium sulfate of the MM was replaced by a mix containing 5 mM Tyr, 5 mM Phe, and 2 mM Trp.*

Values are means of three replicates with the corresponding standard deviations. For each medium, values followed by different superscript letters are significantly different ($P < 0.05$). Colony diameters (cm per 24 h) of wild-type (T6), transformation control (Tp-TC) and Tparo7-silenced transformants (Tparo7-S3 and Tparo7-S4) were measured on agar plates containing PDA or MM supplemented with 2% glucose or 2% glycerol.

Antifungal Activity

The influence of *Tparo7* silencing in the antifungal activity of *T. parareesei* was evaluated using different *in vitro* assays with the phytopathogens FO, BC and/or RS as targets. A lower mycoparasitic behavior was observed for *Tparo7*-silenced transformants than for T6 or Tp-TC in dual confrontation experiments performed between the *T. parareesei* strains and the three targets (**Figure 3** and **Figure S3**). After 4 days of incubation, T6 and Tp-TC were able to overgrow the colonies of FO or BC, whereas Tparo7-S3 and Tparo7-S4 only surrounded them. This absence of FO and BC overgrowth capacity by the silenced transformants persisted even at 10 days of incubation (data not shown).

The antagonistic potential of *T. parareesei* extracellular compounds against these pathogens was evaluated using a membrane assay. After removal of the cellophane sheet containing the *T. parareesei* mycelium, the effect of the total compounds secreted by antagonistic strains on the growth of pathogens was determined. The FO, RS, and BC growth inhibition percentages calculated for the four *T. parareesei* strains are summarized in **Table 4**. The compounds secreted by T6 or Tp-TC displayed a more marked inhibition of FO and RS than those secreted by *Tparo7*-silenced mutants ($P < 0.05$). However, the highest and the lowest inhibition values of BC were respectively obtained from plates where strains Tparo7-S3 and Tparo-S4 had been previously grown.

To analyze the effect of *Tparo7* silencing in the production of CW hydrolytic enzymes by *T. parareesei* strains, we measured protease and chitinase activities on intracellular proteins from 48-h PDB plus 0.5% RS-CWs-grown mycelium (**Table 5**). Lower values of protease activity were observed for the silenced transformants Tparo7-S3 and Tparo7-S4 than for strains T6

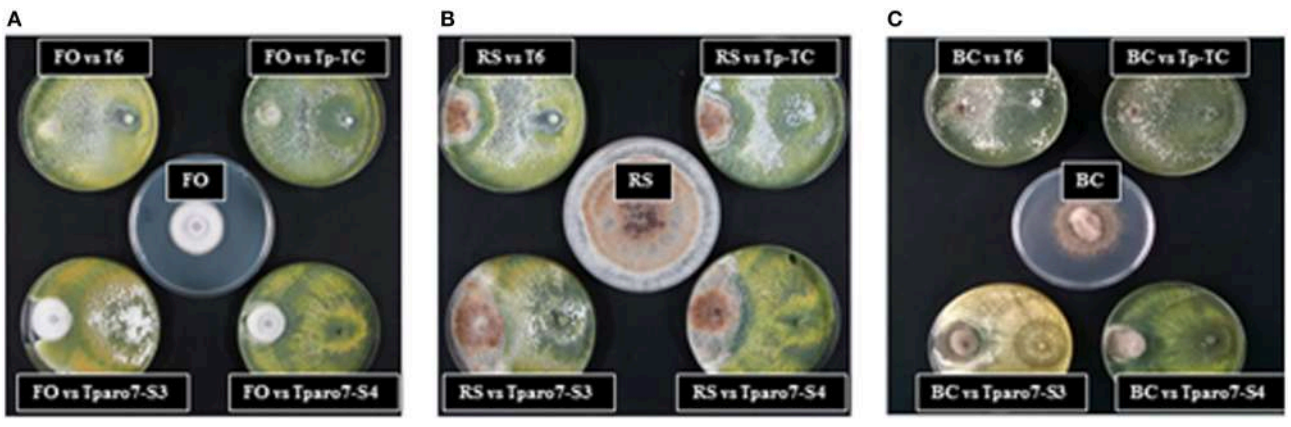

FIGURE 3 | Dual cultures of strains T6, the silenced transformants Tparo7-S3 and Tparo7-S4, and the control transformant Tp-TC of *T. parareesei* and the pathogens *F. oxysporum* (FO) (A), *R. solani* (RS) (B), and *B. cinerea* (BC) (C) on PDA medium. Plates in the center correspond to the growth of each pathogen without *Trichoderma* strains. Plates were incubated at 28°C for 4 days.

or Tp-TC, although such differences were only significant for Tparo7-S4 ($P < 0.05$). However, strain Tparo7-S3 displayed significantly higher chitinase activity than any other strain.

The antifungal activity of *T. parareesei* supernatants was evaluated against FO and BC on 96-well E plates. In these tests, the hyphal growth from conidia of the two target fungi was registered at 24, 48, and 72 h, and unboiled and boiled supernatants were considered to determine the antifungal activity of SMs plus secreted enzymes and SMs, respectively (**Figure 4**). The absorbance values recorded at 0 h did not show significant differences among the five conditions considered in these tests (data not shown). The supernatants from the different *T. parareesei* strains had significant antifungal activity against FO and BC at 72 h. Moreover, Tparo7-S3 supernatants, unboiled and

TABLE 4 | Colony growth inhibition (%) of *F. oxyxporum*, *R. solani* and *B. cinerea* by *T. parareesei* strains grown on cellophane membranes.

	F. oxyxporum	*R. solani*	*B. cinerea*
T6	28.3 ± 0.4^{a}	24.0 ± 0.0^{a}	22.7 ± 1.7^{c}
Tp-TC	28.1 ± 0.4^{a}	24.0 ± 0.2^{a}	26.2 ± 0.8^{b}
Tparo7-S3	26.2 ± 0.3^{b}	11.0 ± 0.1^{b}	29.8 ± 0.2^{a}
Tparo7-S4	16.0 ± 0.1^{c}	2.0 ± 0.1^{c}	8.5 ± 0.5^{d}

Values are means of three replicates with the corresponding standard deviations. For each column, values followed by different superscript letters are significantly different ($P < 0.05$). Strains were wild-type (T6), transformantion control (Tp-TC), and Tparo7-silenced transformants (Tparo7-S3 or Tparo7-S4).

TABLE 5 | Protease and chitinase activities measured in *T. parareesei* strain intracellular protein extracts obtained from 48 h liquid cultures in PDB supplemented with 0.5% *R. solani* cell walls.

	Mean activity (μmol·min^{-1}/mg protein) ± *SD*	
	Protease	**Chitinase**
T6	56.83 ± 0.67^{a}	0.78 ± 0.09^{b}
Tp-TC	57.84 ± 3.80^{a}	0.64 ± 0.03^{c}
Tparo7-S3	48.07 ± 5.83^{ab}	1.93 ± 0.25^{a}
Tparo7-S4	24.65 ± 0.88^{b}	0.90 ± 0.07^{b}

Values are means of three replicates with the corresponding standard deviations. For each activity, values followed by different superscript letters are significantly different ($P < 0.05$).

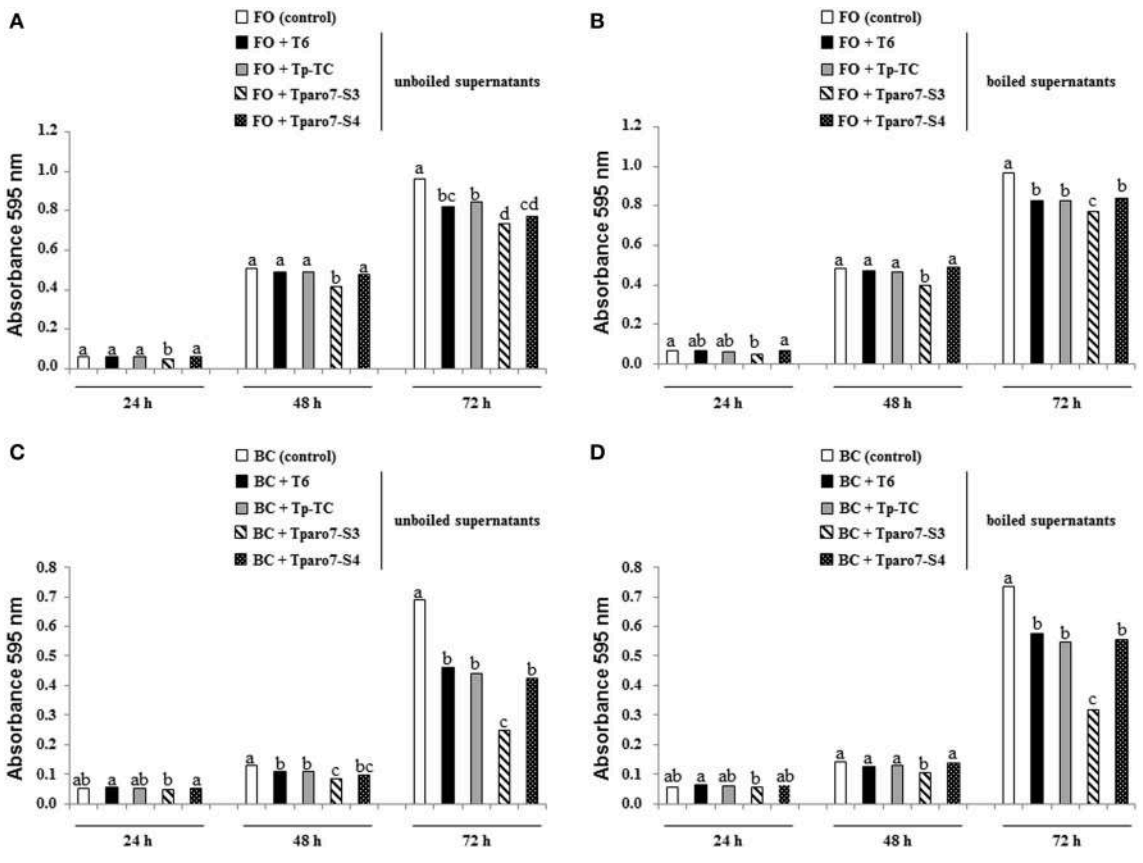

FIGURE 4 | Effect of *T. parareesei* supernatants in the growth of *F. oxysporum* (FO) (A,B) and *B. cinerea* (BC) (C,D). Tests were carried out without (control) or with 50 µl of filter-sterilized supernatant from 48 h-PDB cultures of strains T6, Tp-TC, Tparo7-S3 and Tparo7-S4, previously boiled for 10 min **(B,D)** or not **(A,C)**. Fungal growth was determined after 28°C incubation at 24, 48, and 72 h by measuring absorbance at 595 nm using a microtiter plate reader. Values are means of six replicates. Bars corresponding to every pathogen, supernatant treatment and incubation time marked with different letters are significantly different ($P < 0.05$).

boiled, showed significant higher inhibitory effect against the two pathogens compared to that of T6 or Tp-TC supernatants at 48 and 72 h. In a wide sense, *T. parareesei* supernatants were more effective against BC and SMs looked to be more relevant for their antifungal activity against both pathogens.

Metabolite Production

To analyze the effect of *Tparo7* silencing on shikimate pathway metabolite production, tyrosol, 2-PE and SA were determined in MM and PDB supernatants from 48-h-old cultures (**Table 6**). These metabolites were not detected in MM for any strain. Higher amounts of tyrosol, 2-PE and SA were measured in PDB supernatants of *Tparo7*-silenced transformants than those observed for T6 or Tp-TC. Similar results were obtained in two independent biological experiments.

In order to analyze the biocontrol potential of tyrosol, 2-PE and SA, putatively formed in the metabolic AAA pathway, we tested the effect of these compounds on the mycelial growth of RS, FO, and BC. The growth inhibition percentages of FO, RS and BC by 5, 10, and 30 mM of commercial tyrosol, 2-PE, or SA, concentrations no biologically relevant, are shown in Table S2. In general, growth inhibition values were directly proportional to the concentration of tyrosol, 2-PE, or SA in the culture medium. The growth of these plant pathogens was completely inhibited by 30 mM 2-PE or SA, and inhibition percentages of about 55% were obtained with 30 mM tyrosol.

Tomato Rhizosphere Colonization

The role of *Tparo7* in plant rhizosphere colonization was evaluated on tomato roots by quantitative real-time PCR using T6 and Tp-TC as controls and the two *Tparo7*-silenced transformants. As shown in **Table 7**, the amount of Tparo7-S3 and Tparo7-S4 DNA obtained from tomato roots was significantly lower than those of strains T6 and Tp-TC ($P < 0.05$).

Biocontrol in Tomato

Four-week-old tomato plants previously seed-coated with an aqueous solution (control) or treated with conidia of T6, Tp-TC, Tparo7-S3, or Tparo7-S4 were evaluated in this assay. The *in vivo* assay results revealed that there were significant differences in stem length, aboveground and root dry weights, chlorophyll SPAD index and the leaf area of 4-week-old tomato plants between T6 or Tp-TC and *Tparo7*-silenced strain treatments (**Table 8**). The lowest size, dry weight and chlorophyll value were observed in tomato plants previously seed-coated with *Tparo7*-silenced transformants. These data indicated that *Tparo7* gene silencing negatively affects tomato plants. The results obtained in BC-infected tomato assays with strains T6, Tp-TC, Tparo7-S3 and Tparo7-S4 showed that *Tparo7* silencing also affected the biocontrol activity of *T. parareesei* (**Table 8**, **Figure S4**). The highest necrotic leaf area was observed in *Tparo7*-silenced transformant treatments and no statistically differences were observed among the necrotic lesion sizes observed for the T6 and Tp-TC treatments.

To test whether the tomato response to the different *T. parareesei* treatments involved the differential activation of systemic defense-related genes, we analyzed markers of the SA (*PR-1*), JA (*LOX1*), and ET (*EIN2*) pathways using real-time

TABLE 6 | Concentration of tyrosol, 2-PE and SA in *T. parareesei* T6, Tp-TC, Tparo7-S3, and Tparo7-S4 strain supernatants obtained from 48 h PDB cultures.

	Tyrosol		2-PE		SA	
T6	18.98	19.04	9.49	9.52	0.09	0.12
Tp-TC	20.33	21.01	11.96	12.36	0.08	0.08
Tparo7-S3	33.27	33.60	27.40	27.66	0.73	0.59
Tparo7-S4	32.13	27.21	28.11	23.81	0.15	0.21

Tyrosol and 2-PE are expressed as mg per g of mycelial dry weight, and SA is expressed as μg per g of mycelial dry weight. Values correspond to two independent biological replicates.

TABLE 7 | Colonization of tomato roots by *T. parareesei* wild-type (T6), control transformant (Tp-TC), and *Tparo7*-silenced transformants (Tparo7-S3 or Tparo7-S4)*.

Strain	Tomato actin			*Trichoderma* actin			Ratio****
	Ct	SD	Qty**	Ct	SD	Qty***	
T6	20.82	0.06	144.52	16.87	0.02	24.58	0.17 ± 0.01^{a}
Tp-TC	20.97	0.02	127.65	17.01	0.18	22.07	0.17 ± 0.03^{a}
Tparo7-S3	21.04	0.10	121.25	18.43	0.31	7.74	0.06 ± 0.02^{b}
Tparo7-S4	21.78	0.09	67.43	18.93	0.03	5.38	0.08 ± 0.01^{b}

**Fungal DNA present on the tomato roots 48 h after the inoculation was quantified by real-time PCR. Three replicates were made of each sample. Ct, threshold cycle and SD, standard deviation.*

***Quantity of tomato DNA (ng) referred to tomato actin gene.*

****Quantity of Trichoderma DNA (ng) referred to Trichoderma actin gene.*

*****Proportion of fungal DNA vs. plant DNA. Values are the means of the three replicates with the corresponding standard deviations and values followed by different superscript letters are significantly different ($P < 0.05$).*

TABLE 8 | Effect of tomato seed treatment with *T. parareesei* T6, Tp-TC, Tparo7-S3 or Tparo7-S4 strains or not (control) on tomato plant growth (4-week-old plants) and necrotic leaf area caused by *B. cinerea.**

Treatment	Control	T6	Tp-TC	Tparo7-S3	Tparo7-S4
Stem diameter (mm)	5.31^{a}	5.24^{a}	5.21^{a}	3.81^{b}	4.12^{b}
Stem height (cm)	34.40^{a}	33.80^{a}	36.20^{a}	19.60^{b}	19.50^{b}
Aboveground dry weight (g)	1.81^{a}	1.40^{a}	1.68^{a}	0.49^{b}	0.53^{b}
Root dry weight (g)	0.28^{a}	0.27^{a}	0.29^{a}	0.15^{b}	0.16^{b}
Chlorophyll (SPAD index)	25.70^{a}	24.60^{a}	25.40^{a}	18.70^{b}	20.30^{b}
Foliar area (cm^2)	8.42^{a}	7.09^{a}	7.53^{a}	4.01^{b}	5.53^{b}
Necrotic foliar area (%)*	1.61^{b}	1.59^{b}	1.52^{b}	21.68^{a}	16.85^{a}

Ten plants were considered for each condition. Foliar area and necrotic foliar area were evaluated using ImageJ software.

**One leaf from each plant was inoculated on three leaflets using 10 μl containing 2500 B. cinerea conidia/point and the necrotic leaf area was evaluated after 3 days. In each line, means followed by different superscript letters are significantly different ($P < 0.05$).*

PCR in tomato leaves from 4-week-old plants. Marker gene expression in tomato plants from *in vivo* assays is shown in **Figure 5**. In comparison with the control, *T. parareesei* seed-coated tomato plants showed significantly increased expression levels of *PR-1*, whereas no differences were detected among strains. This result shows that SA pathway was induced at 4 weeks after *T. parareesei* treatment, which indicates a long-term systemic acquired resistance response elicited by *T. parareesei*. At the same time, *EIN2* and *LOX1* were downregulated in tomato seedlings from seeds treated with the *Tparo7*-silenced transformants and no statistically significant differences were observed among their expression levels in control plants and Tp-TC seed-coated plants.

DISCUSSION

Because the use of biofertilizers and biopesticides is an alternative for sustaining high production with low ecological impact, the development of research addressing biological control is currently expanding exponentially. Regarding the biocontrol of plant diseases, *Trichoderma*-based products are the most important biofungicides (Lorito et al., 2010), because selected *Trichoderma* strains have beneficial effects on plants, which can be explained in terms of plant growth promotion and the biological control of plant pathogens (Hermosa et al., 2012). Although *T. parareesei* T6 was able to achieve its best growth at 37°C and this temperature is closely related to the optimal growth temperature range of human pathogens, this species has not been neither isolated nor related to human disease and the continuous use of this strain in the laboratory has not resulted hazardous. In any case, risk assessment studies are needed before considering any *in vivo* application of this species in commercial agriculture (Rubio et al., 2014). The present study focuses on the role a *T. parareesei* CM in biocontrol and the induction of plant responses. This enzyme catalyzes the conversion of chorismate to prephenate, the precursor of the AAA Tyr and Phe, and constitutes a diverted node of the shikimate pathway, a biosynthetic network for primary and secondary metabolites. Contrary to what has been reported for *T. reesei,* with limited biocontrol activity, *T. parareesei* is able to antagonize fungal and oomycete plant pathogens (Atanasova et al., 2010; Rubio et al., 2014). Since the genome of *T. parareesei* has not been annotated and no functional studies of the shikimate pathway have been reported in *Trichoderma*, we applied a genomic approach, taking advantage of the whole genome sequence of *T. reesei* (Martinez et al., 2008). At least eight genes encoding putative enzymes of the shikimate pathway from E-4P and PEP to prephenate were identified in *T. reesei* (**Figure 1**), and the one corresponding to a CM was used to design oligonucleotides to isolate the *Tparo7* gene in *T. parareesei*. As expected, among the CMs previously characterized functionally TpARO7 showed the highest identity with the cytosolic CMs of filamentous ascomycetes (Krappmann et al., 1999). In addition, TpARO7 displayed more sequence identity with CMs from bacteria and plants than that observed

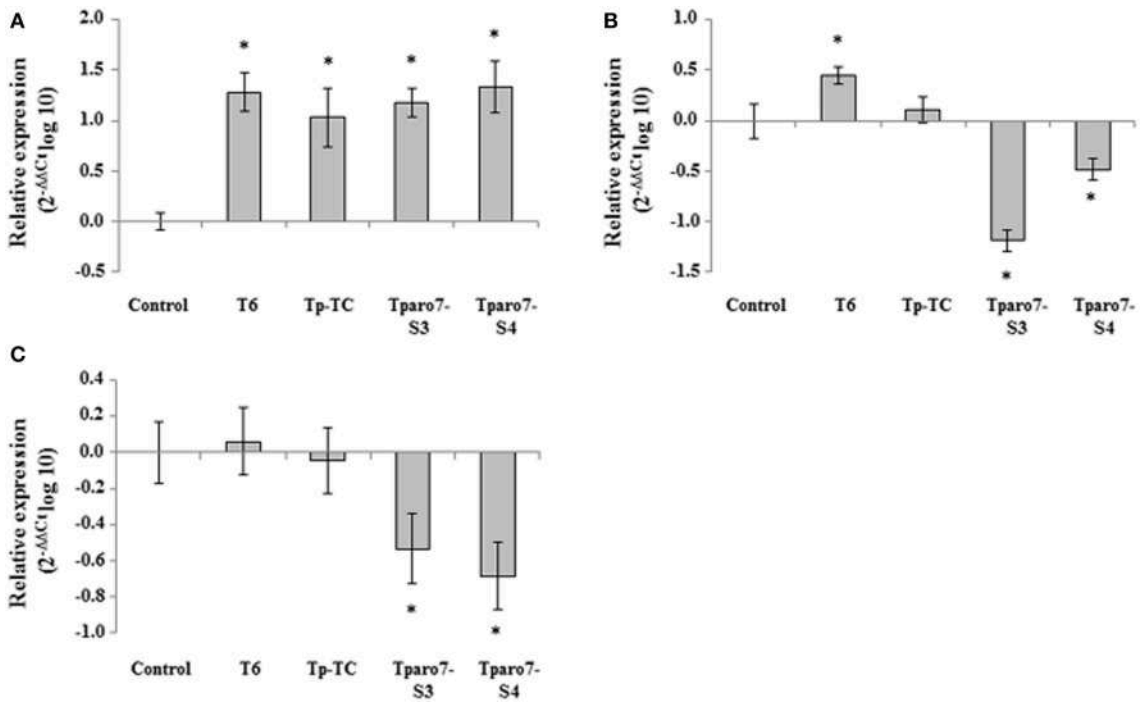

FIGURE 5 | Relative expression of the *PR*-1 (A), *LOX1* (B), and *EIN2* (C) genes in 4-week-old tomato leaves from seeds coated with a conidial suspension of *T. parareesei* T6, Tp-TC, Tparo7-S3, and Tparo7-S4. Values correspond to relative measurements against the transcripts in tomato leaves from untreated seeds ($2^{-\Delta\Delta Ct} = 1$). Tomato *actin* was used as an internal reference gene. Bars represent the standard deviations of the mean of three replicates. Asterisk (*) represents statistically significant differences ($P < 0.05$).

with the secreted Cmu1 of *U. maydis* (Djamei et al., 2011). The catalytic residues R^{20}, E^{27}, R^{162}, K^{173}, T^{250}, and E^{254}, which have been shown to be essential for the activity of some previously reported CMs, were also present in TpARO7.

CMs are normally feedback-inhibited by Tyr and/or Phe and induced by Trp, indicating allosteric regulation by AAA (Krappmann et al., 1999; Tzin and Galili, 2010). The *in vitro* CM activities calculated after the incubation of *T. parareesei* T6 protein extracts in the presence of AAA revealed feedback inhibition by Tyr and induction by Trp. Although *Tparo7* transcript levels were significantly upregulated in strain T6 grown in MM supplemented with chorismic acid or AAA (**Figure S1**), the corresponding CM activities in intracellular protein from mycelia cultured in these media were lower than those calculated in MM (**Table 1**). These results are compatible with the above-indicated regulation of CM by allosteric feedback-control of the different AAA. Since saprotrophic fungal growth has to adapt according to the surrounding environmental conditions, when AAA are scarce their biosynthesis should be regulated. Perhaps this occurred when T6 was incubated under imbalanced AAA sources, since many fungi possess the cross-pathway control system required to overcome an imbalanced amino acids diet, which is a complex process due to several feedback- or cross-feedback-controlled multi-amino acid pathways (Singh et al., 2010).

We generated CM mutant strains by RNA-mediated *Tparo7* gene silencing. This strategy allows the functions of genes to be studied under conditions where they are essential, since small transcript levels are sufficient to keep the organism viable. This approach proved to be adequate to analyze genes functionally in *T. harzianum* (Cardoza et al., 2006). As in previous studies performed with *Trichoderma* silenced transformants (Cardoza et al., 2006; Morán-Diez et al., 2009), different degrees of silencing were observed among the *Tparo7*-silenced mutants at both the transcript and CM activity levels (**Figure 2** and **Table 2**). We observed that *Tparo7* expression and CM activity were related, and only a residual enzymatic activity was detected for the silenced transformant Tparo7-S4, which showed the lowest transcript levels. No correlation was observed between the number of pSIL-ARO7 copies inserted and the gene expression levels and CM activity. In fact, compared to the T6 and the Tp-TC strains, the highest *Tparo7* expression observed in Tparo7-S2 grown in PDB (**Figure S2**) was accompanied by the highest CM activity. This same lack of correlation has also been reported for some other genes overexpressed or silenced in *Trichoderma* (Limón et al., 1999; Montero-Barrientos et al., 2007; Morán-Diez et al., 2009). The differences in *Tparo7* expression and CM activity between T6 and Tp-TC observed in some culture media are indicative of the fact that the insertion of the transformation cassette can affect this key regulation point of the primary metabolism under such culture conditions. For this reason, both strains were included in all tests carried out in the present study, displaying similar behavior in most of them.

As expected for a gene encoding a primary metabolism major node enzyme, *Tparo7*-silenced strains showed lower growth rates than those of T6 or Tp-TC on different culture media (**Table 3**). This phenotype was more evident in MM, mainly when this medium was supplemented with a mixture of AAA. This suggests an allosteric feedback-control of TpARO7 by AAA. Interestingly, Tparo7-S4, the strain with the lowest CM activity, showed the lowest growth rates at early incubation times (see **Figure S3**). However, this silenced mutant presented faster growth than Tparo7-S3 between 24 and 48 h of incubation (**Table 3**). This behavior, observed in several media, could be provoked by the complex regulation of the shikimate pathway just to stay alive despite of its high degree of CM silencing.

It is known that the biocontrol potential of *Trichoderma* varies depending upon the strains confronted (Atanasova et al., 2013). It could be proposed that the lower growth rate of Tparo-S3 and Tparo-S4 could be the cause of the reduced antagonistic ability displayed by these mutants against FO, RS and BC in dual cultures (**Figure 3** and **Figure S3**). However, the limited mycoparasitic efficiency of the silenced mutants against FO and BC cannot be explained on the basis of fitness or substrate competition, but instead by other *Trichoderma* biocontrol mechanisms. *Tparo7* silencing affected CW-degrading enzyme production in terms of reduced protease and increased chitinase activities. Recent studies based on transcriptomic analyses have indicated that proteolysis is a major biological process involved in the mycoparasitism of *Trichoderma* overgrowing its prey (Atanasova et al., 2013; Steindorff et al., 2014). The increased chitinase activity detected in *Tparo7*-silenced strains has also been reported when the *tri4* gene, involved in trichothecene production, was disrupted or silenced in *T. arundinaceum* (Malmierca et al., 2012). It has been documented that exogenous supply of SA or culture media, which contain high levels of SA, induce chitinase activity in plant cells (Schneider-Müller et al., 1994), and that Ca^{2+} plays an important role in the production of chitinase and SA (Schneider-Müller et al., 1994; Kawano et al., 1998). Although Ca^{2+} concentrations were not calculated in the present study, we have observed a correlation between SA production and chitinase activity in the different *T. parareesei* strains. In particular, Tparo7-S3 showed the maximum SA levels accompanied by the highest chitinase activity. In any case, this correlation needs to be further explored in fungi. These results would justify the unexpected high BC inhibition activity detected in membrane assays for Tparo7-S3. In these assays, performed on medium where *T. parareesei* was previously grown for 24 h, the above indicated scarce growth of Tparo7-S4 during the first hours of incubation could explain its low inhibition percentages recorded against the three pathogens (**Table 4**). However, membrane and supernatant assays have different experimental designs and results cannot be compared. In the tests performed with 48 h-PDB supernatants, those from Tparo7-S3 showed the highest antifungal activity against FO and BC, the two pathogens assayed (**Figure 4**). Moreover, taking into account that Tparo7-S4 has less growth than T6 and Tp-TC, its supernatant did not show reduced antifungal activity against these two pathogens at different incubation times. This is in agreement with the increased production of the AAA-derived antifungal compounds 2-PE, tyrosol and SA detected in both *Tparo7*-silenced mutants (**Table 6**). Thus, *Tparo7* silencing has a positive effect on metabolite production in *T. parareesei*. An explanation for this is that CM activity could be compromised

in *Tparo7*-silenced mutants and it has been reported that this enzyme is allosterically regulated by Tyr and Phe (Krappmann et al., 1999; Tzin and Galili, 2010). Thus, the conversion of Tyr and Phe into tyrosol and 2-PE respectively would prevent the allosteric inhibition of CM by these two amino acids. The production of tyrosol and 2-PE has been reported previously in *Trichoderma* spp. (Tarus et al., 2003), and a collateral tyrosol production has been identified in *T. brevicompactum* mutants overexpressing a trichodiene synthase involved in the biosynthesis of trichothecene compounds with antifungal activity (Tijerino et al., 2011). It has also been described previously that tyrosol, 2-PE and SA are part of the biocontrol strategies of yeast and bacteria against phytopathogens (Mercado-Blanco et al., 2001; Tarus et al., 2003; Liu et al., 2014). Bearing in mind the biocontrol assay data, the SM levels resulting from *Tparo7* silencing would affect the production of CW-degrading enzymes and hence the mycoparasitic activity of *T. parareesei*.

Successful colonization is considered to be a major premise for the beneficial effects exerted by *Trichoderma* on plants (Harman et al., 2004; Morán-Diez et al., 2009; Hermosa et al., 2012). Our results concerning tomato root colonization by *T. parareesei* strains are in agreement with the growth rates calculated in different media. However, if the lower root colonization capacity observed for *Tparo7*-silenced mutants as compared to those of the control strains (**Table 7**) were a direct consequence of their limited growth, there would be insufficient data to support the notion that TpARO7 plays a major role in the *T. parareesei* rhizosphere colonization process. Since several reports have shown that *Trichoderma* SMs can affect plant growth and defense (Viterbo et al., 2007; Vinale et al., 2008; Tijerino et al., 2011; Malmierca et al., 2012, 2015), we used *in vivo* assays to explore the role of *Tparo7* in *T. parareesei*-tomato interactions. In comparison with control plants, we observed significantly lower growth parameters in plants whose seeds had been coated with *Tparo7*-silenced mutants (**Table 8**); this could be explained by their higher SA production (**Table 6**). We also observed that exogenous applications of 2–100 μM tyrosol increased tomato seed germination but concentrations higher than 250 μM elicited the opposite effect (data not shown). It has been reported that the effect of exogenous SA on growth depends on the plant species, the developmental stage and the SA concentrations tested, since low amounts of SA can have growth-stimulating effects but higher SA concentrations have been associated with reduced growth and chlorophyll content (Kovácik et al., 2009; Rivas-San Vicente and Plasencia, 2011). We analyzed the production of tyrosol, 2-PE and SA by *T. parareesei* strains, but differences in the biosynthesis of other metabolites could occur after *Tparo7* silencing, since the shikimate pathway is strongly regulated and a huge variety of metabolites can be derived from it. We also observed that tomato plants treated with *Tparo7*-silenced mutants showed reduced protection against BC, which would be in agreement with the observed downregulation of *LOX1* and *EIN2* (**Figure 5**), involved in induced systemic resistance against necrotrophs.

As far as we know this is the first report relating CM silencing with biocontrol. The results concerning CM reported in this article support the hypothesis that SMs produced in the shikimate pathway are involved in the biocontrol potential of *T. parareesei* and some of them appear to be key molecules for maintaining a proper balance in the responses elicited by *T. parareesei* during its interaction with plants. The observed relationships between AAA-derived SM production and CW degrading enzyme activity, already described in plants, need to be explained in fungi and the *T. parareesei* CM-silenced mutants obtained in the present work can be a touchstone to pursuing this goal.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01181

Figure S1 | Expression of *Tparo7* gene in *T. parareesei* T6 by real-time PCR. Total RNA was extracted from mycelia grown for 48 h on PDB and transferred to MM containing 2% glucose (MM) or 5 mM Tyr (MM-Tyr), Phe (MM-Phe), Trp (MM-Trp) or chorismic acid (MM-chorismic acid) for 24 h. Values correspond to relative measurements against the *Tparo7* transcript in T6 grown in MM containing 2% glucose ($2^{-\Delta\Delta Ct} = 1$). *T. parareesei* T6 actin was used as internal reference gene. Bars represent the standard deviations of the mean of three replicates. Asterisk (∗) represents statistically significant differences ($P < 0.05$).

Figure S2 | Southern blot analysis of wild type (T6) and transformant strains. Genomic DNAs were *SacI*-digested and the *phleomycin* gene was used as probe. *T. parareesei* T6 (line 1), Tparo7-S1 (line 2), Tparo7-S2 (line 3), Tparo7-S3 (line 4), and Tparo7-S4 (line 5). *HindIII*-digested λ DNA was used as a marker and molecular sizes are indicated in kbp (line M).

Figure S3 | Dual cultures of strains T6, the silenced transformants Tparo7-S3 and Tparo7-S4, and the control transformant Tp-TC of *T. parareesei* and the pathogen *B. cinerea* (BC) on PDA medium. Plates in the center correspond to the pathogen growth without *Trichoderma* strain. Plates were incubated at 28°C for 18 **(A)**, 48 **(B)**, and 72 **(C)** h.

Figure S4 | Necrotic lesions observed in tomato leaves after *T. parareesei* seed treatment and *B. cinerea* conidia infection. Untreated seed and *B. cinerea*-infected leaves (control) and *T. parareesei* T6-, control transformant Tp-TC-, silenced transformants Tparo7-S3- and Tparo7-S4-treated seed and *B. cinerea*-infected leaves. Image was taken three days after *B. cinerea*-infection.

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Biocontrol agent *Bacillus amyloliquefaciens* LJ02 induces systemic resistance against cucurbits powdery mildew

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Powdery mildew is a fungal disease found in a wide range of plants and can significantly reduce crop yields. Bacterial strain LJ02 is a biocontrol agent (BCA) isolated from a greenhouse in Tianjin, China. In combination of morphological, physiological, biochemical and phylogenetic analyses, strain LJ02 was classified as a new member of *Bacillus amyloliquefaciens*. Greenhouse trials showed that LJ02 fermentation broth (LJ02FB) can effectively diminish the occurrence of cucurbits powdery mildew. When treated with LJ02FB, cucumber seedlings produced significantly elevated production of superoxide dismutase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase as compared to that of the control. We further confirmed that the production of free salicylic acid (SA) and expression of one pathogenesis-related (PR) gene *PR-1* in cucumber leaves were markedly elevated after treating with LJ02FB, suggesting that SA-mediated defense response was stimulated. Moreover, LJ02FB-treated cucumber leaves could secrete resistance-related substances into rhizosphere that inhibit the germination of fungi spores and the growth of pathogens. Finally, we separated bacterium and its fermented substances to test their respective effects and found that both components have SA-inducing activity and bacterium plays major roles. Altogether, we identified a BCA against powdery mildew and its mode of action by inducing systemic resistance such as SA signaling pathway.

Keywords: powdery mildew, biocontrol agent, *Bacillus amyloliquefaciens*, salicylic acid, PR genes, systemic acquired resistance

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Introduction

Cucurbits powdery mildew caused by *Sphaerotheca fuliginea* is a common disease of cucurbits under field and greenhouse conditions in most areas of the world (Perez-Garcia et al., 2009). Sulfur, copper, and various classes of fungicides or combinations of them are widely used to control those infections (Reuveni et al., 1996). Due to long-term extensive use of fungicides, pathogens have gradually evolved resistance to those fungicides. Moreover, the pesticide residues are of major concern because of their detrimental effects on human health and the environment (Zhang et al., 2015). To circumvent those undesirable effects, the application of biocontrol agent (BCA) is considered as a promising alternative treatment that can reduce both environmental pollution and the rise of fungicide resistance (Alamri et al., 2012).

Myriads of mechanisms have been attributed to BCA that keep plant from infections by pathogen (Haas and Defago, 2005). Different types of interaction mechanisms including: phytopathogen external competition, physical displacement of phytopathogens, secretion of anti-pathogen siderophores, synthesis of antibiotics and a variety of small molecules, production of enzymes that inhibit phytopathogens and induction of systemic resistance of the plant (Bargabus et al., 2004).

Generally, upon perception of specific compounds secreted by invasive pathogens, plants can initiate defensive mechanisms to counteract infections through a combination of constitutive as well as induced defenses such as systemic acquired resistance (SAR; Jung et al., 2005). SAR is effective against a wide range of pathogens and requires the synthesis of phenolic signaling compound, salicylic acid (SA; Feys and Parker, 2000). SAR is also known for the coordinate activation of a specific set of PATHOGENESIS-RELATED (PR) genes, several of which code for proteins with antimicrobial activities *in vitro* (Tornero et al., 1997). In addition, another different form of systemic resistance in plants responding to certain non-pathogenic rhizobacteria is referred to as induced systemic resistance (ISR) that is also effective against multiple pathogens (Feys and Parker, 2000). It is of note that ISR is independent of the SA production and PR induction but requires the operation of plant growth hormones jasmonic acid (JA) and ethylene signaling pathways. Although both SAR and ISR are effective against different types of pathogens, it was found that both SAR and ISR require *NPR1* gene in systemic plant defenses, suggesting the interplay of those systemic resistance (Feys and Parker, 2000).

Bacillus amyloliquefaciens was separated from *Bacillus subtilis* as a new species (Priest et al., 1987) and both have been reported as BCAs in controlling cucumber powdery mildew (Chen et al., 2013). As spore-forming bacteria, *B. amyloliquefaciens* possesses several advantages and make them good candidates of BCA. Previous studies showed that the crude protein of antifungal agents could inhibit conidial production effectively (Li et al., 2009). Besides, *B. amyloliquefaciens* produces spores with strong resistance to adverse conditions that provide convenience for commercial uses (Arguelles-Arias et al., 2009). Importantly, their antagonistic effect is mainly dependent on the production of antibiotics and siderophores. *B. amyloliquefaciens* produces various antibacterial and antifungal antibiotics such as surfactin, iturin, and fengycin (Chen et al., 2009). Both iturins and fengycins are recently shown to have major roles in antagonism toward *Podosphaera fusca* infecting melon leaves (Romero et al., 2007).

In this study, we isolated a BCA named LJ02 from greenhouse soil against cucumber powder mildew and characterized it as *B. amyloliquefaciens* using combinatorial analyses. In order to elucidate the protective mechanisms of LJ02, the activity of resistance-related enzymes, the production of SA and the expression of *PR-1* gene in cucumber leaves were monitored after treating plant leaves with 1% LJ02 fermentation broth (LJ02FB). Moreover, we examined the inhibitory ability of LJ02-induced root secretions against pathogen (*Fusarium oxysporum*, *Botrytis cinerea*, and *Alternaria* spp).

Materials and Methods

Isolation of LJ02 and Source of Pathogen Fungi

The LJ02 strain was isolated from greenhouse soil in Tianjin, China. The soil samples were air-dried and sifted through 60 mesh sieve. Five g soil sample was suspended in 45 mL of 0.9% NaCl on a shaker at 200 rpm for 30 min. The suspension was diluted $10^3 \sim 10^4$ times in 0.9% NaCl. 100 μL of the suspension was spread onto sterile LB plates (For 1,000 mL, tryptone: 10 g, yeast extract: 5 g, NaCl: 10 g, agar: 12 g, distilled water: 1,000 mL, pH 7.2 ∼ 7.4) and incubated at 28°C for 48 h. Single colonies were selected and re-streaked for pure culture. The pure cultures were stored in 25% glycerol solution at −80°C. LJ02 and pathogen fungi (*S. fuliginea*, *F. oxysporum*, *B. cinerea* and *Alternaria* spp.) were stored in Laboratory of Plant Protection, Tianjin Agriculture University.

Plant Growth Conditions

Cucumber seeds (*Cucumis sativus* cv *Corona*, Jinchun 4) were obtained from Tianjin Kernel Cucumber Institute. The seeds were surface-sterilized for 10 min in 30% sodium hypochlorite and rinsed three times with distilled water. The seeds were then incubated at 28°C for 24 h on the sterile wet filter paper. Then the sprouted seeds were planted in the mixture of peat soil and vermiculite in 9-cm pots and the seedlings were used for greenhouse trials, detection of resistance-related enzymes, SA production, detecting of fungi quantity and monitoring the expression of *PR-1* gene. As for the inhibition effects on pathogenic fungi, the seedlings were planted in 250 mL erlenmeyer flask containing 20 mL MS solid medium (For 1,000 mL, KNO_3: 1.9 g, $MgSO_4{\cdot}7H_2O$: 0.37 g, NH_4NO_3: 1.65 g, KH_2PO_4: 0.17 g, $MnSO_4{\cdot}4H_2O$: 29 mg, $ZnSO_4{\cdot}7H_2O$: 8.6 mg, H_3BO_3: 6.2 mg, $CoCl_2{\cdot}6H_2O$: 25 μg, $Na_2MoO_4{\cdot}2H_2O$: 250 μg, $CuSO_4{\cdot}5H_2O$: 25 μg, $CaCl_2{\cdot}2H_2O$: 44 mg, KI: 830 μg, glycine: 2 mg, aneurine hydrochloride: 0.4 mg, pyridoxine hydrochloride: 0.5 mg, nicotinic acid: 0.5 mg, Na_2-EDTA: 37.25 mg, $FeSO_4{\cdot}7H_2O$: 27.85 mg, inositol: 0.1 g, agar: 7 g, distilled water: 1000 mL, pH 6.0; Murashige and Skoog, 1962). The MS liquid medium was used to detect the germination rate. All seedlings were cultivated in growth chamber with 16 h day (10,000 lx, 25°C) and 8 h night (20°C) at 60% relative humidity.

Preparation of LJ02FB and Greenhouse Trial

LJ02FB was prepared by the following methods. Strain LJ02 was inoculated into 5 mL of LB liquid medium and cultured to stationary phase. Then 100 μL of LJ02 LB culture was inoculated into 100 mL PDB medium (For 1,000 mL, potato: 200 g, glucose: 20 g, distilled water: 1,000 mL) and cultivated at 28°C for 40 h at 200 rpm. Then LJ02FB [10^9 CFU (colony forming unit)/mL] was diluted 100 times (v/v) into sterilized water (1% LJ02FB). Fresh powdery mildew (*S. fuliginea*, Verhaar et al., 1996) were obtained from infected cucumber leaves in Laboratory of Plant Protection, Tianjin agriculture university. The spores were brushed in sterile water for spore suspension and the concentration was adjusted to 10^6 CFU/mL. The concentration was determined by counting spores using a Neubauer hemocytometer (Xu et al., 2006). To test the efficacy of strain LJ02 against cucurbits powdery mildew, each cucumber seedling was firstly sprayed with pathogen spores

during cotyledon period. As soon as disease spots were observed, the seedlings of treatment group were sprayed with 1% LJ02FB ($\sim 10^7$ CFU/mL, 5 mL). 1% PDB medium (5 mL) was used for control group. Ten seedlings were used as one group for greenhouse experiments and each trial was repeated at least three times. The efficacy on disease severity was investigated for 3 weeks. The disease severity were scored using a modified rating (r) standard, denoting proportions of disease over the whole leaf area, where: 0: 0%, 1: <1%, 3: 2 ∼ 5%, 5: 6 ∼ 20%, 7: 21 ∼ 40%, and 9: >40% (Yan et al., 2006). Then, the disease severity index (DI) and control efficacy (CE) was computed using the formula as follows (Yan et al., 2006):

$$\text{DI}\ (\%) = [\Sigma(rn_r)/9N_t] \times 100$$

Where r is rating value, n_r is number of diseased leaves with a rating of r, and N_t is total number of leaves tested.

$$\text{CE}\ (\%) = [1\text{-}(ck/t)] \times 100$$

Where ck is DI of control group, t is DI of treated group.

PCR Amplification and Sequencing of *gyrB* Gene

Total DNA of strain LJ02 was extracted by using Solarbio DNA kit (Solarbio). Primer pair: up1 (5′-GAAGTCATCATGAC CGTTCTGCAYGCNGGNGGNAARTTYGA-3′) and up2r (5′-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCN GTCAT-3′) were used to amplify the *gyrB* gene (Yamamoto and Harayama, 1995). Amplified fragment were purified by DNA gel purification kit (Omega Bio-Tek) according to the manufacturer's instructions and then sequenced by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd., China. The *gyrB* gene sequence was compared with GenBank database by the BLAST program and the phylogenetic tree was constructed using the neighbor-joining method with the MEGA 5.1 program (Tamura et al., 2011).

Physiological and Biochemical Analysis

Gram staining and spore staining were observed through microscope. Physiological and biochemical properties of strain LJ02 were identified according to Bergey's Manual of Systematic Bacteriology (Vos et al., 2009).

Detection of Activity of Resistance Related Enzymes

The cucumber seeds were cultured in MS solid medium as described above (see *Plant growth conditions*). Each cucumber seedling was sprayed with 1% LJ02FB (5 mL) at the three-leaf stage. 1% PDB liquid medium (5 mL) was applied as a control. Each treatment was repeated three times in all groups. Cucumber leaves were randomly collected at 0.5, 1, 3, 5, 7 and 11 days after treatment to detect the activity of superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL). 0.4 g leaves were ground in liquid nitrogen and homogenized at 4°C in 2 mL of 0.05 mol/L phosphate buffer (pH 6.8). Homogenate (crude enzyme solution) was centrifuged at 4°C at 12,000 rpm for 20 min. The supernatant was used for further analyses.

Detection of SOD Activity

Superoxide dismutase activity was determined using a NBT method as described previously with some modifications (Pokora et al., 2003). 0.05 mL of enzyme liquid was added to the reaction mixture. The tubes without addition of enzyme solution (replaced by phosphate buffer) were taken as control. The SOD activity was measured by optical density (OD) at 560 nm.

Detection of POD Activity

Peroxidase activity was determined as described with modifications (Wang et al., 2012). The mixture was reacted in 2.65 mL of 0.05 mol/L phosphate buffer (pH 6.8). And the condition of bathing was changed into 30°C for 5 min before adding hydrogen peroxide. Control groups were performed in the absence of enzyme liquid. The OD_{470} value was measured for 5 min, and one unit of enzyme activity was defined by the change in absorbance of 0.1 per minute.

Detection of PPO Activity

The activity of PPO was determined according to the method with some modifications (Esterbauer et al., 1977). The reaction mixture contained 0.10 mL enzyme liquid and tubes without addition of enzyme solution were taken as control. Then OD_{495} value of reaction liquid was measured for 5 min, and one unit of enzyme activity was defined by the change in absorbance of 0.1 per minute.

Detection of PAL Activity

Leaves (0.4 g) were ground in liquid nitrogen and homogenized at 4°C in 2 mL of 0.05 mol/L borate buffer (pH 8.8, containing 5 mmol/L mercaptoethanol and 1 mmol/L EDTA). Homogenate was centrifuged at 4°C at 10,000 rpm for 15 min. The supernatant was used for analyses. The activity of PAL was determined as described with some modifications (Li et al., 2008). The reaction mixture contained 3.8 mL of borate buffer, 1.0 mL of 0.02 mol/L L-phenylalanine and 0.2 mL enzyme liquid. The tubes without addition of enzyme solution were taken as a control. Then, all the tubes were bath at 40°C and the OD_{290} value was determined per 15 min until it kept steady, and one unit of enzyme activity was defined by the change in absorbance of 0.1 per hour.

Detection of SA

The cucumber seedlings were grown in the conditions as described above (see *Plant growth conditions*). Two types of treatment were carried out as follows.

LJ02FB Induced SA Production

Each cucumber seedling was sprayed with 1% LJ02FB (5 mL) at three-leaf stage. 1% PDB medium (5 mL) was sprayed as a control. Cucumber leaves (2 g) were randomly collected at 0.5, 1, 3, 5, 7, and 11 days after treatment to detect the free and conjugated SA.

Bacterium and its Fermented Substances Induced SA Production

The LJ02FB culture was precipitated and bacterial pellet was suspended in 10 mM $MgCl_2$ solution (LJ02BC), and its fermented substances were filter sterilized (LJ02FS). The cotyledons of

cucumber seedlings was sprayed with LJ02BC (2 mL) and LJ02FS (2 mL) separately in two groups at three-leaf stage. 1% PDB medium (2 mL) was sprayed in control groups. The upper uninoculated leaves (2 g) of cucumber seedlings and the roots (including main and lateral roots, 2 g) were collected at 0.5, 1, 3, 5, 7, and 11 days after treatment to detect the free SA.

Salicylic acid fractions including free SA and conjugated SA were extracted as described with minor modifications (Palva et al., 1994). The extracts were dissolved in 500 μL methanol and spotted on silica gel plates. Then the plates were developed in a solvent system consisting of petroleum ether (60–90): *n*-hexane: ethyl acetate: acetic acid at the volume ratio of 10:30:15:1. The SA was detected by observing a UV reflected band with an Rf value corresponding to that of the standard SA. The samples were scraped from silica gel plates and dissolved in 1 mL methanol. The suspension was centrifuged at 12, 000 rpm for 5 min and filtered through a 0.22 μm filter and stored at −20°C.

Detection of SA samples was performed using a Shimadzu LC-20AT HPLC equipped with a UV-detector. 20 μL crude extraction of SA was injected into a C-18 reverse-phase column (diameter × length: 4.6 × 150 mm) at 25°C. SA was separated with 80% methanol (v/v) in 0.1% acetic acid solution with a flow rate of 0.8 mL/min and detected under the wavelength of 300 nm.

Detection of *PR-1* Gene Expression

To evaluate the expression of *PR-1* gene in cucumber, each cucumber leave was sprayed with 1% LJ02FB (2 mL) at three-leaf stage. 1% PDB was sprayed as control. The cucumber leaves were sampled 0.5, 1, 2, and 3 days after treatment. The samples were ground by mortar and pestle in liquid nitrogen. Total RNA was extracted using the Total RNA Isolation Kit (Solarbio). One microgram of total RNA was converted into cDNA using Reverse Transcription Reagent Kit (Takara) according to the instructions. The primers for qPCR were used as follows: PR1-forward 5′-TGCTCAACAATATGCGAACC-3′ and PR1-reverse 5′-TCATCCACCCACAACTGA AC-3′ (Alizadeh et al., 2013); 18S-forward 5′-TCTGCCCGTTGCTCTGATG-3′ and 18S-reverse 5′-TCACCCGTCAC CACCATAG-3′ (Wan et al., 2010). The length of amplified fragments were between 100 and 200 bp. PCR reaction mixture (20 μL) consisted of 10 μL SYBR *Premix Ex Taq* (Tli RNaseH Plus), 2 μL of each forward and reverse primer. 2 μL cDNA, 4 μL dH_2O. Then real-time PCR was performed in CFX96 Real-Time PCR Detection System (Bio-Rad) with the following parameters: 95°C for 30 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Gene *18S* was used as the reference gene of cucumber. Relative gene expression was calculated by the Bio-Rad CFX Manager 2.1 software.

Germination Rate of Pathogenic Spore

The exudate of cucumber rhizosphere was collected by the following method. The seedlings were cultured in sterile MS solid medium and then were transplanted into a flask containing 20 mL sterile MS liquid medium when the first leaf sprouted. All seedlings were incubated at 25°C in sterile conditions with illumination intensity of 10,000 lx and illumination time of 16 h/day period. After 2 days, leaves including cotyledon were spotted with 20 μL of the LJ02FB ($\sim 10^9$ CFU/mL) as treatment group. 20 μL of the sterile PDB medium was spotted in control group. After 2 days, 30 μL of the MS liquid medium from LJ02FB-treated group was mixed with 30 μL of pathogen spore suspension (*F. oxysporum*, *B. cinerea*, and *Alternaria* spp., 150 ∼ 200 spores per sample) on the concave glass. Each treatment was repeated three times and the concave glass were incubated at 28°C. The germination of spores was recorded after incubation for 2 ∼ 9 days, respectively.

Detection of Fungi Quantity in the Cucumber Rhizosphere

Counting the fungi in cucumber rhizosphere treated by LJ02FB was conducted by the following steps. The cucumber seedlings were cultured in seedling-raising dish as described above. 1% and 10% LJ02FB were sprayed with 20 μL per leaf at trefoil stage. The CFU of each fungus in the cucumber rhizosphere was determined as described previously (Faheem et al., 2015). Five cucumber plants were pulled out of each group and the soil was shook off and blended from the roots. The soil suspension was prepared by adding one gram of the soil into 9 ml of sterile distilled water and mixing for 10 min. Serial dilutions were subsequently prepared in sterile distilled water. 100 μL of the suspension was spread on selective Martin medium (For 1,000 mL, glucose: 10 g, tryptone: 5 g, K_2HPO_4: 1 g, $MgSO_4 \cdot 7H_2O$: 0.5 g, agar: 15 g, 1% Rose Bangal: 0.33 mL, 1% streptomycin: 3 mL, distilled water: 1,000 mL; Xu and Zheng, 1986) and incubated at 25°C. The number of CFU was recorded on each plate after 3 days. All experiments had at least five replicates.

Inhibition of LJ02-Induced Root Secretions Against Pathogen Fungi

The inhibition against pathogen fungi of LJ02-induced secretions was detected as described previously with minor modifications (On et al., 2015). Briefly, 20 μL of the LJ02FB was dropped onto the leaves including cotyledon under sterile condition at trefoil stage. After treated for 48 h, cucumber seedlings were pulled from the MS solid medium. The MS solid medium containing rhizosphere secretions was made into cake by a sterile puncher (0.5 cm in diameter). Each pathogen fungus (*F. oxysporum*, *B. cinerea,* and *Alternaria* spp.) was also made into fungi cake by a sterile puncher (0.5 cm in diameter) which was then inoculated onto the center of PDA plate. Four pieces of MS solid medium cake were inoculated around the fungi cake at a distance of 2.5 cm (treatment group). The cake made from sterile MS solid medium without root secretions was used as a control. Each treatment was repeated three times and cultured at 28°C for 9 days. Subsequently, the inhibition zone diameter was detected.

Statistical Analysis

The data of disease index, CE, resistance enzyme activities, SA production, germination rate, and fungi pathogen counts in rhizospheres were analyzed by one-way ANOVA. Duncan's multiple-range test was applied when one-way ANOVA revealed significant differences ($P < 0.05$). All statistical analysis was performed with SPSS version 11.5 statistical software (SPSS, Chicago, IL, USA).

TABLE 1 | Disease index and control efficacy of powdery mildew in cucumber.

Treatment	7 days after treatment		14 days after treatment		21 days after treatment	
	DI (%)	CE (%)	DI (%)	CE (%)	DI (%)	CE (%)
1% LJ02FB	3.67 ± 0.55 a	69.80	5.93 ± 0.59 a	86.44	7.80 ± 0.3 a	85.05
Control	12.17 ± 1.01 b	–	43.87 ± 2.76 b	–	53.60 ± 10.34 b	–

Data expressed as the mean ± SD. Different letters indicate significant difference at $P < 0.05$ according to Duncan's multiple range test.

Results

LJ02 is a New Member of *B. amyloliquefaciens*

One-hundred-fifteen isolations were collected to test their antagonistic activity according to their inhibitory activity. One strain was selected and named LJ02. Greenhouse trials showed the CE on cucurbits powdery mildew of ∼70, ∼90, and ∼85% after treated with 1% LJ02FB for 7, 14, and 21 days, respectively (**Table 1**). The results proved that strain LJ02 indeed inhibited the occurrence of cucurbits powdery mildew under greenhouse condition. Therefore, we intended to identify the LJ02 based on its phylogeny, morphology, physiological, and biochemical properties.

Phylogenetic analysis of LJ02 was determined using *gyrB* gene as a reference (Yamamoto and Harayama, 1995). An 1.2 kb *gyrB* gene fragment was amplified from the strain LJ02 and sequenced. The *gyrB* gene sequence of LJ02 was aligned with published sequences of related taxa obtained from GenBank. Phylogenetic tree was therefore constructed and demonstrated high homology to *B. amyloliquefaciens* (**Figure 1**).

Strain LJ02 colonies show light yellow on LB agar medium. Gram staining and microscopic observation showed that LJ02 is Gram-positive, endospore-forming and rod-shaped bacterium with a width of 0.7–0.9 μm and a length of 1.8–3.0 μm, indicative of a typical bacillus morphology (**Figure 2**).

The physiological and biochemical properties were determined and shown in **Table 2**. Specifically, strain LJ02 is aerobic and cannot grow under anaerobic conditions. Strain LJ02 produces H_2S and can use citrate as sole carbon source. It is also positive for catalase, nitrate reduction, and Voges–Proskauer test and negative for oxidase, indole production and egg yolk reaction. LJ02 produces acid from xylose, sorbitol, *L*-arabinose, starch, inositol, sucrose, glucose, galactose, ribose, glycogen, and glycerol. All features are highly identical to those of *B. amyloliquefaciens* described in Manual of Systematic Bacteriology (Vos et al., 2009).

Altogether, the isolated BCA was referred to as *B. amyloliquefaciens* LJ02 based on its morphological, phylogenetic, physiological, and biochemical characterizations.

LJ02FB Induces the Production of Resistance-Related Enzymes in Cucumber Leaves

During initial screening of LJ02, greenhouse trials indicated that LJ02 is effective in controlling cucurbits powdery mildew over 20 days. In view of this long-term effect, we hypothesized that the systemic induced resistance (SIR) may take place when LJ02 was applied. To this end, the activity of systemic resistance-related enzymes was firstly examined.

As is shown in **Figure 3A**, the treatment group had a significant increase in SOD production as compared to the control treatment. The SOD activity of treatment group increased gradually and peaked after 36 h. It is very interesting to notice that the SOD activity retained a higher level even after 11 days treatment, suggesting its major role in disease resistance under our conditions.

As is shown in **Figure 3B**, there was slight difference between the control and treatment group plants at the primary treatment period (<2 days). But the activity of PPO increased rapidly after 2 days and became significant different ($P < 0.05$) from the control at 3rd day.

The rapid increase of POD activity was observed during the first 3 days after treatments and the difference between treatment and control became evident from 7th day (**Figure 3C**). This is probably because of the production of excess H_2O_2 by the action of increased SOD in LJ02FB treated plants.

Both group induced a rapid increase of PAL activity levels in cucumber leaves within 1–3 days. The PAL activities of either group reached the top level at 3rd day, while treatment group induced significantly stronger PAL activity ($P < 0.05$) than the control at the maximum inducing time point (**Figure 3D**).

All those results confirmed that LJ02FB treatment could indeed induce the production of SIR-related enzymes.

LJ02FB Enhances Production of SA in Cucumber Leaves

Salicylic acid is an important plant-produced signaling molecule involved in SAR (Park et al., 2007). It is responsible for inducing tolerance to a number of biotic and abiotic stresses, which is also a trigger for the production of SAR-related enzymes (Korkmaz et al., 2007). Thus, we established a method to detect the concentration of SA in LJ02FB-treated cucumber leaves (**Figures 4A,B**). We extracted the free SA and the sugar-conjugated SA from LJ02FB-treated plant leaves and monitored the trend of SA accumulation in the cucumber leaves in time-course experiments. We found that the accumulation of free SA on cucumber leaves increased rapidly after 5-day treatment as compared to control (**Figure 4C**). In addition, we also observed an increase of conjugated SA (day 3 ∼ 5) with a subsequent decline in production from LJ02FB-treated leaves (**Figure 4D**). Thus, we come to the conclusion that functional SA production was enhanced in cucumber leaves to stimulate SA-mediated defense response, such as the expression of resistance-related enzymes.

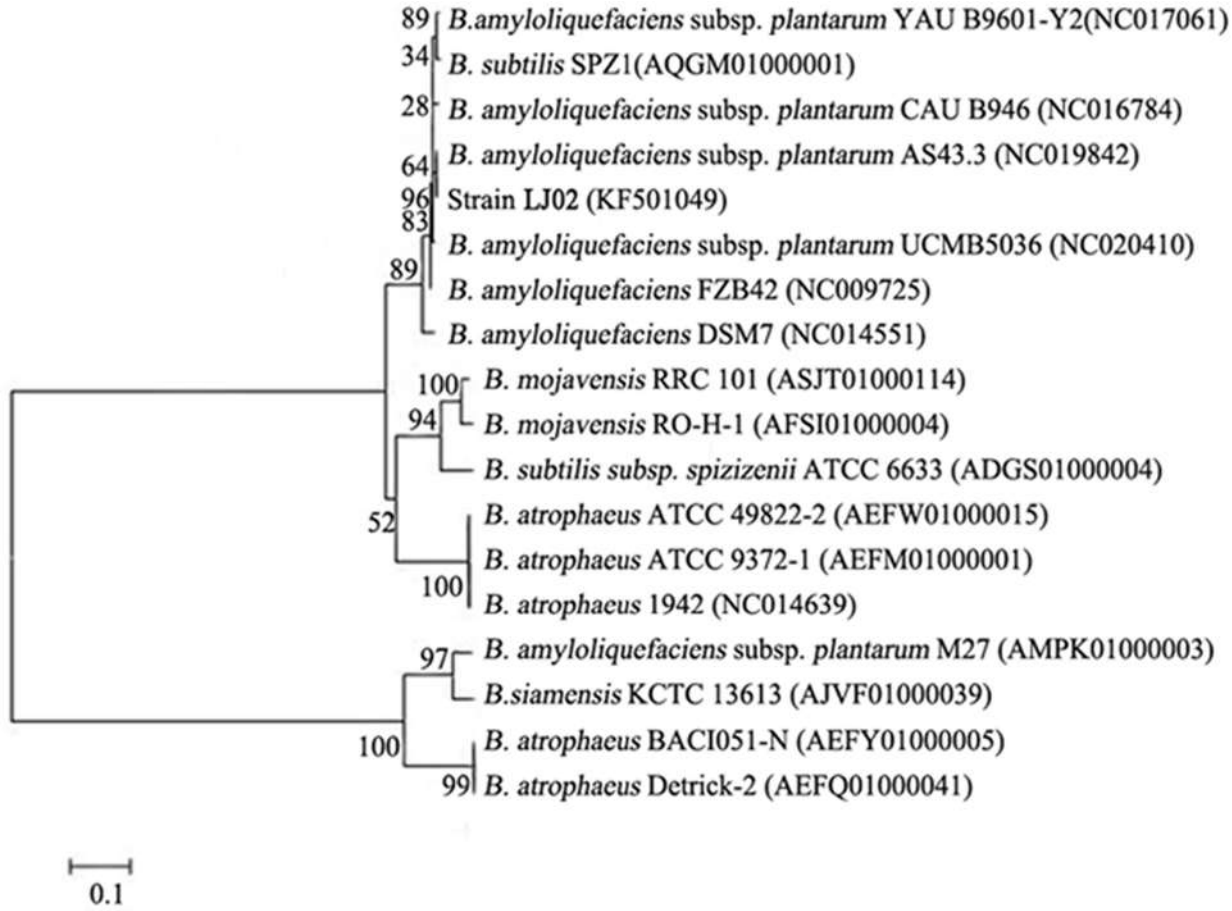

FIGURE 1 | Phylogenetic tree of strain LJ02 and other *Bacillus* species from GenBank based on *gyrB* gene sequences. The tree was constructed by MEGA5.1 by the neighbor-joining method. Genetic distances were computed by Kimura 2-parameter model. The *gyrB* gene sequence of LJ02 was submitted to GenBank database and the accession number is KF501049.

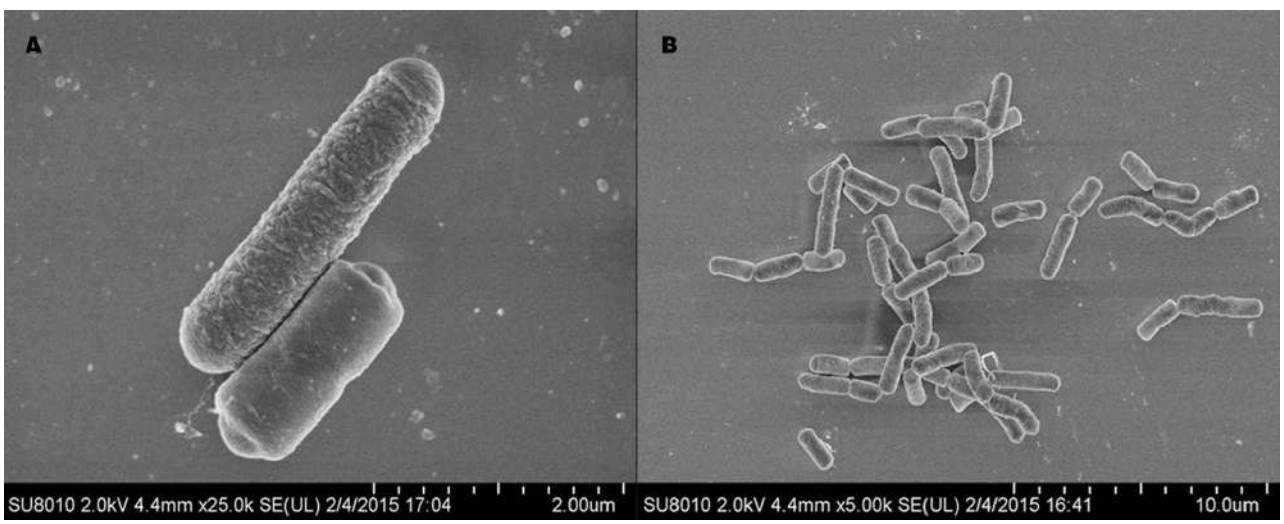

FIGURE 2 | Scanning microscope observation of LJ02 under two resolutions: 25,000× (A) and 5,000× (B). The images were taken from a ultra-high resolution scanning electron microscope SU8010.

TABLE 2 | Physiological and biochemical characteristics of strain LJ02.

Physiological and biochemical index	Strain LJ02	Physiological and biochemical index	Strain LJ02
Catalase activity	+	Indole production	−
Nitrate reduction	+	Growth with 0.001% lysozyme	+
H2S produced	+	Egg yolk reaction	−
Utilization of citrate	+	Anaerobic growth	−
Arginine dihydrolase	−	β-Galactosidase	−
Lysine decarboxylase	−	Oxidase activity	−
Ornithine decarboxylase	−	Methyl red test	−
V. P. Test	+	Temperature for growth range	15–50°C
Phenylalanine deaminase	−	Motility	+
Growth in NaCl range at (%, w/v)	0–10%	Litmus milk test	+
Hydrolysis of		**Utilization of sole carbon source**	
Starch	+	Starch	+
Gelatin	+	Inositol	+
Aesculin	+	*L*-Arabinose	+
Casein	+	*D*-Trehalose	+
Tyrosine	−	Glycerol	+
Urea	−	Ascorbic acid	−
Tween 20	+	Proline	+
Tween 80	−	Cystine	−
Acid produced from		Threonine	−
Xylose	+	Valine	−
Rhamnose	+	Arginine	−
Sorbitol	+	Citric acid	−
Mannitol	+	Sucrose	+
L-Arabinose	+	Xylose	+
Starch	+	Maltose	+
D-Trehalose	+	Tyrosine	−
Inositol	+	Mannitol	+
Sucrose	+	Glucose	+
Fructose	+	Sorbitol	+
Maltose	+	Fructose	+
Glucose	+	Oxalate	−
Mannose	+	Galactose	+
Galactose	+	Ribose	+
Ribose	+	Glycogen	+
Glycogen	+	Rhamnose	−
Glycerol	+	Mannose	−

"+" means positive; "−" means negative.

Expression of SA-Dependent *PR-1* Gene is Induced in LJ02FB-Treated Cucumber Leaves

The enhanced production of total SA in cucumber leaves led us to put forward the hypothesis that defense-related genes are probably induced. To this end, we analyzed the level of transcription of *PR-1* gene, a commonly used maker for SA-mediated expression (Tornero et al., 1997). In order to investigate the effect of LJ02FB on this process, the expression of *PR-1* in cucumber leaves was studied after treated with LJ02FB. As is shown in **Figure 5**, at 2nd day and 3rd day after treated with LJ02FB, the expression of *PR-1* gene in treatment group was significantly higher than that of control group, indicating that SA-mediated defense gene expression is induced in LJ02FB-treated cucumber leaves.

LJ02FB Treatment Elicits a Long-Range Resistance Against Fungi in Cucumber Rhizosphere

In order to further unravel the protective mechanisms of LJ02FB on disease control, the rhizosphere exudates of LJ02FB-treated cucumber were co-cultured with three common pathogen spores. As is described previously, *S. fuliginea* is an obligate parasite which is hard to culture on nutrient medium (Perez-Garcia et al., 2009). Therefore, we detected the diameter of inhibition zone against other pathogen fungi (*F. oxysporum*, *B. cinerea,* and *Alternaria* spp.) through plate cultivation test. Most spores would germinate at 9 h in the preliminary period. However, with the accumulation of antagonistic substance, the germination of all pathogens spores began to decrease significantly at 5th day and reached the lowest level at 8 $\sim$ 9 days. The final germination rate is about 10% for *F. oxysporum* (**Figure 6A**), less than 1% for *B. cinerea* (**Figure 6B**) and less than 2% for *Alternaria* spp. (**Figure 6C**).

Moreover, the quantity of rhizosphere fungi was determined after treated with LJ02FB in cucumber leaves. As is shown in **Figure 6D**, after treated with LJ02FB in cucumber leaves, the number of rhizosphere fungi declined significantly. In line with 7th day, the quantity of fungi at 14th day is still significantly less than that of control group ($P < 0.01$). Based on this findings, we further revealed that the rhizosphere MS solid

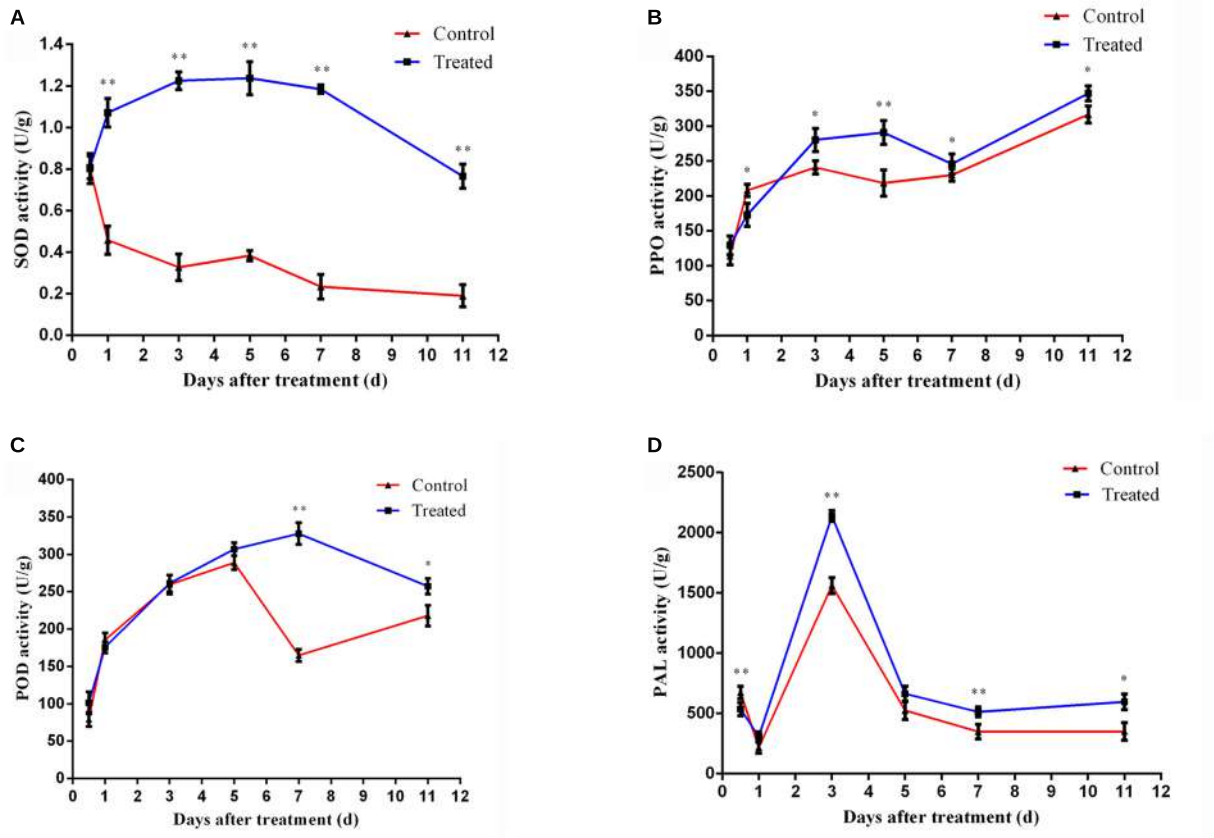

FIGURE 3 | Time course of changes in SOD activity (A), PPO activity (B), POD activity (C), and PAL activity (D) in cucumber leaves of control and treatment group. Error bars indicate standard deviation among triplicates. A one-way ANOVA was performed (*$P < 0.05$; **$P < 0.01$).

medium of LJ02FB-treated cucumber could strongly inhibit the growth of *F. oxysporum*, *B. cinerea*, and *Alternaria* spp. (**Figure 6E**).

All those results showed that LJ02FB could elicit the defense responses from plant leaves to rhizosphere, suggesting a long-range systemic resistance is induced under our test conditions. Besides, the examination of rhizosphere secretions in solid MS medium was proved to be an effective method that identifies potential BCAs with resistance-induction and anti-fungal activities.

Both LJ02 Bacterial Cells and Their Fermented Substances Induce SA Production in Systemic Tissues

To further dissect the functions played by bacterium and its fermented substances of LJ02FB, we separated them by centrifugation and kept bacterial cells in 10 mM $MgCl_2$ solution (LJ02BC) and filtered the supernatants to rule out the contamination of bacterial cells to harvest fermented substances (LJ02FS). We inoculated both fractions onto cucumber cotyledons to treat plants and then extracted the SA samples from upper uninoculated true leaves and in roots after 0.5, 1, 3, 5, 7, and 11 days. In doing so, we could examine the effects of both fractions (LJ02BC and LJ02FS) on the production of SA in cucumber tissues with spatial and temporal scales.

As can be seen from **Figure 7A**, the accumulation of free SA in upper uninoculated cucumber leaves increased rapidly after 3-day treatment as compared to control. Both LJ02BC and LJ02FS could induce the production of free SA in upper leaves throughout all experiments. This confirmed our speculation that LJ02 could stimulate the SA-mediated SAR response in cucumbers. Interestingly, LJ02FS could induce significantly higher amount of free SA at 5th day and declined to control level. This is possibly caused by the degradation of functional elicitors in LJ02FS. However, we observed that LJ02BC could consistently induce ∼twofold increase in free SA production, suggesting that microbe-associated molecular pattern (MAMP) may be responsible for SA stimulation (Newman et al., 2013).

In addition, we also detected an increase of free SA (day 3 ∼ 11) in cucumber roots with both LJ02BC- and LJ02FS-treatment (**Figure 7B**). And it appeared that LJ02BC played a major role in this process, although the amount of free SA was lower as compared to that of leaves. This phenomenon led us to conclude that LJ02 could induce a long-range of defense response by promoting the free SA production, which will then elicit the SA signaling pathways to enhance the immunity of cucumber plants systemically.

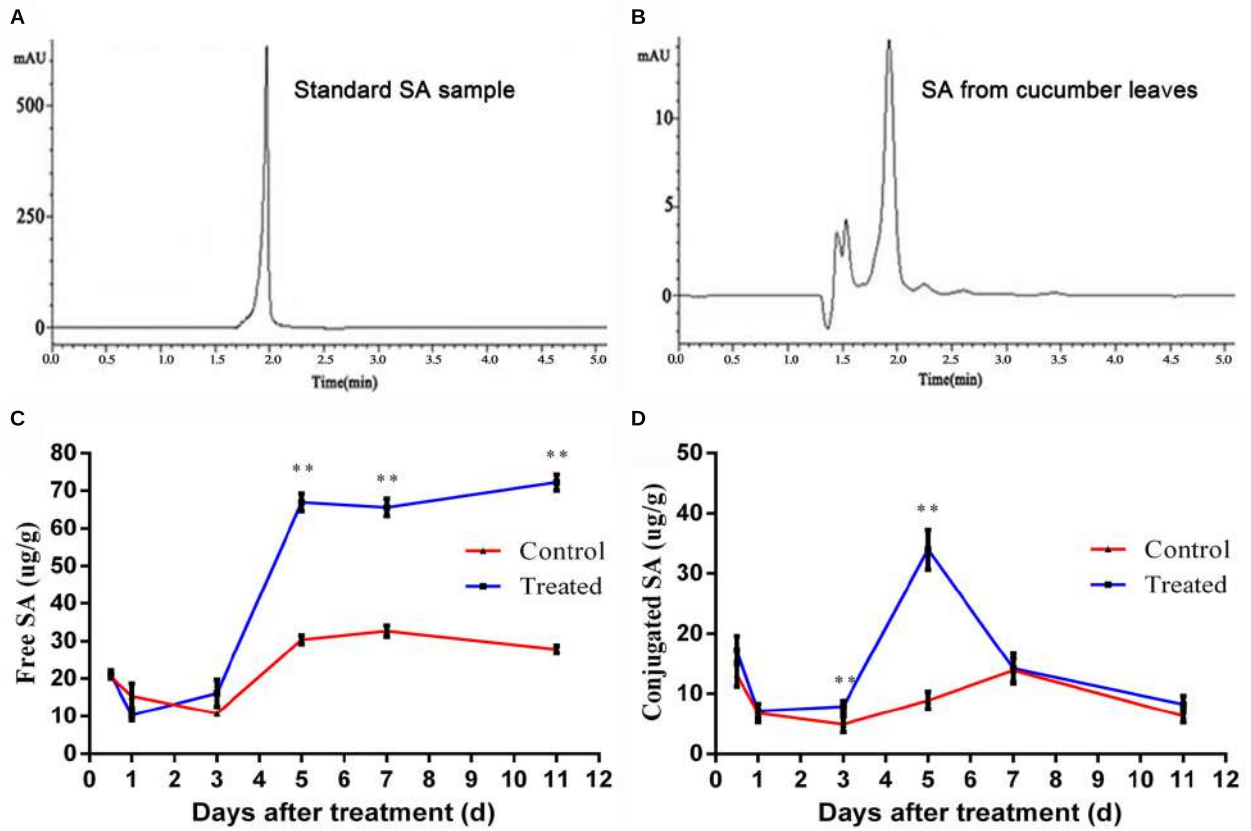

FIGURE 4 | HPLC chromatogram of SA standard (A) and sample (B), temporal changes of free SA (C) and conjugated SA (D) in cucumber leaves of control and treatment group. Error bars indicate standard deviation among triplicates. A one-way ANOVA was performed (*$P < 0.05$; **$P < 0.01$).

Discussion

The search for the BCA against powdery mildew has been a long standing practice for plant pathologists. In this study, we isolated a BCA against powdery mildew and identified it as a new member of *B. amyloliquefaciens*. Interestingly, we further found a large body of evidences that LJ02 could induce SA-mediated SAR as one of its major mode of actions. To be specific, when treated with LJ02FB, cucumber seedlings produced significantly high amount of SOD, POD, PPO, and PAL. The production of free SA and the expression of SA-dependent *PR-1* gene in cucumber leaves were also enhanced markedly after treating with LJ02FB. Moreover, we determined that TJ02FB-treated cucumber leaves secreted resistance-related substance into rhizosphere against a range of fungi pathogens, suggesting that LJ02 could elicit a long-range systemic resistance in cucumber against pathogens. Finally, we further dissect the roles played by LJ02BC and L02FS and found that LJ02 could really elicit SAR response in leaves as well as in roots. Overall, our work provides insights into SA-mediated defense response as a mode of action by *B. amyloliquefaciens* LJ02 against powdery mildew. Furthermore, we have proved that LJ02-induced long-range resistance is an appropriate indicator to examine the biocontrol ability, especially the SAR-inducing activity, of different BCAs using solid rhizosphere medium. Therefore, the established detection method could further promotes the identification of BCAs against agricultural infections or pathogens.

At the first beginning, the identification of LJ02 as *B. amyloliquefaciens* is different from previous studies since we could not distinguish it from *B. subtilis* based on 16S rRNA comparison (data not shown). Therefore, we switched to the other method described by Yamamoto using *gyrB* as a marker to perform phylogenetic analysis (Yamamoto and Harayama, 1995). Although 16S rRNA sequence has been commonly used as a simple method for the identification of microorganisms, it has limitations for constructing bacterial phylogenetic relationships due to the high percentage of sequence similarity between closely related species. At present, *gyrA*, *gyrB*, *rpoB*, and *rpoD* gene sequences have been used for identifying the *Bacillus* sp. and *Pseudomonas* sp. except 16S rRNA gene sequence (Yamamoto and Harayama, 1995). And it was shown that *gyrB* gene sequences provide higher resolution than 16S rRNA gene sequences (Wang et al., 2007). Therefore, it is likely that using *gyrB* as a phylogenetic marker is reliable in our study and could provide clues for bacillus determination.

The main mechanisms of BCAs include the production of antibiotics, competition, plant growth promotion, and the induction of SAR and ISR (Ramarathnam et al., 2011). Generally, it was believed that beneficial bacteria can make the plant more tolerant to pathogens by stimulating ISR, which was shown to protect above-ground plant tissues and acts through roots to

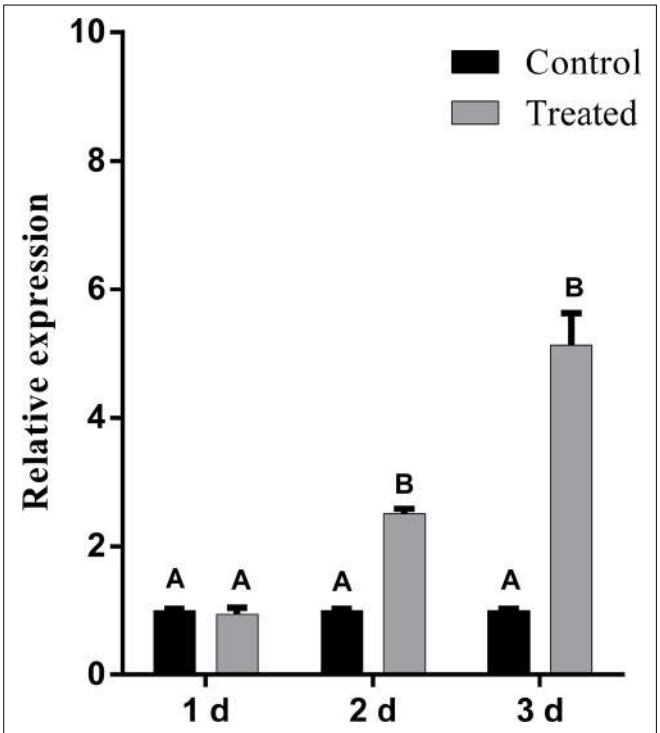

FIGURE 5 | Relative expression of *PR-1* gene in cucumber leaves after treated with LJ02FB (Treated) and PDB (Control). Error bars indicate standard deviation among triplicates. Different letters are significantly difference at $P < 0.01$ according to Duncan multiple range test.

leaves (Van der Ent et al., 2009). Typically, SAR can perceive the invasion of pathogens by initiate defensive responses via the synthesis of SA and the coordinate activation of a large set of PR proteins and resistance-related enzymes (Feys and Parker, 2000). It was also reported that enhanced resistance against phytopathogens by exogenous elicitor application is also associated with defense-related enzymes (Song et al., 2011).

In our study, the activities of resistance-related enzymes including SOD, POD, PPO, and PAL considerably increased after treated by LJ02FB. Interestingly, as an major defensive enzyme, SOD showed significantly elevated activity. The main function of SOD is eliminating the cellular superoxide radical (O_2^-), and the increase of SOD activity can lead to the accumulation of H_2O_2. It is proved that H_2O_2 appears to be a key element involved in disease resistance to pathogens (Ahmad et al., 2010). Therefore, BCA-induced production of SOD could provide an extra protection against pathogen infection of plants. However, the excess production of H_2O_2 will lead to the production of POD that scavenge H_2O_2 (Yamasaki et al., 1997). Additionally, we also found that the activity of PPO was also stimulated during LJ02 treatment. PPOs function as catalyzing the oxygen-dependent oxidation of phenols to quinones and are assumed to be involved in plant defense against pests and pathogens (Li and Steffens, 2002). It was shown that overexpression of PPO in transgenic tomato plants can lead to enhanced disease resistance (Li and Steffens, 2002). PAL was also enhanced during LJ02 treatment. It was shown to catalyze the deamination of L-phenylalanine to produce cinnamic acid, a substrate feeding into several biosynthetic routes to various classes of phenylpropanoid-derived secondary plant products, including SA (Mauch-Mani and Slusarenko, 1996).

The content of free SA is known to increase in dozens of times when plant tissues are affected by incompatible pathogens and elicitors (Leon et al., 1995). And there is compelling evidences that SA acts as a signal molecule in plant disease resistance (Vlot et al., 2009) and the *PR* gene family act as reliable markers of SA-mediated response or SAR (Hunt and Ryals, 1996). Intriguingly, in our study, we detected a significant increase in the content of free SA in cucumber leaves during LJ02 treatment. This is very intriguing since we used the fermentation broth of a beneficial bacterium rather than a pathogen to treat plant leaves. Besides, we found that the production of several resistance-related enzymes has been significantly stimulated upon LJ02 treatment, which was probably caused by the coordinate activation by enhanced SA levels in LJ02-treated plants. Actually, it was reported that PAL and PPO are the main SAR-related enzymes in plants (Jung et al., 2011). As we know, only free SA is involved in the signal transduction in plant disease resistance (Park et al., 2007). Our work demonstrated that the content of free SA in cucumber leaves was kept at a relatively higher level after spraying the LJ02FB. Previous experiments had the same results after they inoculated the pathogens on various plants (Verberne et al., 2000). Furthermore, the expression of *PR-1* gene was detected by qRT-PCR. We found that *PR-1* gene was stimulated after treated with LJ02FB in cucumber leaves at different time points. Among PR genes, *PR-1* expression is a paradigm for the co-regulation of *PR* genes and correlates well with the induction of SAR (Durrant and Dong, 2004).

To further unravel the long-range effect of LJ02FB treatment, we tested the biocontrol activity of root exudates or secretions against common pathogenic fungi. We found extremely evident induced defensive response of root exudates against different pathogens including *F. oxysporum*, *B. cinerea* and *Alternaria* spp. Considerable decrease in spore germination and fungal growth were detected after treated with LJ02FB. This data further confirmed that LJ02 could induce a systemic resistance response against pathogens, perhaps through secreting anti-fungal substances into rhizosphere, possibly through leave-to-root translocation or local synthesis of those substances. This further promoted us to note that LJ02 could induce a long-range defense response from leaves to roots in unknown mechanisms. Subsequently, we found that when we separated the LJ02FB into LJ02BC and LJ02FS, LJ02BC played very important roles in inducing the production of free SA in both upper uninoculated leaves and in roots. Moreover, LJ02FS also has SA-inducing activity with relative lower level as compared to that of LJ02BC, indicating both fractions are important for SA-inducing activity. Those results led us to conclude that SAR is indeed induced by LJ02 and MAMP is likely to be responsible for the triggering of SA-dependent defense such as SA synthesis and the subsequent pathogen inhibition (Porcel et al., 2014). Besides, the long-range elicitation of SA induction in roots may be involved in aforementioned long-range resistance.

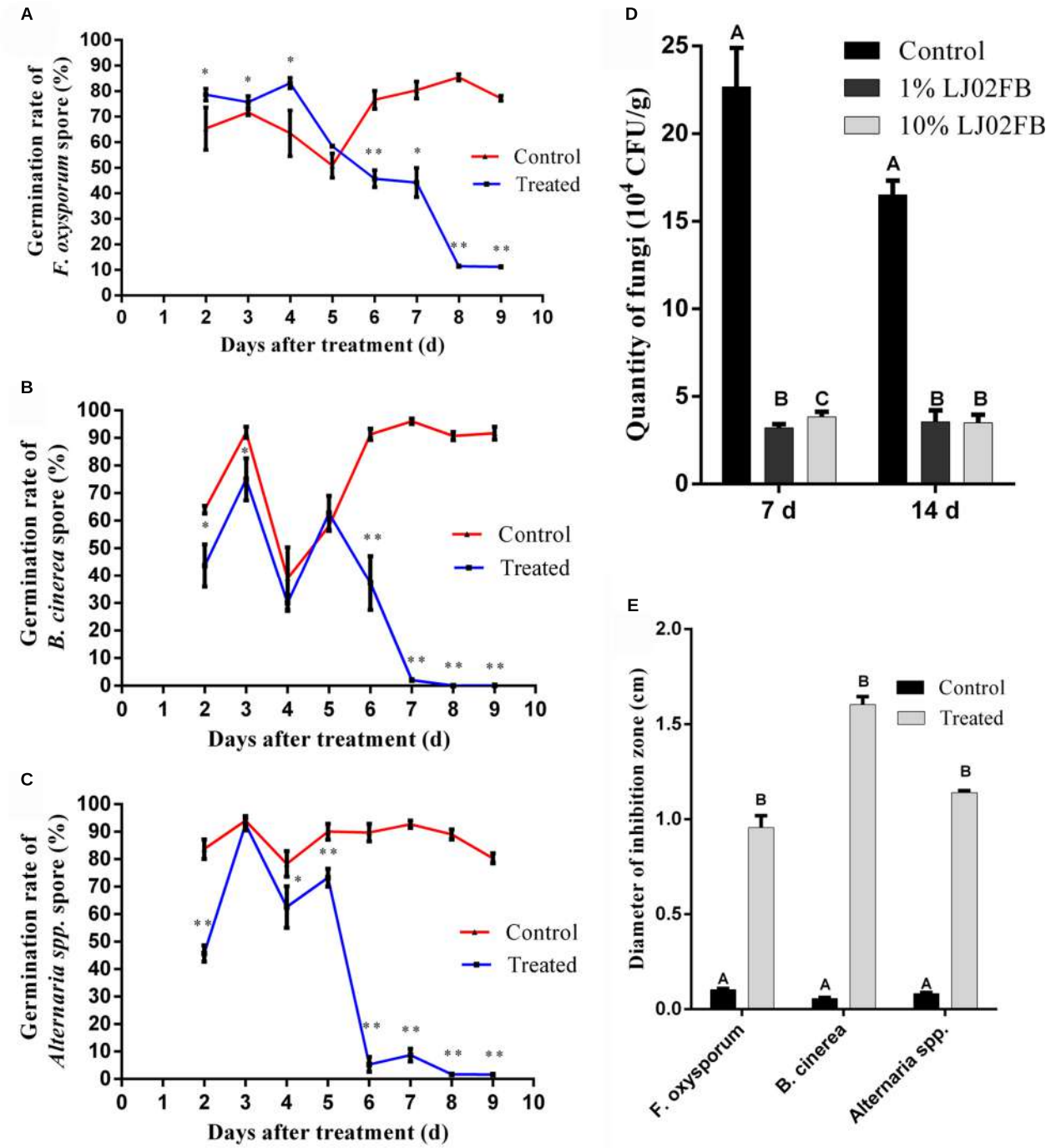

FIGURE 6 | Long-range resistance against fungi in cucumber rhizosphere. Germination rate of *F. oxysporum* **(A)**, *B. cinerea* **(B),** and *Alternaria* spp. **(C)** spores with different treatments. Error bars indicate standard deviation among triplicates. A one-way ANOVA was performed (*$P < 0.05$; **$P < 0.01$). **(D)** The quantity of pathogenic fungi in the rhizosphere of cucumber treated by different concentrations of LJ02FB in leaves. Error bars indicate standard deviation among triplicates. Different letters are significantly difference at $P < 0.01$ according to Duncan multiple range test. **(E)** The comparison of the inhibition zone of cucumber rhizosphere MS solid medium against different pathogenic fungi (cm). Error bars indicate standard deviation among triplicates. Different letters are significantly difference at $P < 0.01$ according to Duncan multiple range test.

Beneficial bacteria and pathogens are initially recognized as harmful invaders in order to limit their spreading (Trda et al., 2015). Plants commonly use plasma-membrane localized pattern recognition receptors (PRRs) to perceive MAMPs/pathogen-associated molecular patterns (PAMPs; Newman et al., 2013). These are conserved signatures of crucial microbial structures, such as cell wall components or motility organs (Boller and Felix, 2009) as well as cyclic lipopeptides found in *B. subtilis*

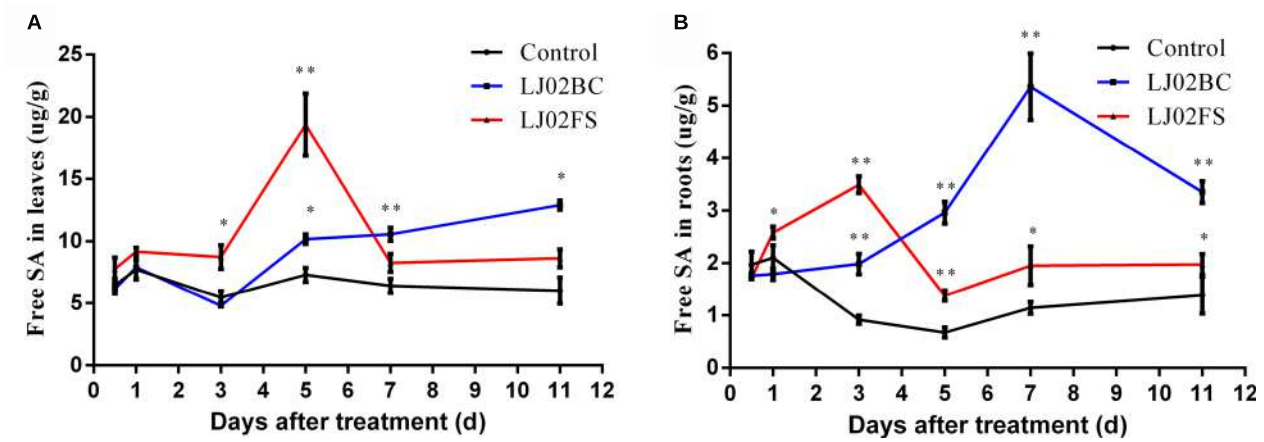

FIGURE 7 | Spatial and temporal detection of SA with separated LJ02FB components (LJ02BC and LJ02FS). Time course of changes in free SA activity in leaves **(A)** and in roots **(B)** of control and treatment group. Error bars indicate standard deviation among triplicates. A one-way ANOVA was performed (*$P < 0.05$; **$P < 0.01$).

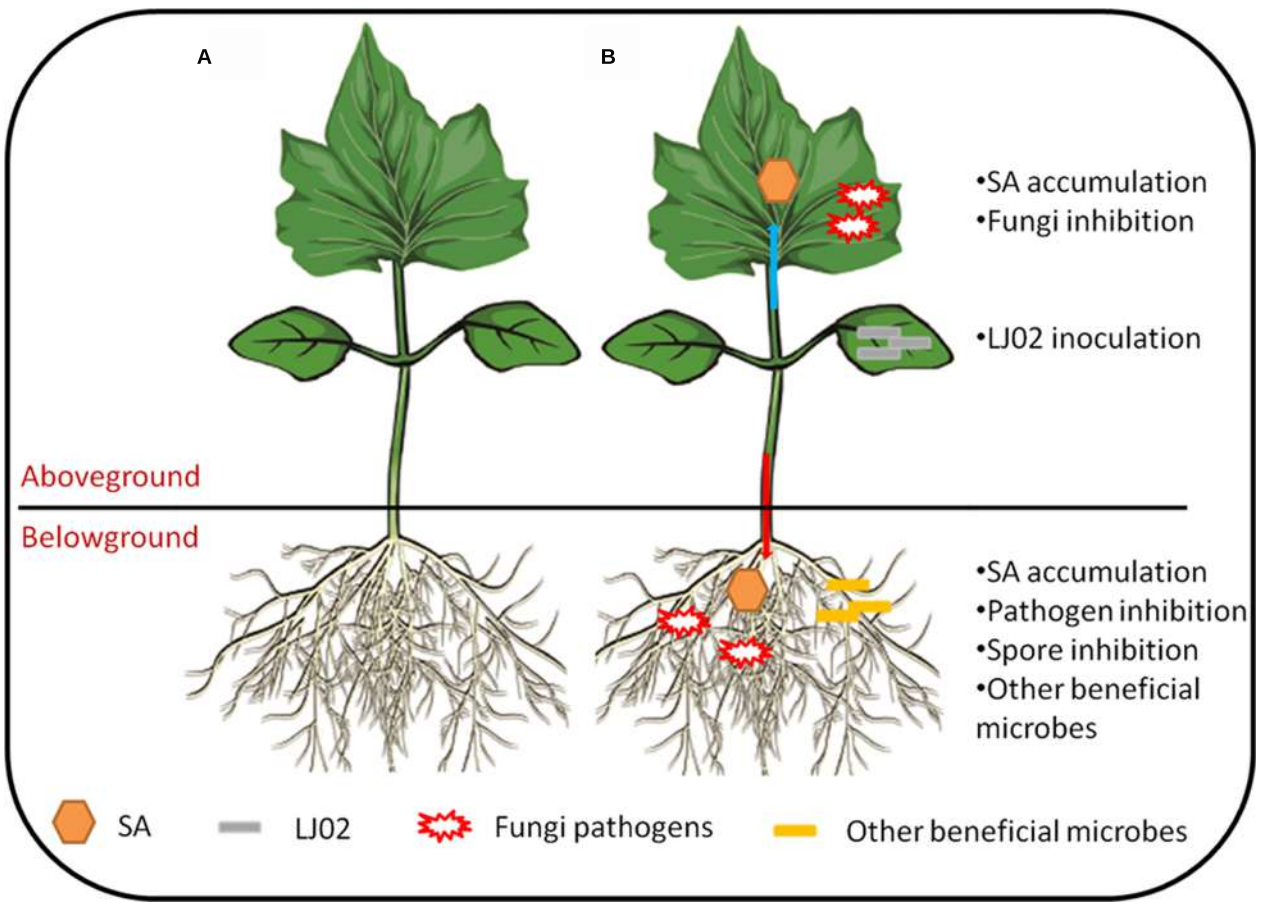

FIGURE 8 | Hypothetical model of LJ02-mediated protection of cucumber plants. (A) A cucumber seedling under normal condition; **(B)** LJ02 inoculation elicits SA-dependent signaling pathway in both directions: AG–AG and AG–BG. For more details, please refer to main text.

(Farace et al., 2015). PRR-mediated microbe sensing gives rise to a wide array of defense responses known as MAMP- or PAMP-triggered immunity (MTI/PTI; Zipfel, 2014). MTI is a defense program with complex early signaling events including ion fluxes, mitogen-activated protein (MAP) kinase cascade activation and the production of reactive oxygen species

(ROS; Garcia-Brugger et al., 2006). SA as a key immune signal is involved in the regulation of downstream defense genes (Robert-Seilaniantz et al., 2011). Also the SA accumulation triggered by MAMPs is a major component of the MAMP-triggered signaling mechanism (Tsuda et al., 2008).

As can be seen from our results (**Figure 7**) that MAMP associated with LJ02 is perhaps the underlying mechanism for the SA accumulation in cucumber tissues since we could detect the increase of SA production when treated with LJ02BC. The LJ02FS is also functional to stimulate the SA production both in upper leaves and roots but not as potent as that of LJ02BC. This is likely to be caused by the degradation of potential elicitors in fermentation broth. *B. subtilis* is known to produce cyclic lipopeptides such as surfactins, iturins and fengycins (Farace et al., 2015). It was reported that purified surfactin, mycosubtilin, and plipastatin could be perceived by grapevine plant cells to stimulate grapevine innate immune responses including SA-mediated pathways (Farace et al., 2015). Therefore, it is conceivably that similar substances will be produced in its close species, *B. amyloliquefaciens* and similar immune responses will be induced. However, the different effects of LJ02BC and LJ02FS on the SA-mediated defense responses may attributed to certain unidentified MAMP factors that functions in association with bacterium itself.

Another interesting finding in our study is that the SA signaling pathways induced by beneficial bacterium such as LJ02 is likely to function from leaves (aboveground, AG) to roots (belowground, BG; **Figure 7B**). Although we did not detect a large amount of SA induced in roots, it is clearly that LJ02 could significant induce its production in a temporal manner. Therefore, LJ02 could employ MAMP to stimulate SA accumulation in both AG (SAR) and BG (SA signaling pathway) and in doing so to exert its biocontrol activities against a wide range of foliar and rhizospheric pathogens. In previous studies, only one-way signal transduction was investigated, such as AG–BG, AG–AG, and BG–BG (Yi et al., 2011). Recently, the bidirectional signal exchanges between AG–BG was identified as another type of induced resistance response. It was reported that foliar attack by the whitefly not only elicited AG resistance against a leaf pathogen, *Xanthomonas axonopodis* pv. *vesicatoria*, but also elevated resistance against the soil bacterium, *Ralstonia solanacearum*. And another interesting finding of this study is that AG whitefly feeding significantly increased the population density of beneficial BG microflora including actinomycetes and saprophytic fungi that may induce systemic resistance (Yang et al., 2011). Very recently, it was discovered that SA could modulate colonization of the root microbiome by specific bacterial taxa, indicating that SA is likely to be important for the colonization or recruitment of beneficial microbes in rhizosphere (Lebeis et al., 2015).

Based on current data and those previous studies, we summarized our results and some hypotheses in **Figure 8**. It can be seen that upon inoculation of LJ02 and in AG part, the upper cucumber leaves synthesized elevated level of SA and at the same time, this could partially explain the inhibition of powdery mildews. In BG part, the long-range resistance responses lead to the inhibition of several pathogens and their spore germination. Besides, the free SA was also increased in roots and it is possible that some beneficial bacteria could also be recruited to rhizospheres to protect plants from pathogenic invasions.

Based on the long-range resistance responses, we have established a method that could easily detect the inhibitory effect induced by foliar BCAs. We proved that induced rhizosphere secretions in solid MS medium by treating plant leaves with BCAs was an effective method to examine the anti-fungal and long-range resistance activities of potential BCAs. Although it is similar to that described for disk diffusion method (On et al., 2015), our new method takes the advantage of the coordinate activation of anti-fungal substances by plant-BCA-pathogen interactions rather than traditional BCA-pathogen interactions. The new approach will broaden our understanding of protective mechanisms by potential BCAs and can be applied to other beneficial microbes.

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frontiers in Microbiology

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Genome mining and metabolic profiling of the rhizosphere bacterium *Pseudomonas* sp. SH-C52 for antimicrobial compounds

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The plant microbiome represents an enormous untapped resource for discovering novel genes and bioactive compounds. Previously, we isolated *Pseudomonas* sp. SH-C52 from the rhizosphere of sugar beet plants grown in a soil suppressive to the fungal pathogen *Rhizoctonia solani* and showed that its antifungal activity is, in part, attributed to the production of the chlorinated 9-amino-acid lipopeptide thanamycin (Mendes et al., 2011). To get more insight into its biosynthetic repertoire, the genome of *Pseudomonas* sp. SH-C52 was sequenced and subjected to *in silico*, mutational and functional analyses. The sequencing revealed a genome size of 6.3 Mb and 5579 predicted ORFs. Phylogenetic analysis placed strain SH-C52 within the *Pseudomonas corrugata* clade. *In silico* analysis for secondary metabolites revealed a total of six non-ribosomal peptide synthetase (NRPS) gene clusters, including the two previously described NRPS clusters for thanamycin and the 2-amino acid antibacterial lipopeptide brabantamide. Here we show that thanamycin also has activity against an array of other fungi and that brabantamide A exhibits anti-oomycete activity and affects phospholipases of the late blight pathogen *Phytophthora infestans*. Most notably, mass spectrometry led to the discovery of a third lipopeptide, designated thanapeptin, with a 22-amino-acid peptide moiety. Seven structural variants of thanapeptin were found with varying degrees of activity against *P. infestans*. Of the remaining four NRPS clusters, one was predicted to encode for yet another and unknown lipopeptide with a predicted peptide moiety of 8-amino acids. Collectively, these results show an enormous metabolic potential for *Pseudomonas* sp. SH-C52, with at least three structurally diverse lipopeptides, each with a different antimicrobial activity spectrum.

Keywords: beneficial microbes, rhizosphere bacteria, antimicrobial peptides, Pseudomonads, genome sequencing, mass spectrometry, biocontrol

Introduction

Pseudomonas species are ubiquitous in aquatic and terrestrial habitats, and are intensively studied for their abilities to promote plant growth and to suppress plant pathogens (Weller, 2007; Berendsen et al., 2012). The main mechanisms involved in plant pathogen control are induced systemic resistance, competition, antibiosis and parasitism (Van Loon et al., 1998; Haas and Defago, 2005; Van Wees et al., 2008; Lugtenberg and Kamilova, 2009). To date, a wide variety of bioactive compounds involved in pathogen control have been identified for Pseudomonads (Gross and Loper, 2009; Raaijmakers et al., 2010; Raaijmakers and Mazzola, 2012). These include siderophores, hydrogen cyanide, 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin, phenazines, 2,5-dialkylresorcinol, quinolones, gluconic acid, rhamnolipids, and various structurally diverse lipopeptides (Gross and Loper, 2009; D'Aes et al., 2010).

Comparative genomics studies of *Pseudomonas* species have shown substantial diversity between the genera, between species, and even between strains belonging to the same species (Silby et al., 2011; Wu et al., 2011; Loper et al., 2012; Redondo-Nieto et al., 2012). Loper et al. (2012) further revealed that most of the genes encoding bioactive compounds map outside the core genome. This includes genes encoding the Non-Ribosomal Peptide Synthetases (NRPSs). NRPSs possess a modular structure and each module is a building block for the stepwise incorporation of an amino acid in the peptide moiety (Gross and Loper, 2009; Marahiel and Essen, 2009). NRPSs are responsible for the production of an array of antimicrobial compounds including lipopeptides (LPs). Intriguing features of LPs are their enormous structural diversity and diverse natural roles in microbial behavior (Raaijmakers and Mazzola, 2012). By the expansion of available genome sequences also the number of identified NRPS gene clusters has increased considerably over the past years. To exploit the hidden genetic and metabolic potential in genome sequences, a number of search tools and approaches have been developed, including regulator-based discovery (Hassan et al., 2010), metabolic networking, peptidogenomics and advanced mass spectrometry methods (Kersten et al., 2011; Watrous et al., 2012). This also led to the discovery of NRPS gene clusters involved in the production of structurally novel LPs (Liu et al., 2014).

Recently, we discovered *Pseudomonas* sp. SH-C52, a strain representative of a larger population of Pseudomonads that contributes to the natural suppressiveness of a soil against the fungal plant pathogen *Rhizoctonia solani* (Mendes et al., 2011). The gene cluster responsible for the activity of strain SH-C52 against *R. solani* is encoded by an NRPS, predicted to synthesize a 9-amino-acid chlorinated LP, designated thanamycin (Mendes et al., 2011). The production and partial structure of thanamycin was resolved by live colony mass spectrometry (Watrous et al., 2012). Next to thanamycin, strain SH-C52 was also found to produce a set of 2-amino-acid LPs, designated brabantamides A-C, which contain a glycosylated 3-hydroxy fatty acid tail. Brabantamide A displays activity against Gram-positive bacteria, including *Staphylococcus aureus* and *Arthrobacter crystallopoietes* (Reder-Christ et al., 2012; Schmidt et al., 2014). The 12-kb gene cluster for brabantamide biosynthesis includes the NRPS gene (*braB*), a glycosyltransferase (*braA*) and a specific FAD-dependent Baeyer–Villiger monooxygenase gene (*braC*). Biosynthesis of brabantamides is complex: a linear di-peptide is formed by BraB after which the sugar moiety is attached by the glycosyltransferase BraA. This glycosylated di-peptide is subsequently rearranged by the brabantamide-specific monooxygenase BraC (Schmidt et al., 2014).

To extend our knowledge on the biosynthetic repertoire of bioactive compounds of plant-associated *Pseudomonas* sp. SH-C52, we sequenced the genome and performed detailed *in silico* as well as metabolomic analyses. Here we show that genome-based phylogeny places strain SH-C52 in the *Pseudomonas corrugata* subgroup of the *P. fluorescens* clade. Next to the known thanamycin and brabantamide gene clusters, *in silico* analysis revealed four additional NRPS gene clusters. We further characterized the antimicrobial activity spectrum of thanamycin and brabantamide and identified the structure, activity and gene cluster of a novel LP with a 22-amino acid peptide moiety designated thanapeptin.

Materials and Methods

Strains and Growth Conditions

The bacterial strains *Pseudomonas* sp. SH-C52 (Mendes et al., 2011), derivatives from this strain, and *Pseudomonas syringae* pv. syringae B728a were grown on Pseudomonas agar F (PSA, Difco) or in King's medium B (KB, King et al., 1954). *Bacillus megaterium* and *Pectobacterium atrosepticum* SCRI1043 were cultured on Luria Bertani (LB) agar plates or in LB broth. When needed, growth media were supplemented with 25 μg/ml gentamycin 100 μg/ml kanamycin, and/or 25 μg/ml tetracycline. All bacteria were grown at 25°C.

Mutants in the thanapeptin gene cluster were obtained by screening the transposon insertion mutant library of *Pseudomonas* sp. SH-C52 for mutants that showed loss of or reduced activity against *R. solani* (Mendes et al., 2011). The initial screening showed the thanapeptin gene cluster mutants (Tn-*tnpA* and Tn-*tnpC*) to have a very minor reduction in the growth-inhibitory activity against *R. solani* (data not shown). The site-directed mutants in the thanamycin gene cluster KO25 and KO26 were obtained previously and were designated in this manuscript as dThaB and dThaC2, respectively. All fungi and oomycetes used in this study were cultured on Potato Dextrose Agar (PDA, Difco, Becton, Dickinson and Company, USA), except for *Phytophthora infestans* strain 88069 and *Phytophthora capsici* LT3239 which were cultured on Rye Sucrose Medium and on V8 medium (Latijnhouwers et al., 2004), respectively. *P. infestans* was cultured at 18°C; whereas all other fungi and oomycetes were cultured at 25°C.

Illumina Genome Sequencing and Assembly

Illumina sequencing was performed by BGI (China) according to their protocols. For this purpose, two libraries with insert sizes of 0.5 and ∼2 kb were constructed and sequenced. In order to ensure the accuracy of follow-up analysis, several steps were performed to filter the raw data: removal of reads with a certain

proportion of Ns' bases or low complexity reads (10% as default); removal of reads with a certain proportion of low quality (≤Q20) bases (40 bases as default); removal of adapter contamination (15 bp overlap between adapter and reads as default); removal of contamination due to duplication. For reads with low sequence quality additional processing was performed, i.e., removal of reads with significant poly-A structure, and removal of reads with a k-mer frequency of 1. During the processing 16% of the original read data was eliminated. Short reads were assembled into genomic sequences using SOAPdenovo, a BGI developed assembler (Li et al., 2010). Using mapping information gaps were filled and single base pairs were proofread. The usage rate of reads was obtained according to read mapping, from which the genome coverage was estimated.

Additional Sequencing, Sequence Adjustments, and Gene Annotation

Sequence information on the thanamycin gene cluster from strain SH-C52 (Mendes et al., 2011) was incorporated in the genome sequence to close gaps in the Illumina acquired assembly. In addition, for the predicted 22 amino-acid NRPS gene cluster, gaps were closed by additional standard Sanger sequencing (Macrogen, Amsterdam). Specific primer pairs were designed for PCR to cover the gaps present in the original Illumina assembly (Table S1). Primers were used for both PCR and sequencing. PCR products were sequenced in both orientations. Gene annotation was performed on the complemented genome sequence by use of RAST (http://rast.nmpdr.org) (Overbeek et al., 2014). The genome sequence and gene annotation are available at the NCBI and EMBL database (accession number CBLV000000000).

Genome Analysis and Comparisons

Genome comparisons were performed by use of nucleotide BLAST (BLASTN) in BioEdit (Ibis Biosciences). Genes identified by Loper et al. (2012) in species from the *Pseudomonas fluorescens* clade were used for BLASTN analysis on the SH-C52 genome sequence. For *Pseudomonas corrugata* CFBP5454 (ATKI00000000) and *Pseudomonas mandelii* JR-1 (NZ_CP005960) these analyses were performed online at the NCBI database. In general, genes were considered to be present when more than 65% identity with the reference sequence(s), and more than 85% of coverage was observed. In addition to the BLAST analysis, secretion systems were identified by screening gene annotations.

A whole-genome phylogenetic analysis was performed with the genomes of species of the *P. fluorescens* group as reported by Redondo-Nieto et al. (2012), with the addition of the genomes of *Pseudomonas corrugata* CFBP5454 (ATKI00000000) and *P. mediterranea* CFBP5447 (AUPB00000000). Phylogenetic trees were built by a Composition Vector approach using the web server CVTree with a *k*-value of 6 (Xu and Hao, 2009). Trees were generated by the Neighbor joining algorithm with *P. aeruginosa* PAO1 as the outgroup. Phylogenetic trees were visualized by MEGA5.1 (MEGA).

For the identification of secondary metabolite gene clusters the genome sequence was analyzed by both the on-line analysis programs NP.searcher (Li et al., 2009) and antiSMASH (Blin et al., 2013).

Thanapeptin Gene Cluster Analysis

The gene cluster identified to encode a NRPS of 22 amino acids, was further analyzed for the domains in the synthetases by the PKS/NRPS predictor (Bachmann and Ravel, 2009). Moreover, the Adenylation (A)-domains predicted by the PKS/NRPS predictor were analyzed by a phylogenetic comparison to known A-domains. MEGA5.1 (MEGA) was used for alignments and subsequent tree construction, using the neighbor-joining method and 500 bootstrap replicates. The combination of the predictions by NP.searcher, antiSMASH, PKS/NRPS predictor and phylogenetic analysis led to a consensus prediction presented in **Figure 5**. The first Condensation (C1)-domain predicted for the thanapeptin gene cluster was also subjected to phylogenetic analysis (as described above), with C1-domains of other NRPS gene clusters, to compare the thanapeptin C1-domain to cyclic and non-cyclic (lipo)peptides NRPSs (De Bruijn et al., 2007).

In vitro Inhibition Assays

For *in vitro* growth inhibition of fungi and oomycetes, SH-C52 and its derivative strains were, using an overnight culture in KB, spot-inoculated (3 μl) at the periphery of 1/5th strength PDA (pH 7.0) plates. After incubation for 2 days at 25°C, an agar plug (4-mm-diameter) from a freshly grown plate with the target organism was transferred to the center of the 1/5th PDA plate. Inhibition of radial growth of the fungus or the oomycete was monitored from 2 days after incubation up to 2 weeks after incubation, depending on the growth speed of the target organism. For *in vitro* bacteria and yeast inhibition assays, bacteria and yeast cells were included, after autoclaving and cooling to 45°C, into the 1/5th PDA medium at a concentration of $\sim 10^5$ cfu/ml. After solidifying and sufficient drying of the plates, SH-C52 and its derivative strains were, using an overnight culture in KB, spot-inoculated (3 μl) at the periphery of the bacteria or yeast containing plates.

Activity of Brabantamide A Against *Phytophthora infestans*

To test both the brabantamide A antimicrobial effect and the effect on phospholipase activity of *P. infestans*, individual *P. infestans* mycelial plugs were transferred to a 24-wells plate (Greiner Bio-One) with each well containing 2 ml rye sucrose broth (Latijnhouwers et al., 2002). Brabantamide A was added to the broth at the concentrations indicated. Brabantamide A was dissolved in DMSO, and DMSO concentrations were normalized for all wells to a final concentration of 1% (v/v). Growth of *P. infestans* was monitored for 6–8 days at an incubation temperature of 18°C. Mycelial mass was determined after drying the mycelium for 2 days at 60°C. Growth experiments were performed twice, each with two replicates per treatment. As similar results were obtained, representative results of one experiment are shown.

To assay the effect of brabantamide A on *P. infestans* phospholipid metabolism, mycelial plugs were labeled overnight with 10 μCi carrier free $^{32}PO_4^{3-}$ (GE Healthcare, Diegem,

Belgium). Brabantamide A treatments were performed simultaneously with the labeling or for 15 min after the labeling. All treatments contained a final concentration of 1% (v/v) DMSO and 0.1% (v/v) of n-butanol. The latter was well below phospholipid metabolism stimulatory concentrations (Latijnhouwers et al., 2002) and was included to detect transient PLD activity (Munnik et al., 1995; Meijer et al., 2011). Incubations were halted by addition of perchloric acid (final concentration 5%) and subsequent freezing in liquid nitrogen. After thawing 3.75 volume of $CHCl_3$/CH_3OH/1M HCl (50:100:1 by vol) and two glass beads (Ø = 3 mm) were added and the samples were again frozen in liquid nitrogen. Samples were thawed and thereafter vigorously shaken for 30 min. Samples were further treated and analyzed as described previously (Latijnhouwers et al., 2002). Radiolabeled phospholipids separated by TLC were detected and quantified by phospho-imaging (Storm, Molecular Dynamics; Sunnyvale, CA, USA).

Mass Spectrometry Analysis

For NanoDESI experiments the instrument setup was according to Watrous et al. (2012). The nanoDESI source coupled to a Thermo LTQ-FT-ICR MS capable of collision-induced dissociation. All analyses were performed in positive ion mode in the mass range of *m/z* from 200 to 2000. Both the primary and the nanospray capillaries were 150 μm o.d. × 50 μm i.d., with solvent being delivered and removed from the liquid bridge at approximate 45° angles. The solvent used was acetonitrile/0.05% formic acid in water (1:1) running at a flow rate of 0.8–2.5 μl/min. The droplet size using this configuration was ∼200 μm in diameter. MALDI imaging was performed according to Liu et al. (2010). For both the MALDI imaging and the NanoDESI experiments, the *Pseudomonas* sp. SH-C52 and its derivatives were grown on 1/5th PDA plates.

For antimicrobial assays and detailed MS analysis of the thanapeptin derivatives, strain *Pseudomonas* sp. SH-C52 was pre-grown overnight in 5 ml LB at 28°C. This overnight culture was used to streak-inoculate (10-μl loop) ISP2 agar plates [∼12 ml/plate, 4 g/l yeast extract (Sigma), 4 g/l dextrose (Sigma), 10 g/l malt extract (Sigma), 7.5 g/l agar (Sigma), 7.5 g/l agar (Teknova)]. Streak-inoculated plates were grown at 28°C for 36–44 h. Thanapeptin was extracted by scraping cells from the plates into solvent (60% acetonitrile/40% water/0.1% formic acid). Extractions were performed by shaking for 1 h. The cell extract was transferred to 50-ml centrifuge tubes and cells were spun down. The extraction was performed on the same cells for a total of 3 times. The supernatant was dried by rotovap and the pellet was retrieved by rinsing three times of 6 ml of the extraction solvent. The extraction fluid was collected in a centrifuge tube and spun down again. Subsequently, the supernatant was dried by rotovap again using a small 20-ml scintillation vial until dry. The vial was then rinsed by three washings with 400 μl of extraction solvent and placed in a 1.5 ml centrifuge tube and centrifuged at 14,000 rpm. The supernatant was injected in the HPLC containing a C18 4.6 × 150 mm 5 μm analytical column with a 250 μl sample loop. Mobile phase A was 100% water with 0.1% formic acid. Mobile phase B was 100% acetonitrile with 0.1% formic acid. The flow rate was set to 1.0 ml/min and a gradient elution was used with 15% mobile phase B ramped to 95% mobile phase B over 45 min. The eluent was collected using a fraction collector set to collect 1 ml fractions. Fractions were checked for purity and the right mass using MALDI (Bruker). Purified thanapeptin and its derivatives were subsequently used in antimicrobial assays, and assayed by MS analysis by a Thermo LTQ-FT-ICR MS capable of collision-induced dissociation.

Thanapeptin Activities

Compounds were tested in interaction with target organisms by different techniques. To test activity against bacteria and the yeast *Rhodoturula pilimanae*, plates containing the target strain were used. Bacteria and yeast were added at a concentration of $\sim 10^5$ cfu/ml to the 1/5th PDA medium. After sufficient drying of the plate, filter discs with the test compounds at indicated concentrations were added to the plate. Plates were incubated at 25°C and growth was monitored for 4 days. Activity against fungal and oomycete strains was tested both in liquid and on plate. For plate assays, thanapeptin derivatives at a concentration of 50 μM were added directly at the border of a 1/5th PDA, after which an agar plug (4 mm diameter) from a freshly grown plate with the target fungal or oomycete strain was positioned in the center of the plate. Hyphal growth was monitored from 2 days after incubation up to 2 weeks after incubation, dependent on the growth speed. Testing of compound activity against fungal and oomycete strains in liquid culture was performed by addition of the compound to 1 ml of liquid 1/5th PDB in a 12 wells culture plate (Greiner Bio-One), in which an agar plug (4 mm diameter) from a freshly grown plate with the target organism was submerged. Growth experiments were incubated at 25°C, except for *P. infestans*, which was cultured at 18°C.

Results

General Genome Features and Phylogeny

Sequencing and assembly showed that the genome size of *Pseudomonas* sp. SH-C52 is ∼6.3 Mb with a GC content of 61.0%. The genome was assembled in 596 contigs and 25 scaffolds, with an estimated coverage of 99.2%. For annotation, 384 contigs of at least 100-bp and covering 6.3 MB were used. In total, 5579 ORFs were annotated with 5523 CDSs and 56 tRNAs (**Table 1**). Previous analysis of the 16S rRNA sequence placed SH-C52 within the *P. fluorescens* clade (Mendes et al., 2011). At that time, however, no conclusive species designation was obtained. Expanding the former genome-wide phylogenetic analysis on *P. fluorescens* species (Redondo-Nieto et al., 2012) with the SH-C52 genome and the recent draft genomes of *P. corrugata* CFBP5454 and *P. mediteranea* CFBP5447, showed that strain SH-C52 clusters within subgroup I of the *P. fluorescens* group. Within subgroup I, SH-C52 and the strains of *P. corrugata* and *P. mediterranea* form a separate clade (**Figure 1**).

In silico Analysis of *Pseudomonas* sp. SH-C52 Primary Metabolism

The metabolic potential of *Pseudomonas* sp. SH-C52 was analyzed by comparing genes from primary metabolism with

TABLE 1 | General genome sequence information of *Pseudomonas* sp. SH-C52.

	Scaffold	Contig > 100 bp
Number	25	384
Total Length (bp)	6,356,481	63,12,590
Max length (bp)	1,572,034	292,658
Min length (bp)	505	102
Sequence GC(%)	61.0	61.0
Gene number	5808	5579
tRNAs	55	56

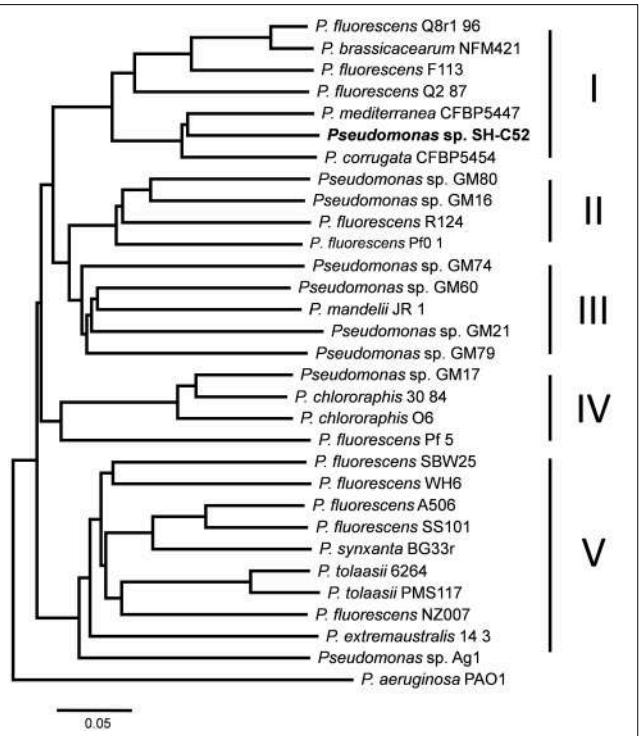

FIGURE 1 | Whole genome phylogenetic analysis of *Pseudomonas* sp. SH-C52. Phylogenetic tree constructed by use of CVtree, and based on the analysis performed previously by Redondo-Nieto et al. (2012), with the addition of strains SH-C52, *P. corrugata* CFBP5454 and *P. mediterranea* CFBP5447.

those known for the *P. fluorescens* clade (Loper et al., 2012). In this analysis, also its close relative *P. corrugata* strain CFBP5454 was included as well as *P. mandelii* strain JR-1 as a representative strain for subgroup III. The comparative analysis showed that for several of the metabolic activities in species of the *P. fluorescens* clade, the gene sequences are also found in strain SH-C52. These include genes involved in catabolism of L-arabinose, mannitol, myo-inositol, and ethanol, as well as genes coding for gelatinase and lipase. The ethanol catabolism genes, however, were only detected in five of the 12 other studied strains of the *P. fluorescens* clade, including subgroup I strains Q8r1-96, Q2-87, and *P. corrugata* CFBP5454. In contrast, genes encoding catabolic pathways for trehalose (10 strains), levan sucrase (7 strains) and L-tryptophan (7 strains, only subgroup IV and V) are present in multiple strains of the *P. fluorescens* clade, but were not detected in the SH-C52 genome (**Table 2**). Interestingly, comparing the SH-C52 genes with genes in the genome of *P. corrugata* CFBP5454 showed a few remarkable differences. Genes for trehalose catabolism were found in *P. corrugata* but not in SH-C52. The same was found for the nitrate reductase gene cluster previously reported for *P. corrugata* strains (Siverio et al., 1993). These differences in the presence of genes involved in primary metabolism further strengthen the phylogenetic delineation (**Figure 1**) where SH-C52 is closely related to, but different from *P. corrugata*.

Genome Analysis of Traits Involved in Plant-microbe Interactions

In silico analysis of the SH-C52 genome for genes involved in the production of phytohormones, volatiles, and plant signaling compounds led to the identification of putative pathways for acetoin/butanediol and for GABA catabolism (**Table 3**). Also the different bacterial secretion systems are of crucial importance for plant-microbe interactions (Korotkov et al., 2012; Russell et al., 2014; Tampakaki, 2014). The genome of SH-C52 contains two gene clusters encoding type II secretion systems with significant sequence similarity (>70%) to the Xcp and Hxc systems of *P. aeruginosa* (**Table 3**). Genes encoding potential substrates of the type II secretion system, such as lipases, proteases, esterases and alkaline phosphatases (Douzi et al., 2012) are well presented in the SH-C52 genome (**Tables 2**, **3**). No genes encoding a type III secretion system were found in the SH-C52 genome, yet two putative effector genes for this secretion system were identified (BN844_1916 and BN844_3678). Type VI secretion systems are conserved among Gram-negative bacteria and are thought to be involved in bacteria-bacteria interactions (Russell et al., 2014). Two gene clusters were found to encode type VI secretion systems of which one (BN844_4205-4227) was typed as a HSI-I locus and the second (BN844_0760-0774) as a HSI-III locus (**Table 3**).

Genome Analysis for Secondary Metabolites

To predict putative secondary metabolites, such as antibiotics, siderophores and other NRPS genes, the genome sequence was first screened by BLAST for ORFs of known compounds produced by other species and strains of the *P. fluorescens* clade (Loper et al., 2012). Only five SH-C52 clusters corresponded with known clusters (**Table 3**). First of all, the gene cluster for hydrogen cyanide production (HCN, BN844_0823-0821) was found in the SH-C52 genome and corresponded to the gene cluster found in eight out of the 10 previously sequenced *Pseudomonas* species (Loper et al., 2012). No pyoverdine synthetase gene cluster was found in SH-C52, which was also the case for *P. corrugata*. This corresponds to the observation that *P. corrugata* strains are known to be pyoverdine negative (Meyer et al., 2002). The siderophore gene cluster identified for SH-C52 is achromobactin (BN844_0513-0523). This gene cluster is not common among members of the *P. fluorescens* species clade, but has been described for *P. syringae* strains (Berti and Thomas, 2009; Owen and Ackerley, 2011) and for two *P. chlororaphis* strains (Loper et al., 2012), and is also found in the

TABLE 2 | Primary metabolism and corresponding genes of *Pseudomonas* SH-C52.

Function	I				II	III	IV			V			
	SH-C52	Pc 5454	Q8r1-96	Q2-87	Pf0–1	Pm JR-1	Pf-5	30–84	O6	SBW25	A506	SS101	BG33R
Gelatinase	BN844_0247	o	o	o	o	o	o	o	o	o	o	o	o
Lipase	BN844_0271	o	o	o	o	o	o	o	o	o	o	o	o
Phenylacetic acid							o	o	o				
Sodium Benzoate			o		o	o	o	o	o			o	
Trehalose		o	o			o	o	o	o	o	o	o	o
Levan sucrase			o	o				o	o	o	o	o	
L-arabinose	BN844_3151-3149	o	o	o	o	o		o	o	o	o	o	o
Nitrate reduction		o	o	o		o			o				
D-serine								o	o				
Denitrification		o	o	o		o							
Ethanol	BN844_0564	o	o	o		o	o						
Sorbitol			o	o		o				o	o	o	o
Mannitol	BN844_0168	o	o	o	o	o	o	o	o	o	o	o	o
myo-Inositol	BN844_0814	o	o	o		o	o	o	o	o	o	o	o
D-xylose	BN844_0249	o	o	o	o	o				o	o	o	o
L-tryptophan							o	o	o	o	o	o	o

**Functions are based on the 10 strains studied by Loper et al. (2012). The roman numbers are indicating subgroups within the P. fluorescens clade as identified by Redondo-Nieto et al. (2012), for which also the gray shades are indicative. Strains studied by Loper et al. (2012) are also included in the table, with the addition of P. corrugata CFBP5454 (Pc 5454) and P. mandelii JR-1 (Subgroup III). For SH-C52 the locus tags for the corresponding genes are given.*

genome of *P. corrugata* strain CFBP5454 (WP_024777665-670, **Table 3**). In addition to the achromobactin gene cluster, an NRPS gene cluster (BN844_2194-2167) was identified with similarity to genes presumably involved in ornicorrugatin biosynthesis. The putative (orni)corrugatin gene cluster identified in SH-C52 was also found in *P. fluorescens* strains Q8r1-96 and SBW25. Ornicorrugatin and the related compound corrugatin are so-called secondary siderophores (Matthijs et al., 2008). For *P. fluorescens*, however, conclusive experiments that link the production of (orni-) corrugatin to its putative gene cluster are still lacking.

Two other gene clusters in SH-C52 were predicted to encode insecticidal toxins. The first gene cluster is related to the Tcc2 toxin for which the genes are also found in the genomes of *P. fluorescens* strains Q8r1-96 and Q2-87. The second insect toxin gene cluster is similar to that of Tcc4 toxin (**Table 3**). No bacteriocin gene clusters could be identified with similarity to those found in other genomes of the *P. fluorescens* clade. However, a prophage gene cluster (BN844_3382-3419) flanked by *cinA* and *mutS*, typical for prophages present in other *Pseudomonas* genomes (Loper et al., 2012), was identified in the genome of SH-C52.

Non-ribosomal Peptide Synthetases

The SH-C52 genome contains six NRPS gene clusters, including the putative (orni-) corrugatin gene cluster (**Figure 2**). The other five clusters include the known NRPS gene clusters for the synthesis of thanamycin (BN844_0670-0673 and BN844_0703-0704, Mendes et al., 2011), and brabantamide (BN844_0705-0707, Schmidt et al., 2014). In close proximity to the thanamycin and brabantamide gene clusters, a third NRPS gene cluster (BN844_0667-0664) was identified (**Figure 2**). This NRPS is predicted to code for a 22 amino-acid lipopeptide with similarity to corpeptin (**Figure 2**) produced by *P. corrugata*. Therefore, in agreement with the naming of cormycin and corpeptin, we designated the predicted 22-amino-acid compound of SH-C52 as thanapeptin. The genome of *P. corrugata* CFBP5454 contains several NRPS genes with similarity to the 22 amino-acid related gene cluster in SH-C52. However, in *P. corrugata* this gene cluster is divided over different contigs. Recently, a partial gene cluster containing a partial NRPS gene, encoding two adenylation-domains and two transport genes, were linked to corpeptin production (Strano et al., 2014). Because of the incomplete nature of the draft genome sequence for strain CFBP5454, it remains unclear if the organization of the gene clusters for cormycin and corpeptin of *P. corrugata* is also similar to that of thanamycin and thanapeptin in SH-C52. However, consistent with the gene organization in SH-C52, a putative brabantamide-like gene cluster was also found in *P. corrugata* down-stream of the putative cormycin gene cluster (**Figure 2**). In addition, the biocontrol strain *P. fluorescens* In5 was recently reported to produce the antagonistic metabolites nunamycin and nunapeptin, also with similarity to cormycin and corpeptin, respectively. Although gene clusters for nunamycin and nunapeptin were presented (Michelsen et al., 2015), the genome information of strain In5 was not provided.

The fourth NRPS gene cluster (BN844_0379-0382) found in SH-C52 is predicted to code for an NRPS that produces an 8-amino-acid lipopeptide, with no significant similarity to other known compounds in general and dedicated databases such as NCBI and the Norine peptide database. This gene cluster

TABLE 3 | Secondary metabolite production and corresponding genes of *Pseudomonas* sp. SH-C52.

		I			II	III	IV			V			
		SH-C52	**Pc 5454**	**Q8r1-96**	**Q2-87**	**Pf0-1**	**Pm JR-1**	**Pf-5**	**30-84**	**O6**	**SBW25**	**A506**	**SS101**	**BG33R**
	ANTIBIOTICS													
	DAPG			O	O			O						
	HCN	BN844_0823-0821	O	O	O	O	O	O	O	O				
	Phenazine								O	O				
	Pyrrolnitrin							O	O	O				
	Rhizoxin							O						
	Pyoluteorin							O						
	HPR								O	O				
	NRPS/SIDEROPHORE/TOXINS													
	CLP*	**Figure 2**	O			O		O			O		O	O
Siderophore	Pyoverdine			O	O	O	O	O	O	O	O	O	O	O
	Pyochelin							O						
	Pseudomonine											O		O
	Achromobactin	BN844_0513-0523	O						O	O				
	Hemophore							O			O		O	O
	NRPS1—MgoA	BN844_4882	O	O	O	O	O		O	O	O	O	O	O
	NRPS3	BN844_2194-2167#	O	O							O			
	Bacteriocin	BN844_3382-3419	O	O	O	O	O	O	O	O	O	O	O	O
Insect toxins	FitD toxin							O	O	O				
	Tcc1											O	O	O
	Tcc2	BN844_3853-3849	2	O	O									
	Tcc3													O
	Tcc4	BN844_2985-2986	O	O		O								
	Tcc5						O			O				
	EXOENZYMES													
	Chitinase 1*					O	O		O	O		O	O	O
	Chitinase 2							O	O	O				
	AprA	BN844_0247	O	O	O	O	O	O	O	O	O	O	O	O
	AprX							O	O	O				
	Protease 1											O	O	O
	Protease 2												O	O
	Protease 3											O		
	Protease 4													O
	Pectate lyase										O			
	SECRETION SYSTEMS													
Type II	T2SS Xcp*	BN844_4825-4833	O		O				O	O				
	T2SS Hxc			O	O	O		O	O	O	O			
	T2SS Hxc-2	BN844_2047-2057									O			
	T2SS novel						O				O	O	O	O
Type III	T3SS 1			O	O									
	T3SS 2										O	O	O	O
	T3SS 3				O									
T3SS effectors	ExoU				O						O	O	O	O
	RopB			O	O									
	RopM			O	O									
	RopAA-1			O	O									
	Novel Effector				O									
	Putative Effectors	BN844_1916, _3678	O								O	O	O	O

(Continued)

TABLE 3 | Continued

			I			II	III	IV			V			
		SH-C52	Pc 5454	Q8r1-96	Q2-87	Pf0-1	Pm JR-1	Pf-5	30-84	O6	SBW25	A506	SS101	BG33R
Type VI	HSI-I	BN844_4205-4227	o	o	o			o	o	o	o	o	o	o
	HSI-II		o		o	o	o		o	o		o	o	o
	HSI-III	BN844_0760-0774	o	o	o	o								
	TSS4									o				
	PLANT COMMUNICATION													
	IAA biosynthesis								o	o				
	IAA catabolism													o
	PAA catabolism							o	o	o				
	ACC deaminase			o										
	Butanediol synthesis							o	o	o				
	Acetoin/Butanediol*	BN844_0610-0604	o	o	o			o	o					
	Acetoin catabolism											o	o	
	GABA catabolism	BN844_4573-4574	o	o	o	o	o	o	o	o	o	o	o	o

Functions are based on the 10 strains studied by Loper et al. (2012). The roman numbers are indicating subgroups within the P. fluorescens clade as identified by Redondo-Nieto et al. (2012), for which also the gray colors are indicative. Strains studied by Loper et al. (2012) are also included in the table, with the addition of P. corrugata CFBP5454 (Pc 5454) and P. mandelii JR-1 (Subgroup III). For SH-C52, the locus tags for the corresponding genes are given. Cyclic lipopeptides (CLP) for SH-C52 are discussed in the text and listed in* ***Figure 2**.*
[#]The SH-C52 NRPS3-corrugatin gene cluster sequence is incomplete and divided over different contigs.

appears to be unique for SH-C52, as it was not found in other genomes. The last NRPS gene cluster identified in SH-C52 shows similarity to *mgoA*-like gene clusters, encoding only one adenylation domain. MgoA or MgoA-regulated compound(s) were proposed to regulate the expression of pathogenicity factors in *P. entomophila* and *P. syringae* (Vallet-Gely et al., 2010; Carrion et al., 2014), although the underlying mechanism is yet unknown.

Thanamycin: Gene Cluster and Antimicrobial Activity

Based on mutant analysis, the thanamycin gene cluster was shown to be important for the growth-inhibitory activity of strain SH-C52 against *Rhizoctonia solani* (Mendes et al., 2011). The thanamycin gene cluster shows similarity to a fragmented NRPS gene cluster in *P. corrugata* CFBP5454 (**Figure 2**), but the incomplete and scattered *P. corrugata* sequences complicate a clear comparison between the thanamycin and cormycin gene clusters. Alignments of the thanamycin NRPSs and the annotated parts of cormycin NRPSs show 85–97% protein identity. To test the antimicrobial activity-spectrum of thanamycin, wild type SH-C52 and two thanamycin biosynthesis mutants (Mendes et al., 2011) were tested for activity against fungi, oomycetes and bacteria. Here, we show that the activity spectrum of thanamycin is not exclusive for *R. solani*, but extends to an array of other fungi (**Figure 3**) and the Gram-positive bacterium *Bacillus megaterium* (data not shown). Also cormycin has been shown to be active against *B. megaterium* and the yeast *Rhodotorula pilimanae* (Scaloni et al., 2004). In contrast, thanamycin has little activity against oomycete pathogens (**Figure 3**) and the Gram-negative bacteria *Pseudomonas syringae* and *Pectobacterium atrosepticum* (data not shown).

Brabantamide: Gene Cluster and Antimicrobial Activity

The gene cluster and the brabantamide compounds were previously identified (Schmidt et al., 2014). Interestingly, a putative brabantamide gene cluster is also present in the genome of *P. corrugata* (**Figure 2**) as well as in the genome of *P. fluorescens* DSM 11579 (Johnston et al., 2013).

In previous studies, brabantamides were shown to have activity against specific Gram-positive bacteria (Reder-Christ et al., 2012; Schmidt et al., 2014). Here, we show that brabantamide A, at a concentration of 50 μM, has activity against the oomycete plant pathogens *Phytophthora capsici*, *Pythium ultimum*, and *P. infestans* (**Figure 4**). No antifungal activity was observed at this concentration (data not shown). For *P. infestans*, mycelial growth was already inhibited at concentrations above 5 μM resulting in a less dense mycelial mat (**Figure 4A**). Previous studies suggested that structural analogs of brabantamide A inhibit phospholipase A2 of rabbit (Thirkettle et al., 2000). When [^{32}P]Pi-labeled hyphae of *P. infestans* were incubated for 15 min with brabantamide, no significant variation in the phospholipid levels was observed (data not shown). However, overnight exposure of *P. infestans* mycelium to brabantamide A led to an increase in the phosphatidylbutanol (PtdBut) levels. This already occurred at a concentration of 1 μM brabantamide A, the lowest concentration tested (**Figure 4**). Other phospholipid levels were not affected based on quantification. The enhanced PtdBut levels indicates a stimulatory effect of brabantamide A on phospholipase D activity. In contrast, no indication was found for the inhibition of phospholipase A2 in this assay.

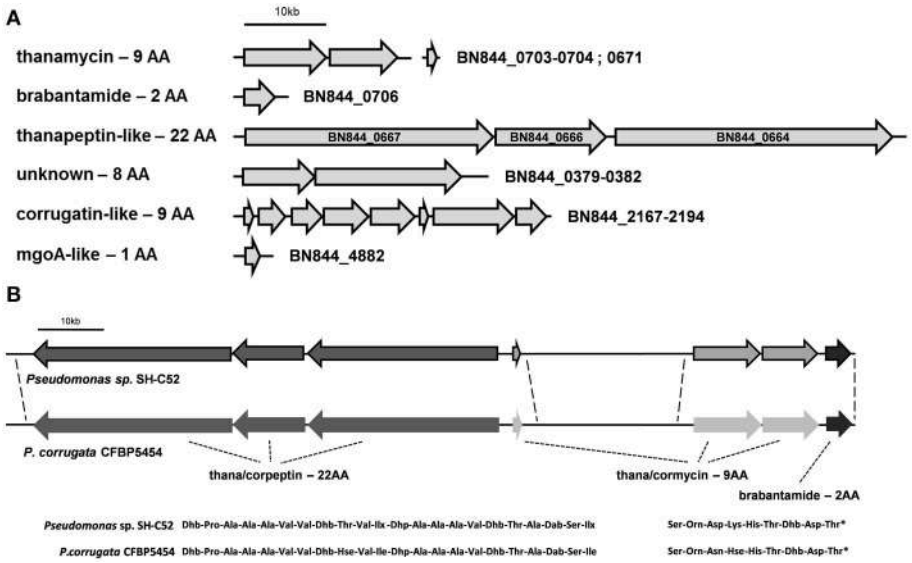

FIGURE 2 | Non-ribosomal peptide synthetase gene clusters present in the genome of *Pseudomonas* sp. SH-C52. (A) The six NRPS gene clusters predicted in the genome of SH-C52. **(B)** A comparison of the SH-C52 thanamycin-thanapeptin-brabantamide cluster with the putative equivalent cluster of *P. corrugata* CFBP5454. Thana/corpeptin related NRPS genes are in dark gray, thana/cormycin NRPS genes are in light gray, and brabantamide genes are in near black. The *P. corrugata* genes are not bordered, as the genome information is scattered over contigs and needs to be confirmed. Dotted lines indicate (expected) sequence conservation. Amino acids are indicated in their standard three-letter annotation. Non-standard amino acids were abbreviated as follows: Hse, homoserine; Ilx, isoleucine or leucine; Dab, 2,4-diaminobutanoic acid; Dhb, 2,3-dehydro-2-aminobutanoic acid; Dhp, dehydro-2-aminopropanoic acid (dehydroalanine). The asterisk in the thana/cormycin structure indicates chlorination of this amino acid.

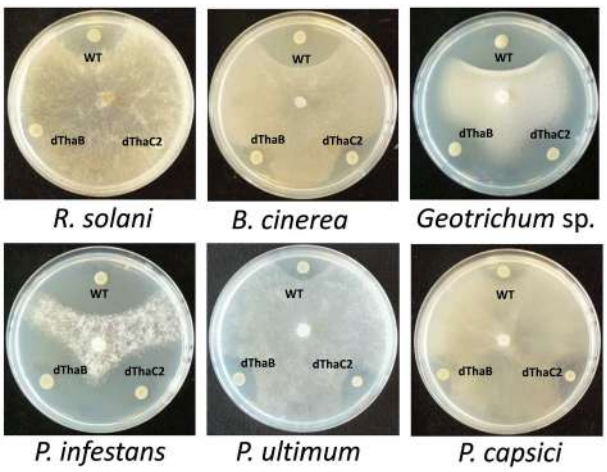

FIGURE 3 | Antagonistic activity of *Pseudomonas* sp. SH-C52. Wild-type (WT) and the thanamycin mutant strains (ThaB, ThaC2) were tested in dual culture assays for activity against fungal plant pathogens **(upper panel)** and oomycete plant pathogens **(lower panel)**.

Thanapeptin: Gene Cluster, Structural Analysis and Antimicrobial Activity

The thanapeptin gene cluster consists of three NRPS genes. BLAST analysis of the protein sequences showed similarity with the NRPSs for the production of the LP syringopeptin of *P. syringae*. Indeed, the first gene of the thanapeptin cluster starts with a specific condensation (C)-domain, a so-called C1 starter domain (data not shown), suggesting *N*-acylation of the first amino acid in the peptide moiety. The three NRPS genes of the thanapeptin gene cluster encode nine, four and nine adenylation (A)-domains, respectively. The prediction of the 22 amino acids activated by these A-domains is shown in **Figure 5**. The C-terminus of the last gene of the cluster encodes two thioesterase (TE)-domains, indicating termination of the thanapeptin synthesis. Despite the similarity between syringopeptin and thanapeptin (**Figure 2**), there also is a clear difference in modular organization as syringopeptin is produced from three NRPS modules, with five, five and twelve adenylation domains, respectively. Comparison with the *P. corrugata* CFBP5454 draft genome sequence shows that the corpeptin gene cluster sequence is incomplete and scattered over contigs. Nevertheless, for the annotated parts, protein identities with the SH-C52 NRPSs ThpA and ThpC ranged between 75 and 95%, whereas for ThpB identities ranged between 60 and 80%.

Using MALDI and live colony NanoDESI mass spectrometry, a specific group of ions was detected for which the parent mass ranged from 2082 to 2150 Da. Comparing the tandem MS spectra of this set of ions indicated these are related peptides, probably produced by the same NRPS gene cluster. Usage of the peptidogenomics approach (Kersten et al., 2011) on the tandem MS data of these ions (Supplementary Data) revealed that they can be linked to the predicted thanapeptin peptide sequence, and thus to the thanapeptin NRPS gene cluster. MS analysis of the extracts from the thanapeptin mutants indeed showed that the

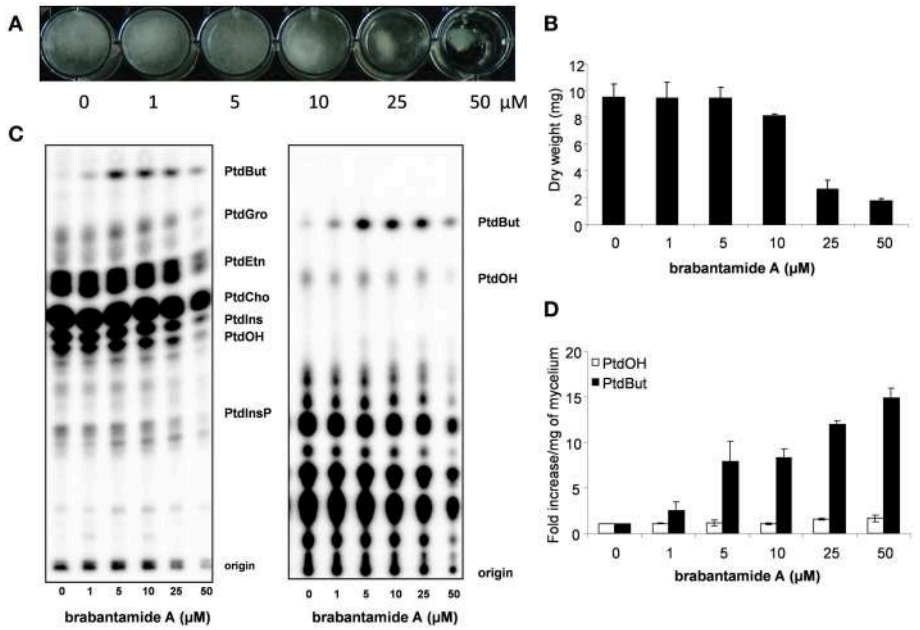

FIGURE 4 | Antimicrobial and phospholipase D stimulating activity of brabantamide A in interaction with *Phytophthora infestans*. **(A)** Growth of *P. infestans* in liquid medium with different concentrations of brabantamide A. **(B)** Dry weight of *P. infestans* grown in liquid medium ($n = 2$). **(C)** Lipids extracted from *P. infestans*, separated by alkaline (left) or ethylactetate (EtAc) TLC (right) and analyzed by phosphoimaging. The origin of the chromatogram, phosphatidylinositolphosphate (PtdInsP), phosphatidic acid (PtdOH), phosphatidylinositol (PtdIns), phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylglycerol (PtdGro) and phosphatidylbutanol (PtdBut) are indicated. **(D)** PtdOH and PtdBut were quantified from the EtAc TLC and presented as the fold increase when compared to control conditions ($n = 2$). Error bars represent standard deviations.

production of all ions of the putative thanapeptin group were absent (data not shown). Further analysis of the tandem MS data of the subsequently purified ion of 2120 Da was in agreement with the *in silico* predicted amino acid sequence for thanapeptin. In addition, tandem MS data provided evidence for cyclization, and resolved the identity of the lipid moiety of thanapeptin (**Figure 5** and Supplementary Presentation 1).

The role of the thanapeptin gene cluster in the activity of SH-C52 against fungi, oomycetes and bacteria was studied by comparing wild-type strain SH-C52 and two independent mutants, each with a single transposon insertion in the thanapeptin NRPS gene cluster. The two mutants lost their antagonistic activity against oomycete pathogens, whereas they still had activity against fungi (**Figure 6**) and bacteria (data not shown). From the wild-type strain, thanapeptin derivatives were purified and seven derivatives were tested for activity against the oomycete *P. infestans*. Substantial differences in anti-oomycete activity were observed between the compound derivatives, with the strongest activity for those with the lowest mass, i.e., compounds with the masses 2082, 2096, 2108, and 2122 Da (**Figure 7A**). In addition, two derivatives with strong activity, 2096 and 2122 Da, were also tested against the oomycete pathogens *Saprolegnia parasitica* and *P. ultimum*. Similar results were obtained as in the assays with *P. infestans*, with the strongest activity for the derivative with a mass of 2096 Da, and slightly lower activity for the derivative with a mass of 2122 Da (data not shown). Subsequently, the activity of the derivative with a mass of 2096 Da was tested at different concentrations against the three oomycete pathogens in a liquid broth. For *P. infestans* (**Figure 7**), a clear reduction in mycelial growth was observed at a concentration of 0.25 µg/ml. For syringopeptin or corpeptin, no anti-oomycete activity has been reported to date (Vassilev et al., 1996; Emanuele et al., 1998). No apparent antifungal activity or activity against Gram-negative bacteria was observed for any of the thanapeptin derivatives, whereas activity was observed against the Gram-positive bacterium *B. megaterium* (data not shown), which is in line with results observed previously for syringopeptin and corpeptin (Vassilev et al., 1996; Emanuele et al., 1998).

Discussion

Pseudomonas sp. SH-C52 was initially studied for its role in the natural soil suppressiveness against the fungal pathogen *R. solani* (Mendes et al., 2011). Here we show by both genome and metabolomic analyses that this plant-associated bacterium has a much greater genomic capacity for the production of secondary metabolites. We showed that *Pseudomonas* sp. SH-C52 can be placed within subgroup I of P. fluorescens species (Redondo-Nieto et al., 2012) as a separate clade together with *P. corrugata* and *P mediterranea*. *P. corrugata* strains have been reported for biological control, for bioremediation and for the production of a range of biomolecules. In contrast, *P. corrugata* strains have also been reported as plant pathogenic bacteria (Catara, 2007).

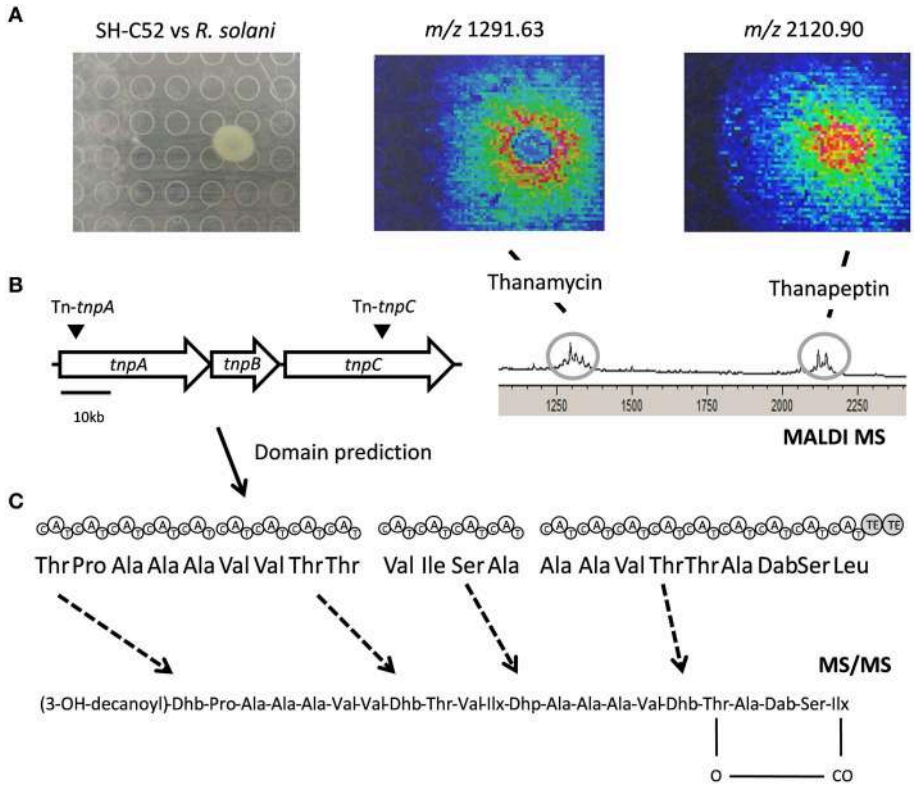

FIGURE 5 | Thanapeptin genetic and chemical analysis. (A) The upper panel shows the interaction of SH-C52 in interaction with *R. solani*, as imaged by MALDI imaging mass spectrometry (IMS). Thanamycin and thanapeptin detection by MALDI IMS are shown. **(B)** The middle panel presents the thanapeptin gene cluster with the position of the transposon insertions, and the modules encoded by the gene clusters and the prediction for the amino acids assembled. On the middle left, the partial spectrometry profile of the MALDI IMS of the SH-C52 and *R. solani* interaction is shown. **(C)** At the bottom the tentative thanapeptin structure obtained from tandem mass spectrometry data is presented. Dashed arrows indicate amino acids that are difficult to predict from genome information, and that needed to be resolved by mass spectrometry.

Indeed, the sequenced strain *P. corrugata* CFBP5454 was isolated because of its pathogenic properties (Licciardello et al., 2007). In contrast, *Pseudomonas* sp. SH-C52 was isolated because of its plant-beneficial and antagonistic properties (Mendes et al., 2011). For *P. corrugata*, regulation of cormycin and corpeptin production is under the control of an *N*-acyl-homoserine-lactone quorum sensing system (Licciardello et al., 2012), for which the genes are conserved in strain SH-C52 (data not shown). For both, the quorum sensing system is located down-stream of the thana/corpeptin gene clusters. The different origin of these strains suggests that structurally identical secondary metabolites can have diverse functions, depending on the niche where they are produced. For instance they can fight off pathogen threats for certain plant niches, as is the case for thanamycin in protecting sugar beet seedlings, or are advantageous to occupy certain niches, as is the case for cormycin during infection of plants.

In addition to thanamycin and thanapeptin, *Pseudomonas* sp. SH-C52 produces a third LP with antimicrobial activity. Previously, brabantamides were shown to be active against specific Gram-positive bacteria (Reder-Christ et al., 2012; Schmidt et al., 2014). Here we report that brabantamide A also has activity against oomycetes. Interestingly, this compound has received considerable attention for the development of the synthetic phospholipase inhibitor darapladib, which is tested for treatment of atherosclerosis (Johnston et al., 2013). This highlights the importance of identifying novel LPs for the development of new medicines. In this respect it is interesting to note that in the genome of *Pseudomonas* sp. SH-C52 a putative fourth LP-NRPS gene cluster is present, with a yet unknown structure and function. Also an *mgoA*-like NRPS gene was identified in the genome of SH-C52. Although the *mgo*-operon was shown to be essential for mangotoxin production in mango-pathogenic *P. syringae* strains (Arrebola et al., 2012), it was later demonstrated that the biosynthesis of mangotoxin occurs by means of the *mbo*-operon (Carrion et al., 2012). This suggests that MgoA likely has a regulatory function (Carrion et al., 2014). An orthologous gene cluster was shown to be essential for insect pathogenicity in *P. entomophila*, however, no pathogenicity factor or compound has yet been linked to this phenotype (Vallet-Gely et al., 2010). The *mgo*-operons are not only present in pathogenic bacteria, but are widespread among *Pseudomonas* species, including species from the *P. fluorescens* clade (Loper et al., 2012). Since the product from MgoA, or the *mgo*-operon, has not been deduced, the function of the *mgo*-operon

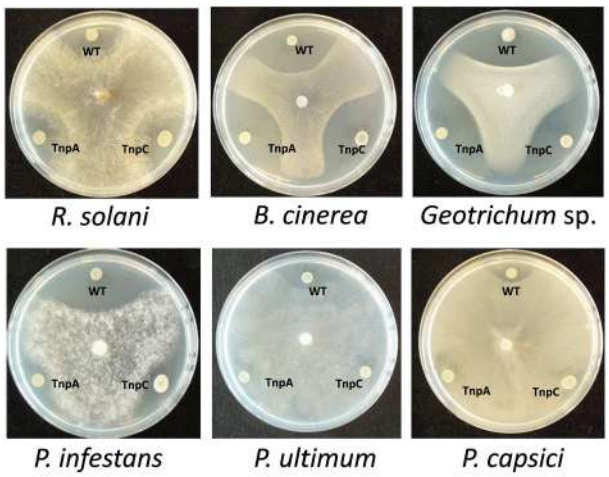

FIGURE 6 | Antagonistic activity of *Pseudomonas* sp. SH-C52. The wild-type (WT) and the thanapeptin mutants strains, Tn-*tnpA* (TnpA) and Tn-*tnpC* (TnpC) were tested in dual culture assays for activity against fungal plant pathogens **(upper panel)** and oomycete plant pathogens **(lower panel)**.

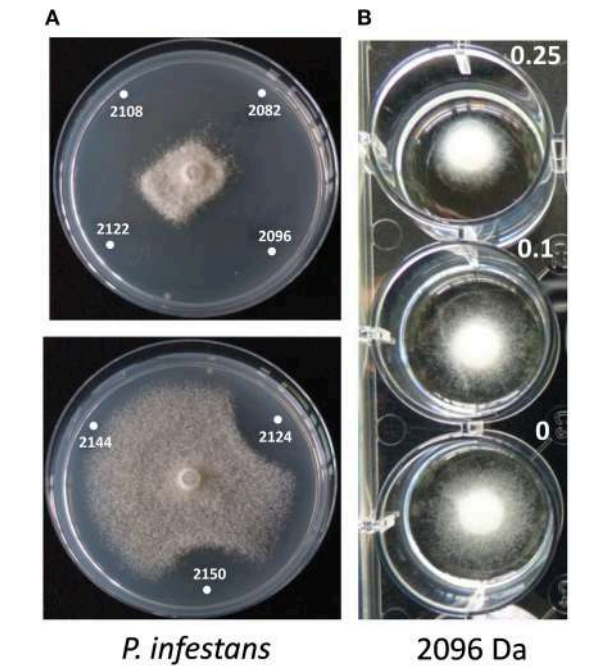

FIGURE 7 | Activity of thanapeptin derivatives against *Phytophthora infestans*. (A) Activity of thanapeptin tested on plate. Numbers indicated in the pictures are the parent mass of the derivate tested in Da. **(B)** For the derivate of 2096 Da the growth-inhibitory activity was tested in liquid broth, at different concentrations, indicated in the picture in μg/ml.

remains an intriguing question. The Mgo-product may act as a *Pseudomonas* specific switch for secondary metabolite production or as a genus- or species-specific regulator involved in different functions.

The production of secondary metabolites often comes at the expense of primary metabolism (Ruiz et al., 2010). Therefore, production needs to be tightly regulated in a temporal and spatial manner. *Pseudomonas* sp. SH-C52 has the ability to produce at least three and likely four structurally different LPs, each with structural variants. LPs have diverse natural functions in motility, biofilm formation, antibiosis, defense or virulence (Raaijmakers and Mazzola, 2012). These functions have to be strictly regulated and cannot be performed all at once. Some LPs are produced at the same time but may have different roles. This is the case for two LPs produced by *Pseudomonas* CMR12a, i.e., sessilin and orfamide that are produced together but involved in respectively biofilm formation and swarming. Regulation takes place by the fact that sessilin hampers the release of orfamide by co-precipitating with orfamide (D'Aes et al., 2014). Although we do see that thanamycin and thanapeptin can be produced simultaneously by *Pseudomonas* SH-C52 (**Figure 5**), we do not see the simultaneous production of the other two LPs. This suggests dissimilar regulatory pathways for the production of these LPs, possibly with negative and positive feedback loops. Three of the LPs produced by SH-C52 have antimicrobial activity *in vitro*, but it remains to be studied if these LPs also have antimicrobial activity *in vivo*. In this respect, future studies will focus on the identification of regulatory pathways and identification of specific extracellular cues that trigger the production of the structurally diverse LPs in SH-C52 in interactions with diverse organisms encountered in the rhizosphere environment.

Author Contributions

MV designed and performed microbiological, molecular and chemical experiments, performed *in silico* genome analysis and drafted the manuscript. HM performed growth inhibition assays with *P. infestans* and experiments to test phospholipase activity. JW designed chemical experiments, helped in chemical analysis, and performed the chemistry and analysis on the thanapeptin structure, ED assisted in microbiological and molecular experiments. RM was involved in acquiring and analysing genome information. YS and HG purified brabantamide A for phospholipase and antimicrobial assays. PD designed chemical experiments and was involved in chemical imaging. JR supervised the work and was involved in the experimental design. All authors contributed to the writing of the manuscript and approved submission.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00693

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Deciphering the conserved genetic loci implicated in plant disease control through comparative genomics of *Bacillus amyloliquefaciens* subsp. *plantarum*

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To understand the growth-promoting and disease-inhibiting activities of plant growth-promoting rhizobacteria (PGPR) strains, the genomes of 12 *Bacillus subtilis* group strains with PGPR activity were sequenced and analyzed. These *B. subtilis* strains exhibited high genomic diversity, whereas the genomes of *B. amyloliquefaciens* strains (a member of the *B. subtilis* group) are highly conserved. A pairwise BLASTp matrix revealed that gene family similarity among *Bacillus* genomes ranges from 32 to 90%, with 2839 genes within the core genome of *B. amyloliquefaciens* subsp. *plantarum*. Comparative genomic analyses of *B. amyloliquefaciens* strains identified genes that are linked with biological control and colonization of roots and/or leaves, including 73 genes uniquely associated with subsp. *plantarum* strains that have predicted functions related to signaling, transportation, secondary metabolite production, and carbon source utilization. Although *B. amyloliquefaciens* subsp. *plantarum* strains contain gene clusters that encode many different secondary metabolites, only polyketide biosynthetic clusters that encode difficidin and macrolactin are conserved within this subspecies. To evaluate their role in plant pathogen biocontrol, genes involved in secondary metabolite biosynthesis were deleted in a *B. amyloliquefaciens* subsp. *plantarum* strain, revealing that difficidin expression is critical in reducing the severity of disease, caused by *Xanthomonas axonopodis* pv. vesicatoria in tomato plants. This study defines genomic features of PGPR strains and links them with biocontrol activity and with host colonization.

Keywords: *Bacillus*, *plantarum*, host colonization, biocontrol, bacterial spot disease, PGPR

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Introduction

Bacteria associated with plant roots that exert beneficial effects on plant growth and development are referred to as plant growth–promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978; Kloepper et al., 2004). *Bacillus* and *Pseudomonas* spp. are predominant among the diverse bacterial genera that have been linked with PGPR activity (Podile and Kishore, 2006). Members of the *B. subtilis* group, including *B. subtilis*, *B. licheniformis*, *B. pumilus, B. amyloliquefaciens*, *B. atrophaeus*, *B. mojavensis*, *B. vallismortis*, *B. sonorensis*, and *B. tequilensis* have been identified as PGPR strains for their capacity to stimulate plant growth and suppress pathogens within rhizosphere and phyllosphere (Kloepper et al., 2004; Hao et al., 2012; Kim et al., 2012). Strains of *B. amyloliquefaciens* are widely used for their positive effects on plant growth (Idriss et al., 2002). Reva et al. (2004) reported that seven *Bacillus* isolates from plants or soil are closely related yet distinct from *B. amyloliquefaciens* type strain DSM7^T. In addition, these strains are more proficient for rhizosphere colonization than other members of the *B. subtilis* group. GB03 (Nakkeeran et al., 2005), INR7 (Kokalis–Burelle et al., 2002), and FZB42 (Chen et al., 2007) are PGPR strains within the *Bacillus subtilis* group that have been widely used in different commercial formulations to promote plant growth.

In addition to promoting plant growth, PGPR strains may exhibit biological control of plant diseases. Antibiosis, through the production of inhibitory bioactive compounds, and induced systemic resistance are widely reported biological control mechanisms of *Bacillus* spp. PGPR strains (Ryu et al., 2004). PGPR *Bacillus* spp. strains produce diverse antimicrobial compounds including antibiotics (Emmert et al., 2004), volatile organic compounds (VOCs) (Yuan et al., 2012), and lipopeptides (Ongena et al., 2007) that are associated with the observed biocontrol activity against plant pathogens. For example, *B. amyloliquefaciens* NJN-6 produces 11 VOCs that provide antifungal activity against *Fusarium oxysporum* f. sp. *cubense* (Yuan et al., 2012). Similarly, *B. subtils* strains produce lipopeptides (e.g., surfactin and fengycin), that induce systemic resistance in bean plants (Ongena et al., 2007).

PGPR strains usually need to colonize plant roots extensively to exert plant growth promoting effects using both direct and indirect mechanisms (Lugtenberg and Kamilova, 2009), extensive root colonization is not required for induced systemic resistance (ISR) (Kamilova et al., 2005). In some PGPR strains, root colonization is a prerequisite for biocontrol activity through antibiosis (Chin et al., 2000). For example, *B. amyloliquefaciens* subsp. *plantarum* FZB42 exerts growth promoting activities through efficient colonization of plant roots (Fan et al., 2011). Previously, it has been demonstrated that over-expression of genes involved in phosphorylation of DegU, a two-component response regulator of *B. amyloliquefaciens* strain SQR9, positively influences root colonization as well as other growth-promoting activities by PGPR strains for controlling cucumber wilt disease (Xu et al., 2014). Moreover, the root colonization capacity of a poor root colonizer can be improved by cloning genes that are required for efficient root colonization (Dekkers et al., 2000). Competitive root colonization by PGPR are controlled by many genes and/or genetic cluster(s) (Dietel et al., 2013), so identification of these genetic loci involved in competitive root colonization are challenging if genome sequences are lacking for those PGPR strains (Lugtenberg and Kamilova, 2009). Analysis of additional PGPR strains will help elucidate the mechanisms of competitive root colonization, antibiosis and ISR of PGPR strains and form a foundation for genetic engineering and other strategies to increase the plant-growth promoting capacity of these bacteria.

In this study, we sequenced the genomes of 12 *Bacillus subtilis* group isolates from diverse locales. Comparative genomic analyses of PGPR strains and control strains of the *B. subtilis* group without any reported biocontrol activity against plant pathogens provides insight into genomic features involved in PGPR activity. PGPR strain AP193, which inhibits growth of plant and animal bacterial pathogens (Ran et al., 2012), is an ideal candidate to evaluate the relative contribution of genes that are predicted to be involved in the biosynthesis of bioactive secondary metabolites that could contribute to biocontrol activity, specifically difficidin (*dfnD* mutant), surfactin (*srfAA* mutant), as well as all polyketides and lipopeptides produced by non-ribosomal peptide synthesis, including difficidin (*sfp* mutant). Mutants were then tested for their ability to inhibit plant pathogens *in vitro* and control bacterial spot disease in tomato.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *E. coli* and *Bacillus* strains were grown in Luria-Bertani (LB) medium; however, for electrocompetent cell preparation, *Bacillus amyloliquefaciens* subsp. *plantarum* AP193 was grown in NCM medium (17.4 g K_2HPO_4, 11.6 g NaCl, 5 g glucose, 5 g tryptone, 1 g yeast extract, 0.3 g trisodium citrate, 0.05 g $MgSO_4{\cdot}7H_2O$ and 91.1 g sorbitol in 1 L deionized water, pH 7.2). For production of secondary metabolites, *Bacillus* cultures were grown for 48 h at 30°C in Tryptic Soy broth (TSB). In addition, ampicillin (100 μg/ml), chloramphenicol (12.5 μg/ml), or erythromycin (200 μg/ml for *E. coli* or 5 μg/ml for *Bacillus*) were used as selective agents in growth media as required.

Sequencing, Assembly and Annotation

Next-generation sequencing of *Bacillus* spp. genomes was performed using Illumina and Roche 454 sequencing platforms. Indexed Illumina libraries were prepared for strains AP71, AP79, and AB01 using Nextera DNA Sample Prep Kit (Epicentre, Madison, WI) and sequences were generated using an Illumina MiSeq with a 2 × 250 paired end sequencing kit. Barcoded Illumina libraries for strains AP143, AP193, and AP254 were constructed using a NxSeq® DNA Sample Prep Kit (Lucigen, Middleton, WI) and sequenced at EnGenCore (Univ. of South Carolina) using the 454-pyrosequencing platform. Genomic DNA library construction and sequencing for *Bacillus subtilis* GB03, *Bacillus pumilus* INR7, *B. mojavensis* KCTC 3706T, *B. tequilensis* KCTC 13622T, *Bacillus siamensis* KCTC 13613T, and *B. sonorensis* KCTC 13918T were conducted at the

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
E. coli K12 ER2925	*dcm-6 dam13::Tn9*	New England Biolabs
B. amyloliquefaciens subsp. plantarum strain AP193	Wild type	Dr. Joseph Kloepper (Department of Entomology and Plant Pathology, Auburn University)
AP193Δ*sfp*	Deficient in lipopeptides and polyketides	This study
AP193Δ*srfAA*	Deficient in surfactin production	This study
AP193Δ*dfnD*	Deficient in difficidin production	This study
Bacillus amyloliquefaciens FZB42	Wild type	Chen et al., 2007
pMK4	*E. coli-Bacillus* shuttle plasmid, rolling circle replicative, Cm^R	BGSC
pNZT1	Replication thermosensitive derivative of the rolling-circle plasmid pWV01 (pG^+ replicon, Em^R)	Xiaozhou Zhang, Virginia Tech
pNZ-sfp	pNZT1 with upstream and downstream sequences of gene *sfp*	This study
pNZ-srf	pNZT1 with knock-out construct of *srfAA*	This study
pNZ-dif	pNZT1 with knock-out construct of *dfn*D	This study

National Instrument Center for Environmental Management (Seoul, Republic of Korea), using the Illumina HiSeq 2000 sequencing platform. Sequence reads were trimmed for quality then assembled *de novo* using the CLC Genomics Workbench (CLCBio, Cambridge, MA). Gene prediction and annotation were performed using GeneMark (Lukashin and Borodovsky, 1998) and the RAST annotation server (Aziz et al., 2008), respectively. The identity of individual open reading frames (ORFs) from secondary metabolite biosynthesis gene clusters was confirmed by BLASTx against the GenBank database. Genome sequence reads for strains AB01, AP71, AP79, AP143, AP193, AP254, GB03 (Choi et al., 2014), INR7 (Jeong et al., 2014), KCTC 3706T, KCTC 13613T (Jeong et al., 2012), KCTC 13918T, and KCTC 13622T were deposited into the Short Read Archive (SRA) at NCBI under the accession numbers SRR1176001, SRR1176002, SRR1176003, SRR1176004, SRR1176085, and SRR1176086, SRR1034787, SRR1141652, SRR1141654, SRR1144835, SRR1144836, and SRR1144837, respectively.

Determination of Average Nucleotide Identity

Average nucleotide identities (ANI) between genomes were calculated using an ANI calculator that estimates ANI according to the methods described previously (Goris et al., 2007).

Phylogenetic Analysis of Bacillus Species

For phylogenetic analysis, the *gyrB* gene sequence for each strain (a list of the 25 strains is presented in **Figure 1**) was retrieved from sequence data. Strains AS43.3, FZB42, YAU B9601-Y2, CAU B946, and 5B6 were used as representative strains of *B. amyloliquefaciens* subsp. *plantarum*; strains DSM7, LL3, and TA208 were used as representative strains of *B. amyloliquefaciens* subsp. *amyloliquefaciens*. The *gyrB* phylogenetic tree was inferred with MEGA5.05 (Tamura et al., 2011) using Neighbor-Joining (Saitou and Nei, 1987) and Maximum Likelihood (ML) methods (Felsenstein, 1981). All positions that contained gaps or missing data were eliminated from the final dataset, resulting in 1911 bp positions of *gyrB* sequence. We used 729,383 bp of DNA to represent the conserved core genome found across 25 strains of the *B. subtilis* group, to generate a phylogenomic tree using RAxML (v 7.2.7) (Pfeiffer and Stamatakis, 2010). The phylogenomic tree was then visualized with iTOL (http://itol.embl.de) (Letunic and Bork, 2011).

BLAST Matrix

The BLAST matrix algorithm was used for pairwise comparison of *Bacillus* PGPR strain proteomes, using methods described previously (Friis et al., 2010). The BLAST matrix determines the average percent similarity between proteomes by measuring the ratio of conserved gene families shared between strains to the total number of gene families within each strain. The absolute number of shared and combined gene families for each strain was displayed in matrix output. This matrix shows the number of proteins shared between each proteome.

Core-genome Analysis

The core-genome of 13 *Bacillus* spp. strains was generated using coding and non-coding sequences. Whole genome sequences from these strains were aligned using progressive Mauve (Darling et al., 2004), which identifies and aligns locally collinear blocks (LCBs) in the XMFA format. LCBs from alignments were collected using stripSubsetLCBs (http://gel.ahabs.wisc.edu/mauve/snapshots/), using minimum lengths of 500 bp. All LCBs were concatenated and converted to multifasta format using a perl script. The same protocol was used to obtain all core sequences, with the exception that the minimum lengths of LCBs were 50 bp, instead of 500 bp. The *Bacillus* spp. core genome was obtained from the comparative alignment of all complete *Bacillus* spp. genomes available in the GenBank as of August 2014 ($n = 81$ genomes). The core genome of the *B. subtilis* group was obtained from comparative analysis of 53 whole genomes of *B. subtilis* strains that included 41 genomes obtained from GenBank and 12 PGPR genomes sequenced in this study. *B. amyloliquefaciens* species-level and *B. amyloliquefaciens* subsp. *plantarum*-level core genomes were generated from 32 *B. amyloliquefaciens* and 28 subsp. *plantarum* genomes. Core genomes were exported to the CLC Genomics Workbench (v 4.9) for evaluation of alignments and annotation using the

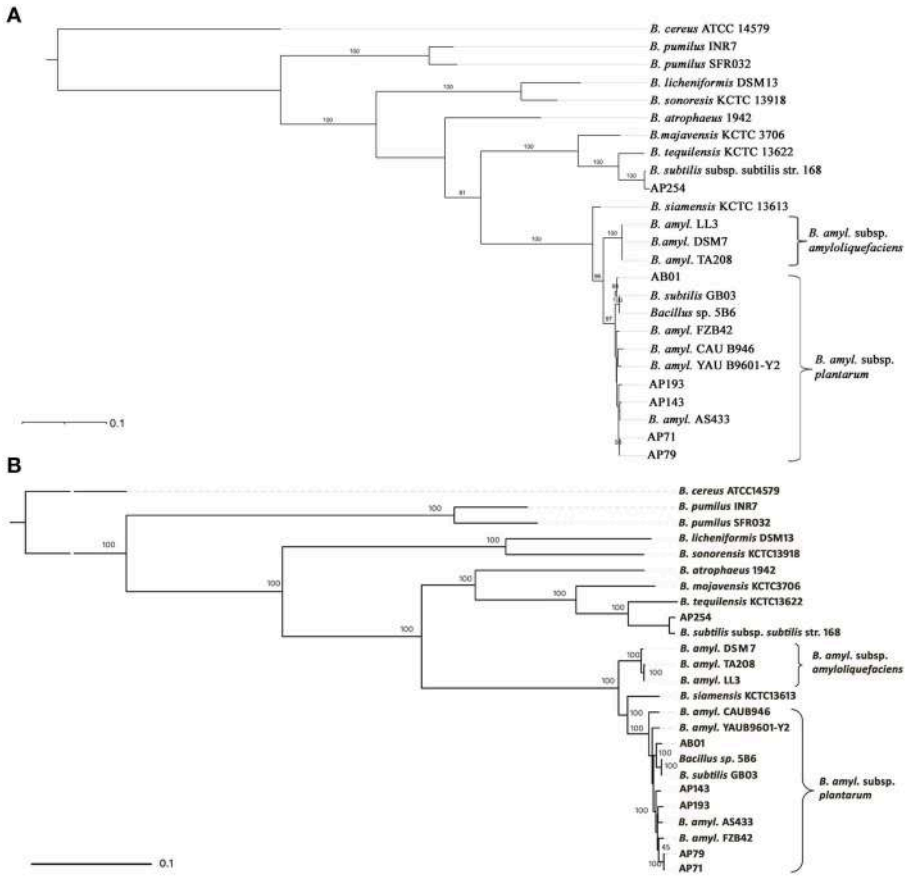

FIGURE 1 | Phylogeny of PGPR *Bacillus* spp. evaluated in this study. (A) Neighbor joining phylogenetic tree based on *gyrB* sequences using *B. cereus* ATCC 14579^T as an outgroup. **(B)** Maximum-likelihood phylogenetic tree of the 25 *B. subtilis* group strains based on 729,383 bp sequence of core genome. Two clusters belonging to *B. amyloliquefaciens* subsp. *plantarum* and *B. amyloliquefaciens* subsp. *amyloliquefaciens* are indicated by brackets.

RAST server (Aziz et al., 2008). The list of *Bacillus* spp. strains used for core genome determination is provided in **Supplemental Table 1**. Additionally, to identify PGPR-specific core genes, raw sequence reads of PGPR strains sequenced in this study were sequentially reference mapped against the genome sequence of non-PGPR strain *B. subtilis* subsp. *subtilis* str. 168 according to methods described previously (Hossain et al., 2013).

Identification of Core Genes Uniquely Present in *B. amyloliquefaciens* subsp. *Plantarum* Strains

The aligned genome sequences of 32 *B. amyloliquefaciens* strains and 28 *B. amyloliquefaciens* subsp. *plantarum* strains (which were included within the *B. amyloliquefaciens* strains) were analyzed using CLC Genomics Workbench to obtain the respective species- and subsp.-level core genomes. Trimmed sequence reads of subsp. *plantarum* strain AP193 were reference mapped against the subsp. *plantarum* core genome to obtain core genome-specific sequence reads. The parameters of reference mapping were as follows: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.5, and similarity = 0.8. Sequence reads mapped to the subsp. *plantarum* core genome were then mapped against the species *amyloliquefaciens* core genome to obtain unmapped sequence reads. These unmapped sequence reads, represent the subsp. *plantarum* core genome that is absent in the *amyloliquefaciens* species-level core genome, were assembled *de novo* using CLC Genomics Workbench then the resulting contigs were uploaded to RAST for gene prediction and annotation. Each ORF, exclusively encoded by the *plantarum* core genome, was further confirmed for uniqueness using BLASTn analysis against the genome sequences of 28 *B. amyloliquefaciens* subsp. *plantarum* and four *B. amyloliquefaciens* subsp. *amyloliquefaciens* strains listed in **Supplementary Table 1**.

Prediction of Secondary Metabolite Biosynthesis Gene Clusters in PGPR Strain AP193

Secondary metabolite biosynthesis gene clusters for strain AP193 were predicted using the secondary metabolite identification tool antiSMASH (Blin et al., 2013). Primer-walking PCR was used to fill gaps between contigs containing gene clusters encoding secondary metabolite biosynthesis. Gene prediction and annotation were carried out by GeneMark

(Lukashin and Borodovsky, 1998) and BLASTx (NCBI), respectively.

DNA Manipulation and Plasmid Construction for PGPR Strain AP193 Mutagenesis

Chromosomal DNA was isolated with the E.Z.N.A. Bacterial DNA Isolation Kit (Omega Biotek, Atlanta, GA) and plasmids were isolated with the E.Z.N.A. Plasmids Mini Kit II (Omega Biotek). Primers used in this study are listed in **Table 2**. Gene deletion constructs were assembled using splicing through overlap extension PCR (Horton et al., 1989). The assembled products were gel purified with Gel/PCR DNA Fragments Extraction Kit (IBI), digested with appropriate restriction enzymes, and cloned into a pNZT1 vector to construct the delivery plasmids for gene replacement.

In vitro Plasmid Methylation Using Cell Free Extract of *Bacillus Amyloliquefaciens* subsp. *Plantarum* AP193

To methylate plasmids prior to transformation into *B. amyloliquefaciens* subsp. *plantarum* AP193, the method developed for *Lactobacillus plantarum* was used with minor modifications (Alegre et al., 2004). Cells from a 100 ml overnight culture of strain AP193 ($OD_{600} = 1.3 - 1.5$) were pelleted by centrifugation (8000 × g), washed with 100 ml of chilled PENP buffer (10 mM potassium phosphate, 10 mM EDTA, 50 mM NaCl and 0.2 mM PMSF, pH 7.0), and then re-suspended to a final volume of 4 ml. Cells were disrupted by performing two bursts (amplitude 50, pulse 3, and watts 25–30) for 5 min each with a pause of 2 min, using a Vibra-Cell sonicator, and cooled with ice to prevent overheating. Cell debris was removed by centrifugation (8000 × g) at 4°C and the extract was collected through decanting. Three milliliter aliquots of extract were mixed with 3 ml of glycerol (100% v/v) and 0.6 ml of bovine serum albumin (1 mg/ml), then stored at −20°C.

The DNA modification assay was performed in a final volume of 100 µl of the following: 53 µl TNE buffer [50 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA], 10 µl S-adenosylmethionine (0.8 mM), 2 µl BSA (5 mg/ml), 25 µl cell free extract derived from strain AP193 and 10 µl plasmid DNA extracted from *E. coli* K12 ER2925 (0.5–1 µg/µl). The mixture was incubated at 37°C for 16 h. Methylated DNA was extracted with a DNA Clean & Concentrator Kit (Zymo Research, CA), then re-suspended in water and stored at −20°C.

TABLE 2 | Primers used in this study.

Name	Sequence (5′ to 3′)
HindIII*sfpLL*	ATCAAAGCTTATACGCTGCTTCTGCCTGAT
SfpLR	CAGATCCGCGATGTGTTCTT
SfpRL	AAGAACACATCGCGGATCTGCGGTCCATATATACTCCGT
PstI*sfpRR*	ATCCTGCAGTGGCGGTTATGCTACAATGA
SfpUp	CGCTTTAACACACGGACTGA
SfpDn	TTTGTAGGAGCGGGAGAAGA
SfpDL	AAAGAGAGGAATCGGGACGA
SfpDR	TGTTTTGACGGGGCTGAT
HindIII*SrfLL*	ATCCAAGCTTATATGTACGGTCCGTCGGAA
SrfLR	GTTCCATTTGCAGCACTTCA
SrfRL	TGAAGTGCTGCAAATGGAACACTGGTCAAGCTGGCTGAAC
PstI*SrfRR*	ATCCCTGCAGGGTGCTTCAGCTCAATTCCT
SrfUp	GCGAAAGAGCGTCTGTAGAA
SrfDn	AGCCGTCATTGTCAGGTCAA
SrfDL	TCGGTCACAGGGAAATCTCT
SrfDR	CTGCTTGCGGTACTGCTCT
XhoI*DifLL*	TCAACTCGAGGGCGATTCTCGGTTTATCTC
DifLR	GATGGAGGATGCCGGTTAC
DifRL	GTAACCGGCATCCTCCATCCAAGAACGCTTTCGGGATT
SpeI*DifRR*	ATCCACTAGTGCCATATCAGATACCGCAGA
DifUp	TGGCTGATAAGCACCTACGA
DifDn	AAATCCGATTACAGGCGAGA
DifDL	ATAAGAAACCCGGTTCGGA
DifDR	TGGCGTGACGTCTCTCATC

Restriction digestion sites are underlined within the sequence of the primers.

Electrotransformation of *B. amyloliquefaciens* subsp. *Plantarum* AP193

For preparation of electrocompetent cells, strain AP193 was grown overnight in TSB, then diluted 100-fold in NCM to inoculate a subculture. The culture was grown at 37°C on a rotary shaker until the OD_{600} reached 0.7. The cell culture was cooled on ice for 15 min and subjected to centrifugation at 8000 × g for 5 min at 4°C. After washing four times with ice cold ETM buffer (0.5 M sorbitol, 0.5 M mannitol, and 10% glycerol), electrocompetent cells were re-suspended in 1/100 volume of the original culture (Zhang et al., 2011). For electroporation, 100 µl of cells were mixed with 100 ng of plasmid DNA in an ice-cold electroporation cuvette (1 mm electrode gap). Cells were exposed to a single 21 kV/cm pulse generated by Gene-Pulser (Bio-Rad Laboratories) with the resistance and capacitance set as 200 Ω and 3 µF, respectively. The cells were immediately diluted into 1 ml of recovery medium (NCM plus 0.38 M mannitol) (Zhang et al., 2011) and shaken gently at 30 or 37°C for 3 h to allow expression of the antibiotic resistance genes. Aliquots of the recovery culture were then spread onto LB agar supplemented with appropriate antibiotics.

Two-step Replacement Recombination Procedure for the Modification of the Strain AP193 Genome

A two-step replacement recombinationwas performed as previously described, with minor modifications (Zakataeva et al., 2010). To integrate the plasmid into AP193's chromosome, a single crossover between the target gene and the homologous sequence on the plasmid must occur. To do this, AP193 that contained a delivery plasmid with thedeletion construct was first grown in LB broth for 24 h at 37°C (a non-permissive temperature for plasmid replication). Next, the culture was serially diluted, plated onto LB agar plates with erythromycin, and incubated at 37°C. Clones were screened by colony PCR using two sets of primers. Each set of primers anneals sequences specific to one of the homologous fragments and to

the chromosomal region just outside of the other homologous fragment (**Table 2**). If PCR products had a reduced size, relative to the wild-type genotype for either primer set, this indicated successful chromosomal integration of the plasmid. In the second step, clones of the integrant were cultured with aeration in LB at 30°C for 24–48 h to initiate the second single-crossover event, resulting in excision of the plasmid, yielding erythromycin sensitive (EmS) clones with either a parental or a mutant allele on the chromosome. Colony PCR was used to examine the presence of desired mutations by primer sets that flank the deleted sequence (**Table 2**).

Construction of Strain AP193 Mutants Defective in Secondary Metabolite Biosynthesis

All mutant strains generated in this study are indicated in **Table 1**. The disruption of the *dfnD* gene was achieved as follows: DNA fragments corresponding to positions −867 to +247 and +643 to +1570 with respect to the *dfnD* translation initiation site were PCR amplified using AP193 genomic DNA as a template. The two fragments were then assembled by fusion PCR. A frameshift mutation was introduced during fusion to ensure complete disruption of the gene. The deletion construct was digested with XhoI and SpeI, then cloned into pNZT1, yielding pNZ-dif. The plasmid was methylated *in vitro* as described above and introduced into strain AP193 by electroporation. Once introduced into strain AP193, plasmid pNZ-dif generated the isogenic mutant AP193Δ*dfnD* by two-step replacement recombination.

To generate the *sfp* deletion mutant, DNA fragments corresponding to positions −781 to +29, with respect to the *sfp* translation initiation site, and +95 to + 935, with respect to the *sfp* translation termination site, were PCR amplified using AP193 genomic DNA as template, assembled by fusion PCR, digested with HindIII and PstI, and cloned into pNZT1 to construct pNZ-sfp. The plasmid pNZ-sfp was used to generate mutant AP193Δ*sfp* using procedures described above.

The Δ*srfAA* mutant was obtained as follows: DNA fragments corresponding to positions +5375 to +6091 and +6627 to +7366, with respect to the *srfAA* translation initiation site, were PCR-amplified, fused by fusion PCR, digested with HindIII and PstI and cloned into pNZT1 as pNZ-srf. Similarly, a frameshift mutation was introduced during the fusion of the upstream and downstream fragments of the target deletion sequence to ensure complete disruption of the gene. The plasmid pNZ-srf was used to generate mutant AP193Δ*srfAA* using procedures described above.

In vitro Antimicrobial Activities of PGPR Strain AP193 and its Mutants against Plant Pathogens

Plant pathogens *Pseudomonas syringe* pv. tabaci, *Rhizobium radiobacter*, *Xanthomonas axonopodis* pv. vesicatoria, and *Xanthomonas axonopodis* pv. campestris were grown in TSB until the OD_{600} reached 1.0. The wild type strain AP193, as well as the three isogenic mutants Δ*dfnD*, Δ*sfp*, and Δ*srfAA* developed in this study, were grown at 30°C in TSB for 48 h at 220 rpm. Cultures were then centrifuged at 10,000 × g for 2 min then supernatant was passed through a 0.2 μm nylon filter (VWR, PA). For antibiosis assays, 100 μl of an overnight culture for each plant pathogen was spread onto TSA plates (Thermo Scientific, NY) separately then sterile cork borers (10 mm diameter) were used to bore wells in agar plates. Filtered supernatant of AP193 and its three mutants were separately added to fill wells. Plates were allowed to dry and then incubated at 30°C overnight. Zones of inhibition were measured and compared between mutants and wild-type strain AP193 to determine their antimicrobial activities against plant pathogens.

LC-MS Analysis of Bacterial Supernatants

Bacterial cultures were grown in 2 ml TSB for 72 hours and then cells were removed by centrifugation at 10,000 × g for 10 min, followed by 0.2 μm filtration of the culture supernatant. Samples were analyzed by direct injection from *m/z* 50 to 1200 on a ultra-high pressure liquid chromatography/QTof-mass spectrometer (Waters Acquity UPLC and Q-Tof Premier, Milford, MA) operated at a spray voltage of 3.03 kv and the source temperature of 100°C. The MS analysis was conducted in negative ion mode with a mobile phase of 95% acetonitrile, 5% water, and 0.1% formic acid.

In vivo Antibiosis of Strain AP193 and its Mutants against a Plant Pathogen

Rutgers tomato seeds (Park Seed, USA) were sown in Styrofoam trays. Three weeks after planting, seedlings were transplanted into a 4.5 inch square pot with commercial potting substrate (Sunshine mix, Sun Gro Horticulture, Agawam, Maine). Three days after transplanting, plants were sprayed with sterile water or PGPR cell suspensions (10^6 CFU/ml) that had been washed three times prior to being resuspended in sterile water and normalized at an $OD_{600} = 1.0$ before being serially diluted. PGPR-inoculated plants were placed into a dew chamber at 100% humidity in the dark for 2 days at 24°C then transferred to the greenhouse. One day later, plants were challenge-inoculated with *X. axonopodis* pv. vesicatoria by spraying approximately 10 ml of a 10^7 CFU/ml pathogen suspension over each plant. Pathogen-inoculated plants were placed in the dew chamber for 2 days then placed in the greenhouse. Plants were watered once daily. Disease severity ratings and harvest were conducted after 14 days of challenge-inoculation. For disease severity rating, four compound leafs were selected from the bottom of each plant. The disease severity of each of the compound leaves was determined by rating the disease severity of each leaflet and calculating the average rating for the compound leaf. Leaflets were rated using a 0–4 rating scale, where 0 = healthy leaflet, 1 =< 20% necrotic area of the leaflet, 2 = 20–50% necrotic area of the leaflet, 3 = 51–80% necrotic area of the leaflet, 4 = 80–100% necrotic area of the leaflet. In addition, dry shoot and root weights were determined. The experimental design was a randomized complete block with 10 replications per treatment. The experiment was conducted twice.

Data Analysis

All data were analyzed by an analysis of variance (ANOVA), and the treatment means were separated by using Fisher's protected least significant difference (LSD) test at $P = 0.05$ using SAS 9.3 (SAS Institute, Gary, NC, USA).

Results

Genome Statistics and Genetic Relatedness of *Bacillus* Species

Genome sequences of 12 different PGPR *Bacillus* spp. strains were determined using next-generation sequencing. The summary statistics for each *Bacillus* spp. genome sequences and their assemblies are presented in **Table 3**. The approximate sizes of *Bacillus* spp. genomes ranged from 2.95 to 4.43 Mbp with an average genome size of 3.93 Mbp, which is similar to the 4.09 Mbp average genome size of complete *B. subtilis* genomes available in GenBank (April, 2015). The percent G+C content of the 12 PGPR *Bacillus* spp. strains ranged from 41.3–46.6%, averaging 45.15%, which is similar to the average percent G+C content of the *B. subtilis* genome sequences available in GenBank (43.72%) (March, 2015). Pairwise average nucleotide identities (ANI), a newly proposed standard for species definition in prokaryotes (Richter and Rosselló-Móra, 2009), were calculated for 13 *Bacillus* PGPR strains to determine their interspecies relatedness among *Bacillus* species. The ANI values for PGPR *Bacillus* spp. strains AB01, AP71, AP79, AP143, AP193, and GB03 against *B. amyloliquefaciens* FZB42 (Chen et al., 2007) were greater than 98% (data not shown), indicating that these PGPR strains are affiliated with the *B. amyloliquefaciens* species. The 98.88% ANI of PGPR strain AP254 to *B. subtilis* subsp. *subtilis* strain 168 suggests that AP254 is affiliated with *B. subtilis* (data not shown). The pairwise ANI comparison of PGPR strains INR7, KCTC 3706T, KCTC 13613T, KCTC 13918T, and KCTC 13622T against each other produce ANI values less than 95% (data not shown) suggests that they are distantly related to each other and represent diverse *Bacillus* species.

Phylogenetic Relationship of *Bacillus* Strains

A phylogenetic analysis based on *gyrB* gene sequences showed sufficient resolution among *Bacillus* taxa and was consistent with ANI comparisons. Strains AP71, AP79, AP143, AP193, AB01, and GB03 were grouped together with reference strains of *B. amyloliquefaciens* subsp. *plantarum* with high bootstrap support, indicating that they are affiliated with subsp. *plantarum*. The three strains of *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7, TA208, and LL3 clustered as a single clade, separated from strains of subsp. *plantarum*, supporting the division of two subspecies in *B. amyloliquefaciens* (Borriss et al., 2011). The placement of strain AP254 with *B. subtilis* subsp. *subtilis* strain 168 as a single clade with strong bootstrap support suggests its affiliation with members of the *B. subtilis* group (**Figure 1A**). A *gyrB* gene based phylogenetic tree constructed using Maximum Likelihood (ML) methods was also concordant with the phylogeny constructed using Neighbor-Joining methods (data not shown). In addition to the *gyrB*-based phylogeny, we constructed a phylogenomic tree using 729,383 bp of core genome sequences present within the genome of 25 *B. subtilis* group isolates to provide a more refined phylogenetic placement of PGPR strains. The topology and allocation of strains to clades in the *gyrB* phylogeny was similar to the phylogenomic tree (**Figure 1B**). One notable difference is that the topology of the tree regarding the position of strain *B. siamensis* KCTC13613 differs significantly between the *gyrB*-based tree and the phylogenomic tree, with the *gyrB* based phylogeny placing KCTC13613 in a separate clade whereas the phylogenomic tree included it within a monophyletic group that includes strains of *B. amyloliquefaciens* subsp. *plantarum.*

BLAST Matrix

Genome wide proteome comparisons of 13 PGPR *Bacillus* strains using an all-against-all BLASTp approach demonstrated that PGPR *Bacillus* spp. strains are highly diverse, as indicated by gene family similarity between PGPR *Bacillus* spp. genomes ranging from 32-90% (**Supplemental Figure 1**). Consistent with the phylogenetic analysis, high similarity was found among strains AP71, AP79, AP193, AB01, GB03, and FZB42, with proteomic similarity ranging from 70 to 90%.

Core-genome Analysis

Analysis of genome sequence alignment using progressive Mauve determined that the core genome of 13 PGPR *Bacillus* spp. strains

TABLE 3 | Summary of draft genomes of *Bacillus* species sequenced used in this study.

Isolates	Number of contigs (>1 kb)	Size (total bp in assembly)	%G+C	NCBI BioProject number	NCBI short read archive accession no.	Approx. sequence coverage (×)	Number of predicted ORFs
AB01	20	3,903,296	46.4	PRJNA239317	SRX475739	44	3944
AP71	198	4,278,192	45.7	PRJNA239317	SRX475740	15	4531
AP79	47	4,236,770	45.8	PRJNA239317	SRX475741	31	4368
AP143	146	2,956,670	46.6	PRJNA239317	SRX475742	24	3324
AP193	152	4,121,826	46.3	PRJNA239317	SRX475807	37	4159
AP254	59	4,048,419	43.8	PRJNA239317	SRX475808	29	4717
GB03	26	3,849,547	46.5	PRJNA227787	SRX380920	560	3928
INR7	44	3,681,709	41.3	PRJNA227786	SRX447924	750	3857
KCTC 3706T	17	3,935,582	43.7	PRJNA227789	SRX447926	895	4140
KCTC 13613T	23	3,779,696	46.3	PRJNA161489	SRX450083	500	3915
KCTC 13918T	32	4,428,962	45.5	PRJNA227788	SRX450084	1000	4704
KCTC 13622T	33	3,981,302	43.9	PRJNA227791	SRX450086	1000	4299

contains 1,407,980 bp of genomic DNA which encode 1454 ORFs (data not shown). Comparison of core genome sequences of the genus *Bacillus*, subgroup *B. subtilis*, species *B. amyloliquefaciens*, and subspecies *plantarum* demonstrated that as the number of genomes increases, the number of different subsystems within each respective core genome decreases (**Figures 2A–C**). The highest numbers of subsystems in each of the core genome categories, except for the genus *Bacillus* core genome, was devoted to carbohydrate metabolism. These findings suggest that strains from the genus *Bacillus* use diverse carbon sources. In addition, the core genome for the genus *Bacillus* has more subsystems devoted to RNA, DNA, and protein metabolism compared to carbohydrate metabolism (**Figures 2A–C**).

The genome alignment from 28 different subsp. *plantarum* strains, including six subsp. *plantarum* strains sequenced in this study, identified 2,550,854 bp of core genome sequence that is predicted to encode 2839 ORFs (**Supplemental Table 2**). The genome alignment of 32 *B. amyloliquefaciens* strains, including 28 subsp. *plantarum* strains, identified 2,418,042 bp of core genome sequence predicted to encode 2773 ORFs (**Supplemental Table 3**).

The genome alignment of 53 strains of *B. subtilis* group, including the 12 strains sequenced in this study, identified 578,872 bp of core genome sequence predicted to encode 674 ORFs (**Supplemental Table 4**). The number of protein coding genes present within the genome of *Bacillus* spp. (∼4000) and the low number of ORFs (674) encoded by their core genomes suggests a large amount of genomic plasticity among *Bacillus* genomes that experience frequent gene acquisitions and losses. It was observed that the *B. amyloliquefaciens* core genome was devoid of mobile genetic elements, such as prophages, transposable elements, and plasmids (data not shown). Furthermore, the *B. subtilis* core genome was also devoid of genes or genetic clusters linked with iron acquisition and metabolism, secondary metabolite biosynthesis, signal transduction and phosphorus metabolism (**Figures 2A–C**).

In this study, the genus *Bacillus* core genome was also determined by analyzing all complete genome sequences from the genus *Bacillus* currently available in GenBank. Our study determined that the genus *Bacillus* contains 194,686 bp of core sequence predicted to encode 201 different ORFs (**Supplemental Table 5**). The predicted functions present in all *Bacillus* strains are limited to the following subsystem features: cofactor synthesis, vitamin synthesis, prosthetic groups and pigments biogenesis, cell wall and capsule biogenesis, membrane transport, RNA metabolism, nucleoside metabolism, protein metabolism, regulation and cell signaling, DNA metabolism, respiration, amino acids and derivatives, sulfur metabolism, and carbohydrate utilization.

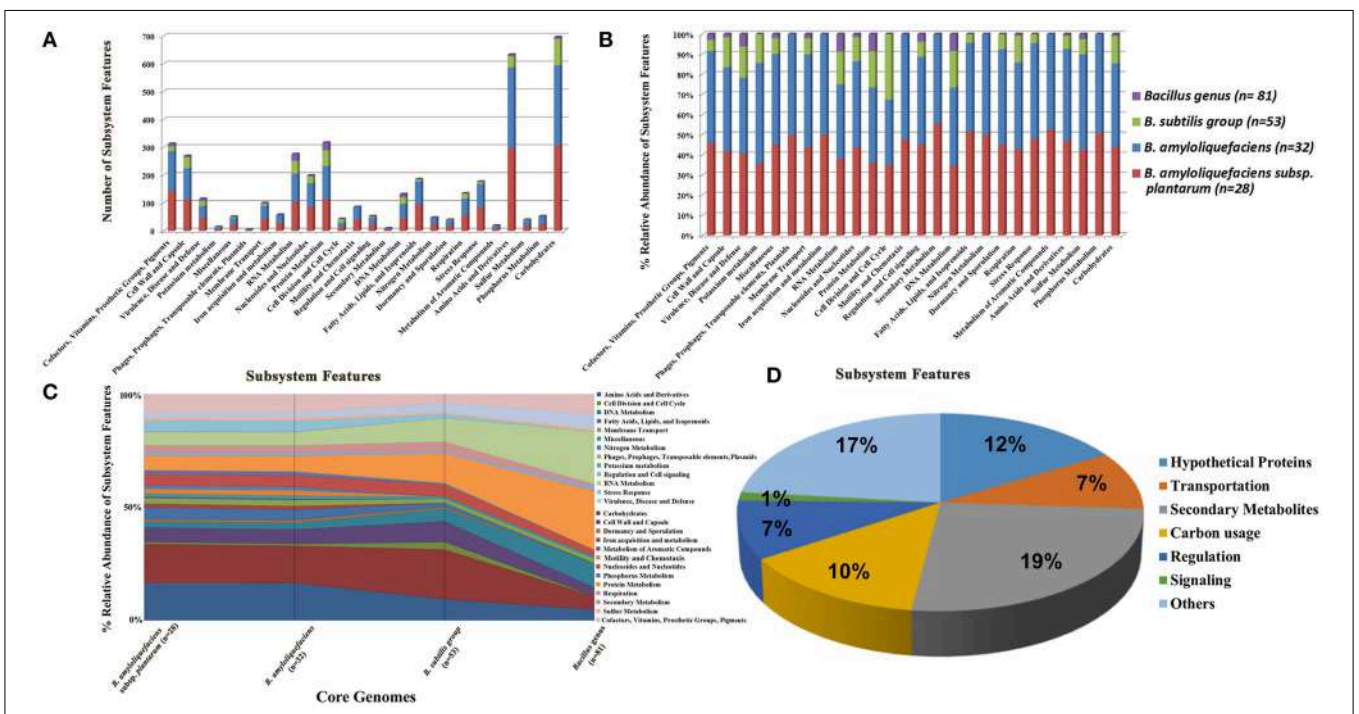

FIGURE 2 | The distribution of different subsystem categories of four different core genomes specific to genus *Bacillus* (*n* = 81), *B. subtilis* subgroup (*n* = 53), species *B. amyloliquefaciens* (*n* = 32) and subsp. *plantarum* (*n* = 28). (A) The total counts for genes within different subsystem categories for each of the core genomes. **(B)** The % relative abundance of the genes within different subsystem categories for each of the core genomes. **(C)** Represents the change in % relative abundance of subsystem categories as the number of genomes is reduced after inclusion of additional genomes for core genome prediction. **(D)** Categories of functions encoded by the 73 *B. amyloliquefaciens* subsp. *plantarum*-specific genes present in the *B. amyloliquefaciens* subsp. *plantarum* core genome but absent in the *B. amyloliquefaciens* species-level core genome. The number beside each subgroup of the pie figure represents the number of genes encoding the function.

Comparative Analysis of Core Genes Uniquely Present in *B. amyloliquefaciens* subsp. *Plantarum*

Comparison of PGPR-specific genomes with that of non-PGPR *B. subtilis* subsp. *subtilis* str. 168 did not identify any genes other than essential housekeeping genes that were conserved within the genomes of PGPR strains (data not shown). Comparative analysis of core genomes from 28 *B. amyloliquefaciens* subsp. *plantarum* and 32 *B. amyloliquefaciens* species identified 193,952 bp of sequences that are present within the subsp. *plantarum* core genome but absent in the *B. amyloliquefaciens* core genome. Among these genetic loci there were 73 genes shared by all 28 *plantarum* strains but were not present in any strains of subsp. *amyloliquefaciens* (**Supplemental Table 6**). The putative functions of these genes includes transportation (7 genes), regulation (7 genes), signaling (1 gene), carbon degradation (10 genes), synthesis of secondary metabolites (19 genes), and hypothetical proteins (12 genes) (**Figure 2D**). Some of these gene products may be involved in interactions with plants and rhizosphere competence of subsp. *plantarum* strains (e.g., pectin utilization). For instance, genes required for uptake and use of D-galacturonate and D-glucuronate are shared among genomes of *B. amyloliquefaciens* subsp. *plantarum* strains. In addition, genes required for biosynthesis of the polyketides difficidin and macrolactin were consistently found in PGPR subsp. *plantarum* strains, suggesting their relevance in the biocontrol activities of these strains.

Gene Clusters Encoding Secondary Metabolite Biosynthesis and Natural Competency in Strain AP193

Due to our observations of beneficial interactions between PGPR strain AP193 and both plant and animal hosts (Ran et al., 2012), we selected this strain for more intensive genome analysis. Assembly of strain AP193 genome sequences *de novo* resulted in 152 contigs larger than 1 kb, with a combined length of 4,121,826 bp. Analysis of AP193 contig sequences, using the antiSMASH secondary metabolite prediction program, suggests that gene clusters were present that are responsible for synthesis of three different polyketides: bacillaene, macrolactin and difficidin. In order to provide complete sequences for these biosynthesis pathways, the gaps between contigs 5 and 6, contigs 33 and 38, as well as contigs 27 and 28 were filled using PCR, followed by DNA sequencing. Each of the gene clusters in AP193 are collinear to their counterparts in *B. amyloliquefaciens* FZB42; a naturally competent plant root-colonizing *B. amyloliquefaciens* isolate with the ability to promote plant growth and suppress plant pathogens (Chen et al., 2007). The percent amino acid identities of the proteins encoded by those clusters were within the range of 98–100% when compared with those of FZB42. Secondary metabolite biosynthesis gene clusters involved in non-ribosomal synthesis of cyclic lipopeptides surfactins, fengycin and bacillomycin D and of the antimicrobial dipeptide bacilysin present in FZB42 were also detected in the AP193 genome. The percent amino acid identities of the AP193 proteins encoded on those clusters to the FZB42 homologs ranged from 98 to 100%. The lack of natural competency of the PGPR strain AP193 prompted us to determine the presence of competence-related genes within this strain. We searched the AP193 genome sequences for the presence of competence related genes found within the genome of FZB42, and observed that all of the genes required for encoding the structural components of the competence system found in strain FZB42 are present within the genome of AP193 with 98 to 100% identity (data not shown); however, genes *comQ*, *comX*, and *comP* are involved in regulating quorum-sensing in *B. amyloliquefaciens* FZB42 (Chen et al., 2007) were absent within the genome of strain AP193 (data not shown). The absence of *comQ*, *comX*, and *comP* may be responsible for the lack of natural competency for strain AP193.

AP193 Secondary Metabolites Inhibit the Growth of Multiple Bacterial Plant Pathogens *In vitro*

Antimicrobial activities of strain AP193 and its mutants AP193Δ*dfnD* (deficient in the production of difficidin), AP193Δ*srfAA* (deficient in surfactin production), and AP193Δ*sfp* (unable to produce polyketide or lipopepetide due to a deletion of *sfp* gene encoding 4′-phosphopantetheinyl transferase) were tested against plant pathogens *Pseudomonas syringe* pv. tabaci, *Rhizobium radiobacter*, *Xanthomonas axonopodis* pv. vesicatoria, and *Xanthomonas axonopodis* pv. campestris. The AP193 wild type strain demonstrated strong antimicrobial activity, whereas the AP193Δ*sfp* mutant was devoid of an inhibitory effect against those plant pathogens (**Figure 3**), underlining the contribution of lipopeptides and/or polyketides in the bioactivity of AP193. This also indicates that the dipeptide bacilysin, whose synthesis is independent of Sfp, was not involved in antagonistic activity expressed *in vitro*. The AP193Δ*srfAA* mutant conferred antimicrobial activity similar to wild-type to *P. syringe* pv. tabaci, *R. radiobacter*, *X. axonopodis* pv. vesicatoria, and *X. axonopodis* pv. campestris (**Figure 3**), suggesting that surfactin has no putative role in the antibacterial activity of AP193 against those plant pathogens under the conditions tested in this study. These findings also demonstrated that surfactin neither influences the antimicrobial compound biosynthesis in AP193 nor does it inhibit antibacterial activities of the antibacterial compounds produced by AP193. Difficidin acts as the major antibiotic in antagonism of AP193 against plant pathogens *P. syringe* pv. tabaci, *R. radiobacter*, *X. axonopodis* pv. vesicatoria, and *X. axonopodis* pv. campestris as indicated by the lack of the inhibitory effect of the AP193Δ*dfnD* mutant against those plant pathogens (**Figure 3**).

We further confirmed that the AP193Δ*dfnD* and Δ*sfp* mutants lacked synthesis of difficidin by conducting LC-MS analysis of the cell-free TSB culture supernatants from wild-type AP193 and each of these mutants. As reported previously, only the deprotonated form of oxydifficidin was detectable in bacterial supernatants using MS in the negative mode ($[M - H]^- = 559.3$) (Chen et al., 2006), with a molecular mass of 559.3 detected in supernatants of the wild-type AP193 culture but not observed from the culture of the Δ*dfnD* mutant (**Figure 4**) or from the Δ*sfp* mutant (data not shown). The Δ*srfAA* mutant exhibited difficidin synthesis as in the wild-type AP193 culture (data not

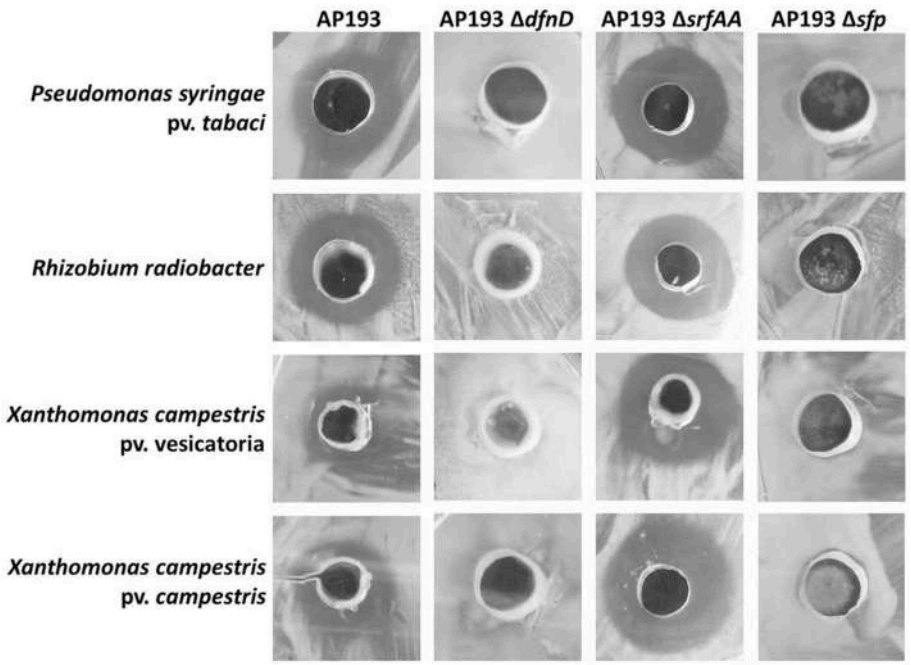

FIGURE 3 | Antimicrobial activities of *Bacillus* sp. AP193 and its mutants Δ*srfAA*, defective in surfactin expression, Δ*dfnD,* defective in difficidin expression, and Δ*sfp,* defective in the expression of multiple secondary metabolites (including difficidin) against plant pathogens *Pseudomonas syringe* pv. tabaci, *Rhizobium radiobacter*, *Xanthomonas axonopodis* pv. vesicatoria and *Xanthomonas axonopodis* pv. campestris as demonstrated with an agar diffusion assay.

shown). These findings demonstrate the importance of difficidin in the biocontrol activity of subsp. *plantarum* strains against plant pathogens.

Strain AP193 Secondary Metabolites Control Bacterial Spot Caused by *X. axonopodis* pv. Vesicatoria in Tomato Plants

To determine the role of bioactive compounds produced by strain AP193 in providing protection against plant diseases, the AP193 wild-type strain and its AP193Δ*dfnD*, AP193Δ*sfp*, and AP193Δ*srfAA* mutants were applied to tomato plants several days before those plants were subsequently inoculated with plant pathogen *X. axonopodis* pv. vesicatoria. Both AP193 wild-type and AP193Δ*srfAA* significantly ($P < 0.05$) reduced disease severity of bacterial spot on tomato plants compared to the disease control (**Table 4**). Additionally, the application of strain AP193 significantly increased the root dry weight of the plants (**Table 4**). Unlike AP193 wild-type and its AP193Δ*srfAA* mutant, strains AP193Δ*sfp* and AP193Δ*dfnD* neither protected tomato plants from severe bacterial spot caused by *X. axonopodis* pv. vesicatoria nor improved plant growth (**Table 4**), further supporting the importance of difficidin for plant disease protection. These findings are in agreement with the *in vitro* antibiosis pattern of AP193 wild-type strain and its AP193Δ*dfnD*, AP193Δ*sfp*, and AP193Δ*srfAA* mutants demonstrated against plant pathogen *X. axonopodis* pv. vesicatoria.

Discussion

PGPR *Bacillus* spp. strains are used worldwide to improve crop yields and to protect against plant diseases. In this study, 12 PGPR genomes were sequenced, including *B. subtilis, B. pumilus*, *B. amyloliquefaciens*, *B. mojavensis*, *B. siamensis*, *B. sonorensis*, and *B. tequilensis*. These data were analyzed using ANI, *gyrB*-based phylogenies and core genome-based phylogenies to resolve taxonomic affiliation of *Bacillus* spp. strains. Our findings demonstrate that half of the strains sequenced in this study are affiliated with *B. amyloliquefaciens* subsp. *plantarum*, including strain GB03 that was formerly designated as *B. subtilis*. Previously, *B. siamensis* type strain KCTC 13613T was proposed as a novel species (Sumpavapol et al., 2010), but a *Bacillus* core genome-based phylogenomic analysis (**Figure 1**) revealed that *B. siamensis* KCTC 13613T is instead affiliated with *B. amyloliquefaciens* subsp. *plantarum*. This finding supports the results of Jeong et al. (2012) that determined the close affiliation of *B. siamensis* type strain KCTC 13613T to *B. amyloliquefaciens* subsp. *plantarum* based on ANI. These findings also support the continued use of core genome-based phylogenomic approaches to provide better phylogenetic resolution than analyses that use a single housekeeping gene (e.g., *gyrB*). Phylogenies based on *gyrB* and core genome sequences demonstrate that *B. amyloliquefaciens* subsp. *plantarum* are highly similar, but comparison of their proteomes demonstrates that they are closely related, yet distinct, and may exert plant growth-promoting activities through different mechanisms.

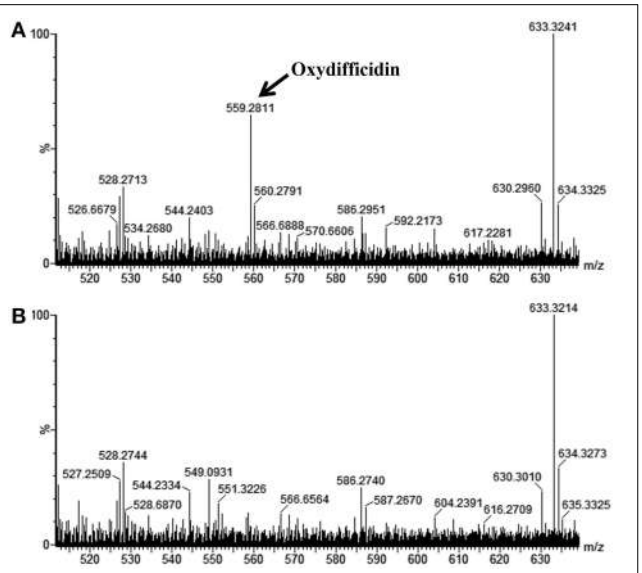

FIGURE 4 | LC-MS spectra for metabolites from cell-free supernatants of (A) wild-type *B. amyloliquefaciens* AP193, and (B) its isogenic *dfnD* mutant, when grown in TSB for 72 h. Note that in negative ion mode that only the deprotonated form of oxydifficidin was detected in bacterial culture supernatants at a *m/z* 559.3.

TABLE 4 | Effects of plant growth-promoting rhizobacteria (PGPR) strains on severity of bacterial spot disease and plant growth.

Strain[ab]	Disease severity[c]	Shoot dry weight (g)	Root dry weight (g)
Disease Control	2.11 a	2.07 bc	0.378 c
AP193	1.30 b	2.18 b	0.453 a
AP193Δ*srfAA*	1.48 b	2.16 b	0.423 abc
AP193Δ*sfp*	2.31 a	2.18 b	0.405 abc
AP193Δ*dif*	2.06 a	2.00 c	0.389 bc
Healthy Control	0.00 c	2.38 a	0.435 ab
LSD	0.35	0.15	0.050

[a] *The experimental design was a randomized complete block with 10 replications per treatment. The experiment was conducted twice. Values followed by the same letter were not significantly different (P = 0.05) according to Fischer's protected LSD.*

[b] *One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/ml) 1 week after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/ml) 3 days after inoculating PGPR.*

[c] *Disease severity ratings and harvest were done 14 days later. For disease severity rating, four compound leafs were selected from the bottom of each plant. The disease severity of each of the compound leaves was determined by rating the disease severity of each leaflet and calculating the average rating for the compound leaf. The leaflet was rated using a 0–4 rating scale, where 0 = healthy leaflet, 1 =< 20% necrotic area of the leaflet, 2 = 20-50% necrotic area of the leaflet, 3 = 51–80% necrotic area of the leaflet, 4 = 80–100% necrotic area of the leaflet, or fully dead leaflet.*

B. amyloliquefaciens subsp. *plantarum* strain AB01 was isolated from the intestine of channel catfish (Ran et al., 2012), but its affiliation with plant-associated strains may suggest transient presence within a fish gastrointestinal tract; however, given that the fish feed is soy-based it is likely that the plant-based diet was also a factor in the growth of this strain within a fish intestine. Similarly, *B. siamensis* type strain KCTC 13613T was found to be closely affiliated with *B. amyloliquefaciens* subsp. *plantarum* and was isolated from salted crab, rather than a plant-associated source. The efficacy of strains AB01, AP193, and other plant-associated strains as probiotics in fish shows the capacity for biocontrol of animal and plant pathogens as well as an overlap in host colonization (Ran et al., 2012).

With rapid advances in sequencing technologies it is now possible to extend genomic analysis beyond individual genomes to analyze core genomes (Medini et al., 2008). In this study, core genomic analyses were conducted on PGPR strains from species affiliated with the *B. subtilis* group. This analysis identified 73 genes exclusively present among all subsp. *plantarum* that are absent in subsp. *amyloliquefaciens* strains. This small number of subsp. *plantarum*-specific genes agrees with a previous report that identified 130 subsp. *plantarum*-specific genes using a limited number of genome sequences from subsp. *plantarum* strains (He et al., 2012). Of these 73 *plantarum*-specific genes identified in this study, many are predicted to be important for plant-associated and soil-associated functions. For example, genes that are required for the use of D-galacturonate and D-glucuronate were found in the pool of *B. amyloliquefaciens* subsp. *plantarum*-specific core genes. This observation is consistent with the absence of these genes in the genome of *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 (Rückert et al., 2011), a strain without any reported PGPR activity. Pectin, a complex polymer found in plant tissues, is broken down to D-glucuronate and D-galacturonate which then serves as a carbon source for bacterial growth (Nemoz et al., 1976). This pectin could potentially serve as a nutrient source for efficient root colonization of PGPR through competitive nutrient uptake. Therefore, the presence of genes that enable D-galacturonate and D-glucuronate utilization could be advantageous for *B. amyloliquefaciens* subsp. *plantarum* for plant growth-promoting activity through efficient root colonization.

Since many of the PGPR strains are from the *B. subtilis* group, the core genome estimation was expanded to include a larger number of *B. subtilis* strains. Increasing the number of *Bacillus subtilis* genomes analyzed to 53 resulted in a 579,166 bp core genome that is predicted to encode 674 ORFs. This smaller number of predicted genes reflects genomic diversity among the *B. subtilis* group. This finding demonstrates that the number of ORFs found in the *B. subtilis* group core genome is close to the number of *B. subtilis* ORFs that are considered as indispensable for growth in complex media (610 ORFs) (http://www.minibacillus.org/project#genes).

To validate a gene's involvement in plant-related processes, it is essential to construct isogenic mutants that are devoid of those genes. Therefore, we deleted genes from PGPR strain AP193 to evaluate the role of secondary metabolite biosynthesis gene clusters in the biological control of plant pathogens. To do this, a methylated shuttle vector pNZT1 (Zakataeva et al., 2010) with gene deletion constructs delivered targeted genetic modifications to AP193, demonstrating the efficacy of *in vitro* methylation of plasmids by cell-free extract in circumventing a restriction system that was presumed to have prevented transformation through electroporation.

Difficidin is a highly unsaturated 22-membered macrocylic polyene lactone phosphate ester with broad-spectrum antibacterial activity (Zimmerman et al., 1987). Difficidin expressed by strain FZB42, together with the dipeptide bacilysin, are antagonistic against *Erwinia amylovora*—the causative agent of fire blight disease in orchard trees (Chen et al., 2009). This study using an isogenic mutant AP193Δ*dfnD* demonstrated for the first time that difficidin solely, not in conjunction with any other polyketides or dipeptides, exerts *in vitro* antibacterial activity against plant pathogens, such as *Pseudomonas syringe* pv. tabaci, *Rhizobium radiobacter*, *Xanthomonas axonopodis* pv. vesicatoria and *Xanthomonas axonopodis* pv. campestris. We also demonstrated, by using isogenic mutant AP193Δ*dfnD*, that difficidin expression is responsible for control of bacterial spot disease in tomato plants caused by *X. axonopodis* pv. vesicatoria. Taken together, these findings demonstrate that difficidin is the most important strain AP193 secondary metabolite for biological control of plant diseases due to bacterial pathogens. In addition, the construction of the *sfp* gene deletion allowed investigation of multiple secondary metabolites produced by AP193 and their individual contributions to biocontrol activity. The *sfp* deletion mutant lost antagonistic activity against each pathogen that was susceptible to the AP193 wild-type strain. Mutants with the *sfp* deletion are expected to lose the ability to synthesize difficidin in addition to other metabolites. Because the lack of antimicrobial activity of AP193Δ*sfp* is consistent with that of the AP193Δ*dfnD* mutant, this therefore suggests that difficidin is the primary metabolite responsible for *in vitro* inhibition of bacterial pathogens. In contrast, the surfactin mutant retained antimicrobial activity against all plant pathogens tested, demonstrating that surfactin is neither critical for *in vitro* antibiotic activity nor influences the synthesis or secretion of other secondary metabolite biosynthesis in this *Bacillus* spp. strain; however, surfactin may influence plant growth promoting activity since it has been observed that surfactin of *B. subtilis* elicits ISR in plants (Ongena et al., 2007) and is expressed in the plant cells colonized by FZB42 (Fan et al., 2011).

By studying the contributions of genetic loci that are conserved among top-performing PGPR strains we continue to uncover the relative contributions of genes in plant colonization, growth promotion, and/or pathogen biocontrol. In particular, future investigation of genes related to the uptake and use of pectin-derived sugars will help determine the relative importance of these genes for colonization of plants and persistence within this microbiome. Comparative genomic analysis of *Bacillus* spp. PGPR strains has led to a better understanding of gene products and provides a foundation to develop application strategies that result in greater plant growth promotion and biocontrol activity.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00631

Supplemental Table 1 | List of *Bacillus* strains used for core genome determination.

Supplemental Table 2 | Nucleotide and protein sequences of annotated ORFs from the core genome of 28 strains of *B. amyloliquefaciens* subsp. *plantarum*.

Supplemental Table 3 | Nucleotide and protein sequences of annotated ORFs from the core genome of 32 strains of *B. amyloliquefaciens.*

Supplemental Table 4 | Nucleotide and protein sequences of annotated ORFs from the core genome of 53 strains from *B. subtilis* group.

Supplemental Table 5 | Nucleotide and protein sequences of annotated ORFs from the core genome of 81 strains of genus *Bacillus*.

Supplemental Table 6 | Nucleotide and protein sequences of annotated 73 ORFs commonly present within the core genome of *B. amyloliquefaciens* subsp. *plantarum*. This dataset is the basis for the protein categorization presented in **Figure 2D**.

Supplemental Figure 1 | BLAST matrix of the 13 PGPR *Bacillus* spp. strains. This matrix reveals the pairwise proteomic similarity among the 13 strains. In addition, this matrix presents the absolute number of gene families preserved between any two strains along with the total number of families between them and is used as a basis for the color intensity. The green color represents the % homology between proteomes, and the red color represents % homology within proteomes.

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Analyzing the antagonistic potential of the lichen microbiome against pathogens by bridging metagenomic with culture studies

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Naturally occurring antagonists toward pathogens play an important role to avoid pathogen outbreaks in ecosystems, and they can be applied as biocontrol agents for crops. Lichens present long-living symbiotic systems continuously exposed to pathogens. To analyze the antagonistic potential in lichens, we studied the bacterial community active against model bacteria and fungi by an integrative approach combining isolate screening, omics techniques, and high resolution mass spectrometry. The highly diverse microbiome of the lung lichen [*Lobaria pulmonaria* (L.) Hoffm.] included an abundant antagonistic community dominated by *Stenotrophomonas, Pseudomonas,* and *Burkholderia.* While antagonists represent 24.5% of the isolates, they were identified with only 7% in the metagenome; which means that they were overrepresented in the culturable fraction. Isolates of the dominant antagonistic genus *Stenotrophomonas* produced spermidine as main bioactive component. Moreover, spermidine-related genes, especially for the transport, were identified in the metagenome. The majority of hits identified belonged to *Alphaproteobacteria*, while *Stenotrophomonas*-specific spermidine synthases were not present in the dataset. Evidence for plant growth promoting effects was found for lichen-associated strains of *Stenotrophomonas.* Linking of metagenomic and culture data was possible but showed partly contradictory results, which required a comparative assessment. However, we have shown that lichens are important reservoirs for antagonistic bacteria, which open broad possibilities for biotechnological applications.

Keywords: lichen, antagonistic bacteria, plant growth promotion, *Stenotrophomonas,* spermidine

Introduction

Plant pathogens and the diseases they cause are major threats to humanity. Each year we globally lose over one third of the total harvest to bacterial and fungal pathogens. The past two decades have seen an increasing number of virulent infectious diseases in plants (Fisher et al., 2012), and human activity is intensifying pathogen dispersal as well as reducing diversity in agricultural systems (Schmid et al., 2011). However, microbial diversity is a key factor in avoiding pathogen outbreaks (Mendes et al., 2012; van Elsas et al., 2012). Therefore, biocontrol of plant pathogens is

a promising solution to control plant pathogens (Berendsen et al., 2012; Berg et al., 2013; Berg, 2015) because it was also shown that it enhances general microbial diversity (Erlacher et al., 2015a). Naturally occurring antagonists toward plant pathogens play an important role for biocontrol approaches. In natural ecosystems, which often contain a high proportion of antagonistic microorganisms, such antagonists potentially function in stabilizing the community, but might also protect the community against pathogen outbreaks (Opelt et al., 2007; Zachow et al., 2008; Grube et al., 2015). However, the ecology of naturally occurring antagonistic microorganisms is only partly understood and not yet exploited.

Lichens, which are classic examples of self-sustained symbioses, are interesting models for antagonism studies because within these mini-ecosystems the cooperation between microbial partners facilitates stability and longevity under extreme ecological conditions although they are often attacked by allochthonous bacteria and fungi (Lawrey and Diederich, 2003; Bates et al., 2011; Mushegian et al., 2011). While the lichen-specific structure is provided by fungal symbionts, which also is the naming component of the symbiosis, green algae, and/or cyanobacteria are incorporated into specific layers or compartments and contribute with photosynthetically fixed carbohydrates to the symbiosis (Nash, 2008). Lichen-associated bacteria were only recently shown to be highly diverse and omics approaches have indicated that they are functional contributors to robustness of the lichen holobiome (Grube et al., 2009, 2015). The intricate association of members of different organismal kingdoms in well-delimited and long-living symbiotic structures – as symbiotic hotspots of terrestrial life – highlights lichens as a veritable treasure chest for interorganismal communication, regulation, and bioactivity in general (Boustie and Grube, 2005; Boustie et al., 2011). Conditioned by the slow growth of many lichens and difficulties in culturing the symbionts, biotechnological exploitation of lichens was lagging behind other natural resources. With the advent of modern technologies, however, the secondary metabolism and antagonistic potentials in lichens receive new impulses, and this will particularly apply to culturable bacterial partners. Although, lichens are equipped with various secondary compounds with antagonistic effects (Oksanen, 2006; Lawrey, 2009; Boustie et al., 2011), we hypothesize that only a diverse protective microbiome can efficiently maintain stability over longer periods to prevent pathogen attacks.

The objective of this study was to analyze the antagonistic potential of the lichen microbiome against model pathogens by a novel approach bridging metagenomic with culture techniques. Model pathogens associated with human, lichen and plant diseases were accessed to screen for a broad spectrum of antagonistic activity. Furthermore, we utilized the lung lichen *Lobaria pulmonaria* (L.) Hoffm., which is one of the fastest growing leaf-like lichens (MacDonald and Coxson, 2013) and used as indicator species of undisturbed forests and air pollution (Rose, 1976; Scheidegger, 1995). We also characterized the most active as well as the most abundant lichen-associated antagonists *Stenotrophomonas,* which were already identified as versatile antagonists from plant origin (Ryan et al., 2009; Alavi et al., 2014; Berg and Martinez, 2015). Beneficial *Stenotrophomonas* strains produced osmoprotectans and spermidine in response to eukaryotic hosts (Alavi et al., 2013). In our study we applied multidisciplinary techniques to link metagenomic data with those obtained from bacterial cultures. Moreover, we could show that lichens are important reservoirs for antagonistic bacteria, which can also be used for biological control approaches to protect plants against biotic and abiotic stress.

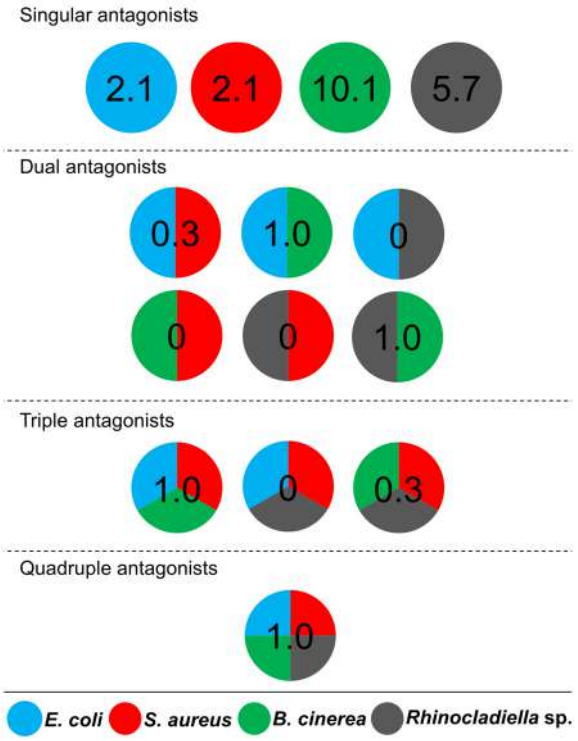

FIGURE 1 | Distribution of antagonistic bacteria isolated from multiple *Lobaria pulmonaria* thalli. Dual-culture experiments with 388 bacterial cultures were used to identify antagonists against four different model pathogens. *Escherichia coli* and *Staphylococcus aureus* were used as models for human pathogens, *Botrytis cinerea* and *Rhinocladoniella* sp. were used as models for plant and lichen pathogens, respectively. Results are presented in a schematic illustration of antagonists, targeting a specific pathogen (indicated by relative numbers and different colors, respectively). Divided circles indicate particular combinations of pathogens, which were inhibited by an assigned percentage of *Lobaria*-associated isolates. Singular antagonists: *Lobaria*-associated isolates that inhibited only one model pathogen; Dual antagonists: isolates that inhibited two distinct model pathogens; Triple antagonists: isolates that inhibited three distinct model pathogens; Quadruple antagonists: isolates that inhibited four distinct model pathogens.

Materials and Methods

Sampling Strategy and Isolation of Lichen-Associated Bacteria

Lichen thalli of *L. pulmonaria* were sampled from three different locations in Austria (Tamischbachgraben, N47°32′40″, E14°37′35″, Johnsbach, N47°38′07″, E14°44′45″, and St. Oswald

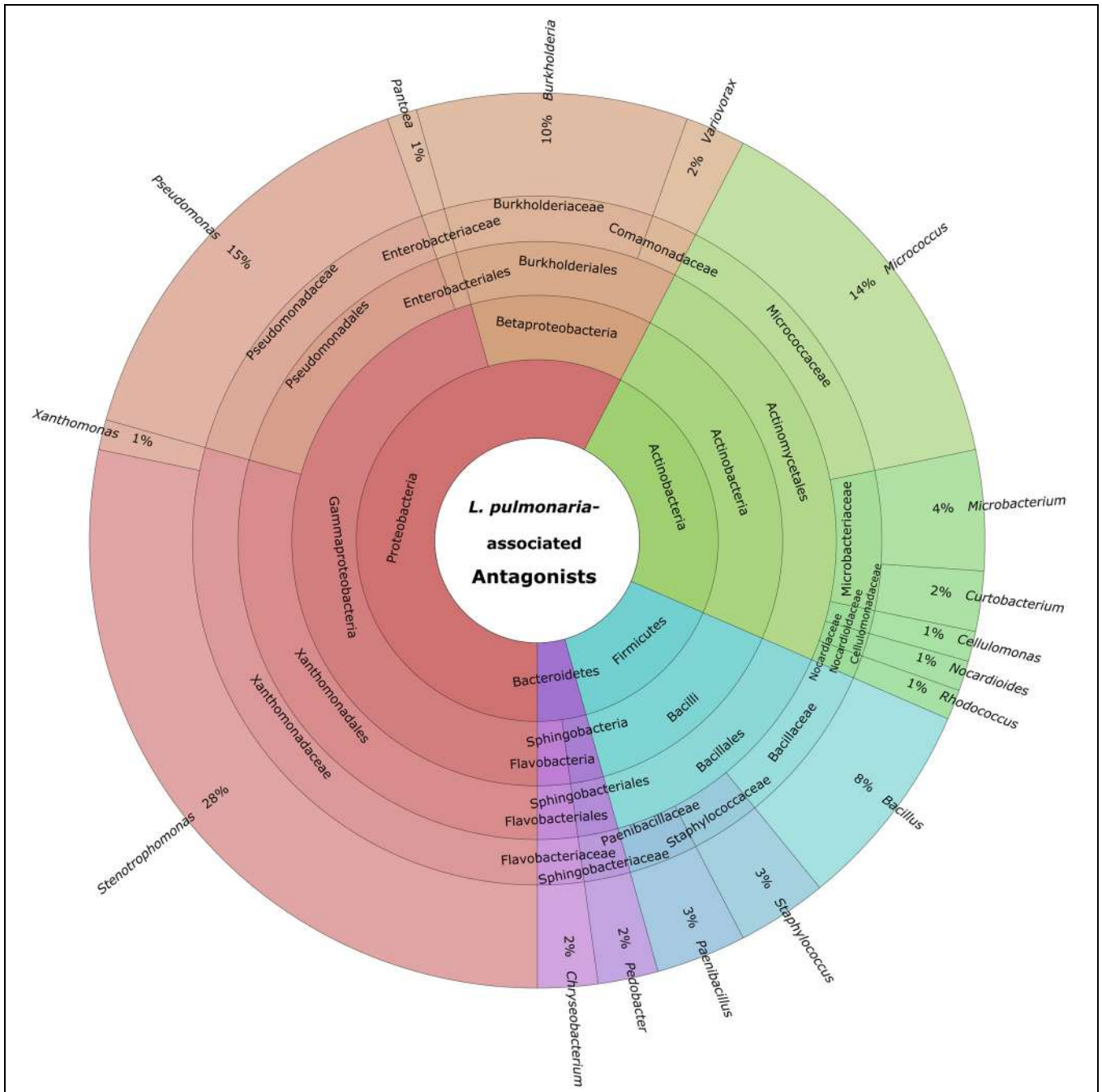

FIGURE 2 | Summary of identified antagonistic bacteria in a multi-level chart (http://sourceforge.net/p/krona). The displayed taxa were shown to inhibit growth of one or more of the utilized model pathogens: *B. cinerea*, *E. coli*, *S. aureus,* and *Rhinocladionella* sp. The inner circles represent higher taxonomic ranks, while more detailed taxonomic ranks (up to genus level) are presented in the outer circles.

ob Eibiswald, N46°44′ 50″, E15° 04′ 26″) after visual inspection to avoid contamination by lichenicolous fungi and other organisms. Five separate lichen thalli were sampled from each sampling site. The samples were stored on dry ice and were, shortly after, ground with mortar and pestle. A homogenate was prepared using sterile 0.85% NaCl in a 1:10 (w/v) ratio, together with a lab stomacher (BagMixer; Interscience, St Nom, France). Diluted fractions were plated on R2A agar (Carl Roth, Karlsruhe, Germany), R2A agar with 25 μg ml^{-1} cycloheximide, starch casein agar (SCA; Küster and Williams, 1964) and ISP2 agar (Shirling and Gottlieb, 1966). Bacterial colonies were randomly picked within 5 days of incubation at room temperature (RT) and a total of 388 isolates was obtained. The isolates were stored in glycerol stocks at −70°C prior to cultivation-based experiments.

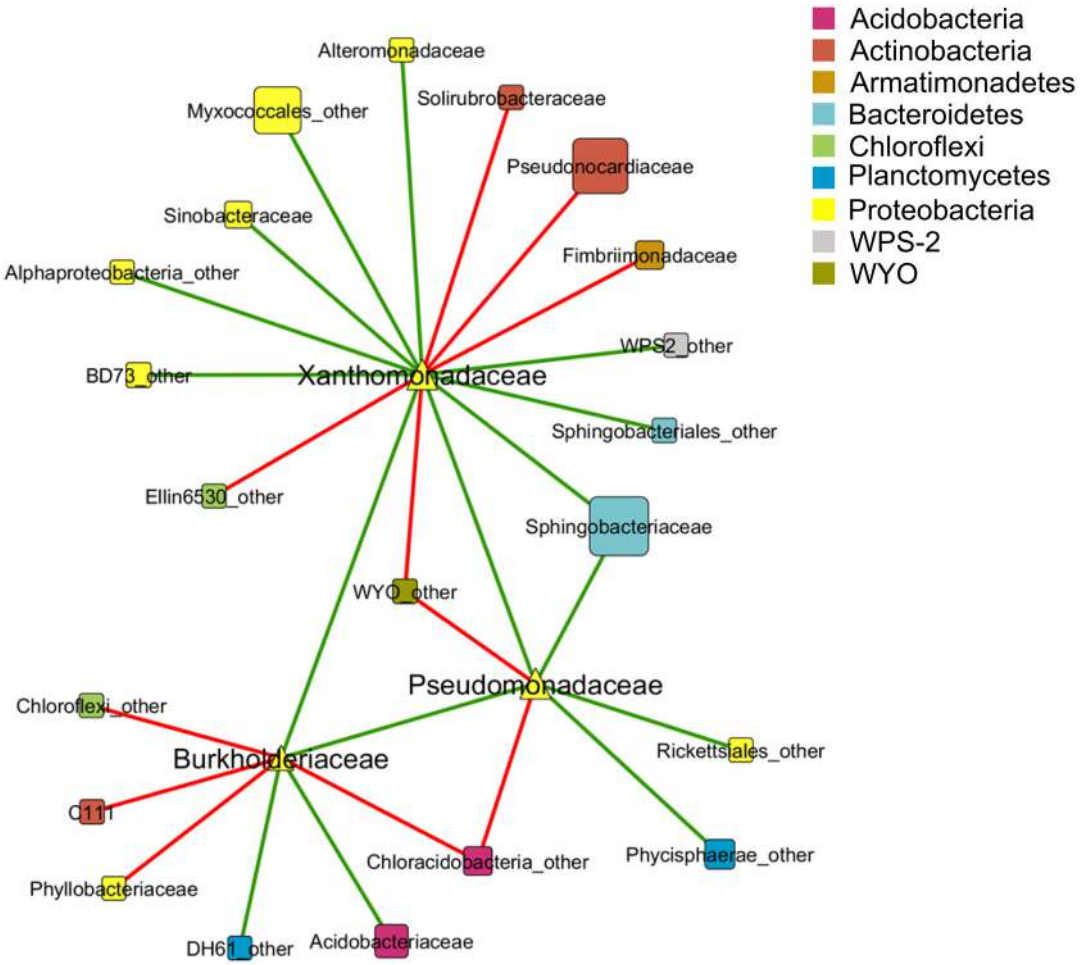

FIGURE 3 | Co-occurrence pattern between taxa of the lichen-associated microbiome. Nodes represent taxa at family level which are colored by phylum affiliation. Node size reflects sequence abundance of each taxon. The edges represent Spearman correlations in green (positive correlation, $R > 0.6$) and red lines (negative correlation, $R < -0.6$).

Screening of Isolates for *In Vitro* Antagonistic Activity Toward Particular Bacteria and Fungi

Dual-culture experiments were carried out as confrontation assays, using different media and target organisms according to Berg et al. (2002) and Opelt et al. (2007). Lichen-associated isolates were spotted on solid media pre-inoculated with *Escherichia coli* XL1 and *Staphylococcus aureus* ATCC 25923 and assessed for inhibition zones after 4 days of incubation at 30°C. Antagonistic activity against the fungus *Botrytis cinerea* Pers. (in-house culture collection) was tested by dual culture on Waksman agar (WA), according to Berg et al. (2002) and assessed after 5–7 days incubation at 20°C. Cultures of the lichen-pathogenic fungus *Rhinocladiella* sp. (culture collection of Lucia Muggia; Institute of Plant Sciences, University of Graz) were homogenized and re-suspended in sterile 0.85% NaCl. In the following step, 50 μL aliquots from one batch were used to inoculate each well of 24-well plates which contained solid potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany). Subsequently, 100 μL culture filtrate obtained from each lichen-associated isolate was added to particular wells. After 3 weeks of incubation, the wells were checked for growth reduction. All experiments were conducted with three independent replicates.

Amplicon Library Preparation and Co-Occurrence Analysis

Amplicon libraries obtained by Aschenbrenner et al. (2014) were used to extract distinct taxa for additional studies. The utilized 454-pyrosequencing data was obtained from lichen samples from the same sampling sites that were used for isolation of lichen-associated bacterial cultures. Out of the 454-amplicon dataset 15 thallus samples (five for each sampling site) were used for a co-occurrence analysis. Therefore OTUs (Operational Taxonomic Units) were clustered with UCLUST (Edgar, 2010) at 95% similarity (correlates with the taxonomic genus level). Mitochondrial, chloroplast, and *Nostoc* sequences were excluded as well as all OTUs with less than three sequences. Co-occurrence patterns were created with calculated Spearman correlations

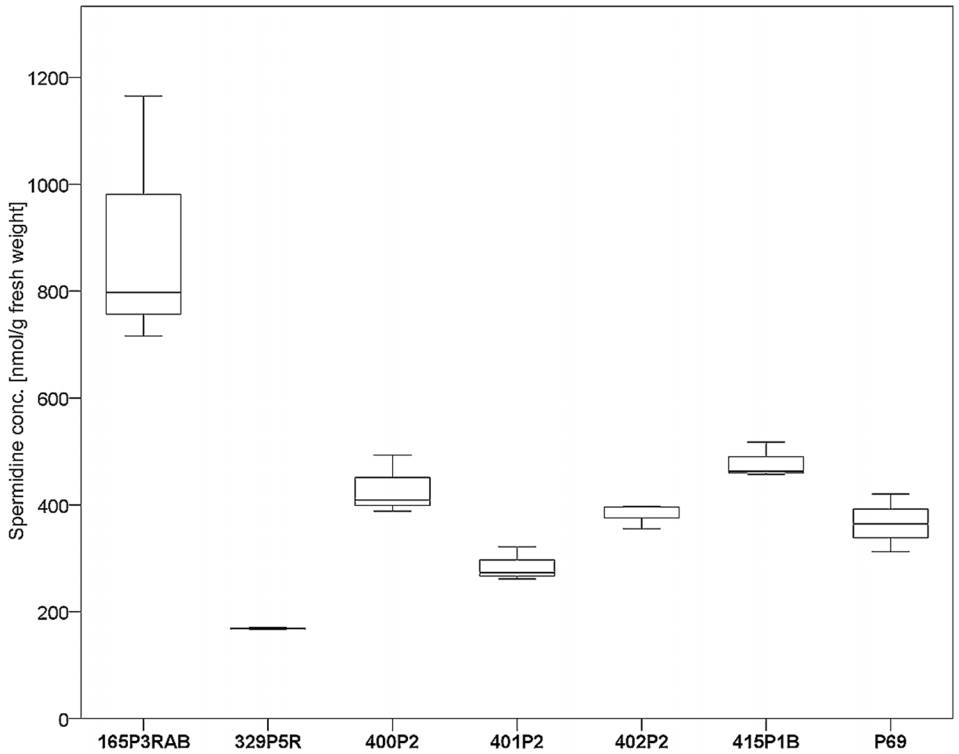

FIGURE 4 | Analysis of internal spermidine concentrations and lichen-associated *Stenotrophomonas* sp. isolates. The isolates were cultivated on solid cultivation media followed by cell disruption and quantification of internal spermidine levels ($n = 21$). A total of six *Lobaria*-associated isolates were utilized together with one plant-associated isolate (P69) in a comparative approach. *Stenotrophomonas* sp. 165P3RAB was shown to contain the highest internal spermidine concentration ($p < 0.1$) compared to all other isolates, while isolate 329P5R had the lowest concentration ($p < 0.01$).

between taxa at family level (>0.6 and <-0.6; R environment version 3.1.2[1]). Only families, which showed a correlation to *Pseudomonadaceae, Xanthomonadaceae,* and *Burkholderiaceae* were considered for further analysis and visualized as network with Cytoscape (organic layout; version 3.2.1; Saito et al., 2012). Node size within the network reflects the sequence abundance of each taxon and nodes were colored according to phylum affiliation.

16S rRNA-Based Identification of Antagonistic Bacteria and Phylogenetic Analysis

Primer pair 27F/1492r was used to amplify specific 16S rRNA gene fragments from antagonistic bacterial cultures. Subsequent sequencing and BLASTn searches within the 16S ribosomal RNA sequence database (NCBI) were conducted for identification of antagonists. These sequences were later trimmed to the hypervariable V4 region to allow alignments with 454-pyrosequencing data of the same rRNA region. QIIME 1.6.0 (Quantitative Insights Into Microbial Ecology, Caporaso et al., 2010) and the implemented Greengenes database (DeSantis et al., 2006) was used to search for bacterial OTUs in the corresponding amplicon dataset (Aschenbrenner et al., 2014) that were assigned to the three most dominant proteobacterial genera within prior identified antagonists. OTUs which could not be assigned to any particular phylum or to species level within *Proteobacteria* were additionally analyzed with Seqmatch (RDP database) for taxonomic assignment. The representative sequences of these additionally identified OTUs were used for further phylogenetic analyses. The phylogenetic tree was constructed with V4-trimmed 16S rRNA sequences from antagonistic bacteria cultures and an amplicon subset. Sequences were aligned with MEGA6 (Tamura et al., 2013) and processed for bootstrapped neighbor-joining with PHYLIP work package v.3.695[2]. Confidence levels for the internal branches were assessed by bootstrap analysis with 100 re-samplings. FigTree v.1.4.0[3] was used for annotation and final graphic visualization of the phylogenetic tree. All utilized 16S rRNA gene fragment sequences from isolate and amplicon sequencing were deposited at GenBank[4] (accession numbers: KP739786–KP739797 and KR611621–KR611709).

Metagenomic Mining for Specific Genes of Interest

All metagenome-based analyzes were carried out on the assembled dataset described in a previous study by Grube

[1]http://www.r-project.org

[2]http://evolution.genetics.washington.edu/phylip

[3]http://tree.bio.ed.ac.uk/software/figtree

[4]http://www.ncbi.nlm.nih.gov/genbank

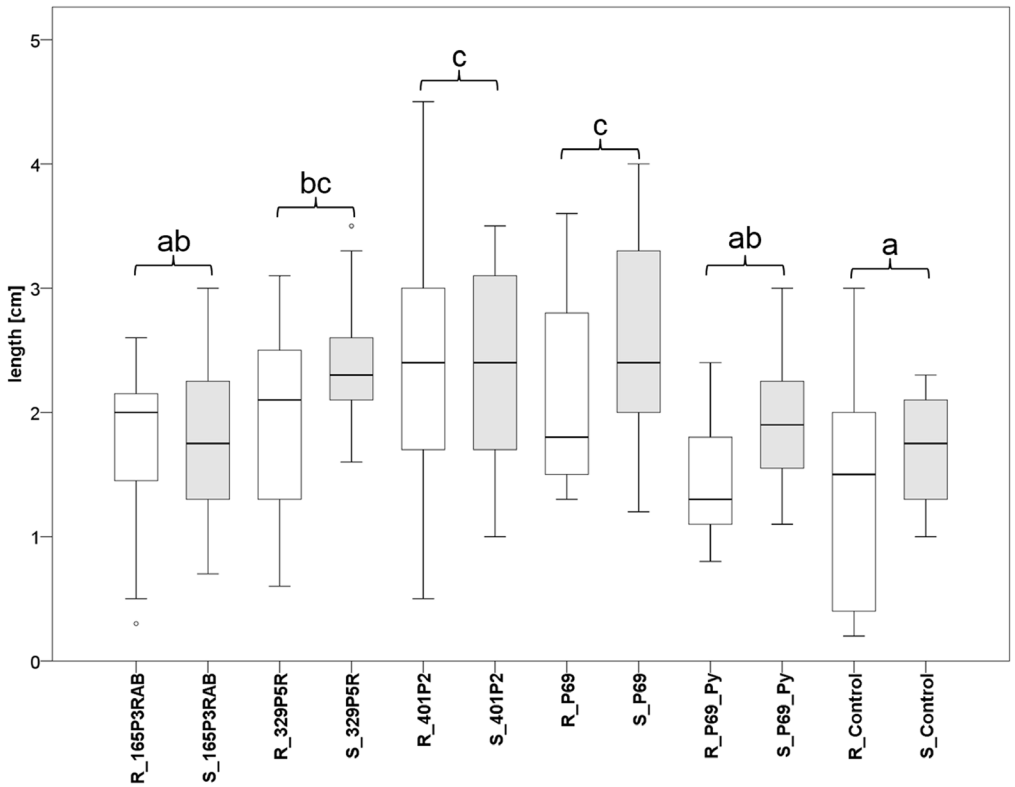

FIGURE 5 | Greenhouse experiment with *Stenotrophomonas*-primed tomato seeds and two distinctive controls (P69_Py and Control). Three *Lobaria*-associated isolates (165P3RAB, 329P5R, and 401P2) were utilized together with a plant-associated isolate (P69). Root (R; $n = 64$) and stem (S; $n = 66$) lengths were measured after 2 weeks plant growth under limited water supply. White (root lengths) and gray (stem lengths) plot pairs represent the treatments with specific isolates and complementary controls. Statistical analysis was employed to identify significant ($p < 0.05$) differences in combined root and stem lengths. Different letters were used to differentiate between statistically discriminative groups.

et al. (2015). CLUSTER CONTROL (Stocker et al., 2004) was used to search with the blastn algorithm for specific spermidine synthase matches (NCBI accession numbers: NC_010943.1, NC_011071.1, NC_015947.1, and NC_017671.1) within the dataset (368,424 contigs). MEGAN (v4.70.4) was used to retrieve taxonomic classification and relevant SEED functions.

Metabolite Extraction from Bacterial Cultures Grown on Solid Medium

Bacterial cultures were washed from several densely colonized Nutrient Agar (NA; Sifin, Berlin, Germany) plates after 48 h incubation at 30°C and homogenized in 9 mL 0.85% NaCl solution. The homogenate was centrifuged for 20 min, 2,000 *g* at 4°C. This step was repeated two times to remove residual media from bacterial cells. The pellet was re-suspended in 2 mL ddH_2O followed by centrifugation for 15 min, 18,000 *g* at 4°C. Precooled 90% methanol at −70°C was used for reproducible extraction and to avoid further degradation of metabolites. Subsequently, 1 mL was added to each pellet and the bacterial cells were mechanically disrupted with glass beads for 2 × 45 s at 6 m/s. Followed by a final centrifugation step for 15 min, 18,000 *g* at 4°C, 100 µl of each supernatant was collected and immediately placed in a deep freezer at −70°C until further analysis. Three independent biological replicates were prepared for each isolate.

Preparation of Culture Supernatants for Spermidine Quantification

Bacterial cultures were used to prepare overnight cultures (ONC) in fluid Nutrient Broth II (NBII; Sifin, Berlin, Germany) medium. These ONCs were used to inoculate 50 mL NBII flasks, which were then incubated at 30°C, 120 rpm for 48 h. In the following step, 2 mL aliquots were taken from the cultures and centrifuged for 20 min, 18,000 *g* at 4°C. The supernatants were filtered with 0.25 µm filters and immediately placed in a deep freezer at −70°C until further analysis. Three independent biological replicates were prepared for each isolate.

Quantification of Specific Bacterial Metabolites with High Resolution Mass Spectrometry

Samples were analyzed in nine biological/technical replicates with a combined HPLC-hybrid quadrupole-orbitrap mass spectrometer (Q Exactive; Thermo Scientific, Bremen, Germany). A Luna 5u NH2 100A 250 × 4.6 column (Phenomenex,

Aschaffenburg, Germany) was used to separate different metabolites from the cell extracts. Formic acid (0.1%, v/v) in acetonitrile was used as solvent A and aqueous formic acid (0.1%, v/v) as solvent B. Starting conditions for the gradient elution were 10% A and 90% B. The conditions were gradually changed to 80% A and 20% B within 15 min. This step was followed by 5 min at 10% A and 90% B for readjustment to initial conditions. The eluent flow was maintained at 0.8 mL/min together with a column temperature of 25°C. Sample analysis was carried out with negative ion ESI detection. ESI conditions were set to 3.2 kV spray voltage and 350°C capillary temperature. Scans were recorded in the range 100.0–300.0 m/z with the AGC target set to 500,000 and maximal accumulation time of 200 ms. The resolution was adjusted to 200,000. Altering full MS-SIM and targeted MS^2 cycles were employed and a specific inclusion mass of 146.16517 amu was selected. Standard calibration was obtained with 0, 0.02, 0.03, 0.04, 0.05, 0.1, and 0.2 μM spermidine standard (Duchefa Biochemie, Haarlem, The Netherlands) diluted in 0.2 mM HCL.

Plant Growth Experiments with *Stenotrophomonas*-Primed Seeds

Overnight cultures with selected *Stenotrophomonas* isolates were used to inoculate main cultures in fluid NBII. In addition to the lichen-associated isolates, a plant-associated isolate *Stenotrophomonas rhizophila* P69 (Minkwitz and Berg, 2001; Wolf et al., 2002) was also utilized for comparisons. After 2 h of growth at 30°C and 120 rpm the fluid cultures were diluted to 5×10^6–1×10^7 cells per mL in sterile 0.85% NaCl solution. Tomato (*Solanum lycopersicum* L. cv. Kremser Perle; Austrosaat, Graz, Austria) seeds were surface sterilized with 4% NaHClO for 10 min followed by drying at RT. The sterilized seeds were put into the respective bacterial suspensions and incubated for 4 h at 120 rpm and RT. The control samples were put in 0.85% NaCl solution without bacteria. Ground and homogenized seeds from each inoculum were plated on NA to test priming efficiency after the incubation time. The remaining seeds were planted in sterile soil (150 g/tray) with vermiculite (1:3 ratio) and watered with 30 mL sterile H_2O. Beside the control with non-primed seeds an additional control with P69-primed seeds was added. Therefore, 60 μL 5-sec-butyl-2,3-dimethylpyrazine (Sigma–Aldrich, Steinheim, Germany) was supplemented into 30 mL sterile H_2O used for irrigation after planting. The closed trays were placed without further irrigation for 2 weeks into a greenhouse with 12 h day/night cycles and a constant temperature of 24°C. Root ($n = 64$) and stem ($n = 66$) lengths of the plantlets were assessed separately for all samples.

Statistical Analysis

Statistical analyses were performed with SPSS v.20.0.0 (SPSS Inc, Chicago, IL, USA). Data were tested for normal distribution with the Kolmogorov–Smirnov test. Sets with normally distributed data were analyzed with univariate ANOVA and Duncan tests at $p < 0.05$. The *t*-test was employed for statistical analysis of data that was not normally distributed ($p < 0.05$ and $p < 0.1$).

Results

Antagonistic Bacteria within the *Lobaria* Microbiome

Cultivable bacteria, which have been isolated from 15 *L. pulmonaria* samples, were tested in dual-culture assays against the bacterial model pathogens *E. coli*, *S. aureus*, the plant pathogen *B. cinerea* and the lichenicolous fungus *Rhinocladoniella* sp. to determine the general antagonistic potential. In these experiments, lichen-associated antagonists were shown to primarily target lichen and plant pathogenic fungi (20.1% of all isolates) while a lower proportion was directed against bacterial model pathogens (7.7% of all isolates). A total of 95 isolates (24.5%) showed inhibition of growth of at least one of the model pathogens (**Figure 1**). Singular antagonists (active against only one target microorganism) were dominated by *Stenotrophomonas* spp. (31% of singular antagonists) followed by *Pseudomonas* spp. (19%) and *Burkholderia* spp. (12%; Supplementary Figure S1). Dual antagonists (active against two microorganisms) comprised nine isolates. *Stenotrophomonas* and *Micrococcus* were represented by three isolates and *Chryseobacterium*, *Microbacterium,* and *Paenibacillus* by only one isolate, respectively. Nine bacterial strains inhibited the growth of either three or four model pathogens simultaneously. These cultures were identified at genus level as *Bacillus* (five isolates), *Micrococcus* (one isolate), and *Paenibacillus* (three isolates). A complete taxonomic breakdown for all identified antagonists was visualized in **Figure 2**.

Comparison of the hypervariable V4 rRNA region from the most abundant proteobacterial antagonistic isolates: *Stenotrophomonas* spp., *Pseudomonas* spp., and *Burkholderia* spp. with filtered OTUs from an amplicon library constructed with *Lobaria* samples from the same sampling sites, revealed high homology of sequences within the same genus (Supplementary Figure S2). Specific branches (bootstrap values > 70%) were detected for amplicon- and isolate-based sequences. OTUs that were assigned to *Stenotrophomonas* sp., *Pseudomonas* sp., and *Burkholderia* sp. comprised, respectively, 0.06, 0.56, and 0.09% of all analyzed OTUs in the amplicon library. Interestingly three of the highly active antagonistic genera (*Bacillus*, *Micrococcus,* and *Paenibacillus*) were not substantially represented in the amplicon library.

A co-occurrence pattern between different taxa at family level was created with the calculated Spearman correlations based on 15 lichen thallus samples. In total, 24 correlations between the families *Pseudomonadaceae*, *Burkholderiaceae,* and *Xanthomonadaceae* to other taxa within the microbiome could be detected and were visualized as a co-occurrence network (**Figure 3**). Most correlations (10 out of 24) were found within the phylum *Proteobacteria* followed by *Actinobacteria* (3). The strongest positive correlations (Spearman correlation > ±0.7) showed *Xanthomonadaceae* with *Pseudomonadaceae* and *Alteromonadaceae*, all assigned to *Gammaproteobacteria*, whereas the strongest negative correlations were found between *Burkholderiaceae* (*Betaproteobacteria*) and *Phyllobacteriaceae* (*Alphaproteobacteria*) and a family within the class

Chloracidobacteria (*Acidobacteria*) which was not further classified in the utilized database.

In addition, the abundance of antagonistic taxa was extracted from the *Lobaria* metagenome. The proportion of retrievable antagonistic genera was determined for *Stenotrophomonas* (0.22% of all bacteria within the metagenome), *Pseudomonas* (1.14%), *Burkholderia* (2.81%), *Xanthomonas* (0.43%), *Nocardiodes* (0.10%), *Rhodococcus* (0.18%), *Bacillus* (0.08%), and *Staphylococcus* (0.02%). The remaining antagonistic taxa could not be retrieved at genus level. However, they comprised at family level together 2.14% of all bacteria. Altogether, antagonistic taxa comprised 7.12% of the total bacterial community. Neither the genus *Cellulomonas* nor the family *Cellulomonadaceae* was present in the assembled metagenomic dataset.

Spermidine Production *In Vitro* and Spermidine-Related Genes within the *Lobaria*-Associated Metagenome

Genes coding for spermidine synthases were analyzed from the *Lobaria* metagenome and taxonomically assigned. In addition, spermidine production and secretion was analyzed *in vitro*.

Seven antagonistic *Stenotrophomonas* sp. isolates were cultivated on solid agar plates and in liquid media prior to the extraction of spermidine. The detection limit for spermidine on the utilized instruments was determined to be <30 nM. Externalized spermidine levels detected in liquid cultivation media were in the range between 8.2 and 10.5 μmol/g fresh weight. Extracellular spermidine concentration differences between utilized *Stenotrophomonas* isolates were not statistically significant (Supplementary Figure S3). Conversely, the same isolates were shown to contain different internal spermidine concentrations after cultivation on solid media (**Figure 4**). The lowest internal spermidine concentration was found to be 168 nmol/g fresh weight, which was around fourfold lower than the highest observed concentration. Isolate 165P3RAB was found to contain significantly higher spermidine concentrations than all other isolates. In contrast, *Stenotrophomonas* isolate 329P5R contained the lowest spermidine concentrations.

For BLASTn searches, reference sequences for spermidine synthases from four different *Stenotrophomonas* strains were utilized. Additionally, SEED assignments were searched for related functions. *Stenotrophomonas*-specific contigs that contain known spermidine synthases were not found in the utilized metagenome, while three other bacteria-derived contigs were present. Two spermidine synthase contigs that were retrieved with SEED-based analysis were assigned to *Proteobacteria*. One contig was assigned to *Burkholderiaceae* and the other contig to *Acetobacteraceae*. A third contig could not be assigned to any taxon. Conversely, spermidine putrescine transporter permeases were more abundant in the metagenome. A total of 50 contigs were assigned to this specific transporter protein (Supplementary Figure S4). More than a half of these contigs were assigned to bacteria (58%), while 42% remained unassigned to a specific kingdom. The hits were predominantly associated with *Proteobacteria* (52%) and more specifically to *Rhizobiales* (28%).

Stenotrophomonas Treatments Increased Plant Growth of Tomato Under Greenhouse Conditions

Tomato (*Solanum lycopersicum* L.) seeds were inoculated with three lichen-associated and one plant-associated *Stenotrophomonas* isolate and stress protecting agent as reference (Alavi et al., 2013) to analyze the effect of bacterial inoculants on plants. The primed seeds were grown with limited irrigation for 2 weeks in sterile soil. Two control types were implemented to evaluate growth promotion effects by the inoculants. One control (P69_Py) was supplemented with 60 μL 5-sec-butyl-2,3-dimethylpyrazine per tray during the initial irrigation of *Stenotrophomonas* P69-primed seeds. This heterocyclic compound was found to limit the growth of *Stenotrophomonas* isolates in previous experiments. Correspondingly, the growth of P69-primed samples that were treated with 5-sec-butyl-2,3-dimethylpyrazine (R_P69_Py and S_P69_Py), was similar to non-primed control samples (**Figure 5**). Also, the plant growth was not enhanced by isolate 165P3RAB, which was shown to contain the highest internal spermidine concentrations when compared to both implemented controls. *Stenotrophomonas* isolates 329P5R, 401P2, and P69 enhanced the plant growth significantly when compared to both controls. These isolates were shown to produce low internal spermidine concentrations in previous experiments. Treatments with the lichen-associated isolate 401P2 and the plant-associated isolate P69 resulted in similar plant growth.

Discussion

The lichen symbiosis was discovered as reservoir for antagonistic bacteria. Interestingly, it was possible to transfer selected isolates from lichens to cultivated plants while maintaining beneficial effects. In addition, we have shown the usability as well as limits of various applied techniques to efficiently screen for specific characteristics and how to reasonably couple classic microbiology with high-end techniques in a comprehensive approach. Starting from a culture collection and dual-culture experiments to screen for active antagonists, the approach was expanded with detailed specification of continuously filtered isolates.

The microbiome involved in the lichen symbiosis is highly diverse (Aschenbrenner et al., 2014) and was identified as bioresource for antagonistic bacteria. *L. pulmonaria* is predominately colonized by *Alphaproteobacteria* (Grube et al., 2015), in particular by various members of *Rhizobiales* (Erlacher et al., 2015b), which harbor many bacterial genera known for a beneficial host-microbe interaction especially with plants. Interestingly, all antagonistic genera identified for lichens – *Stenotrophomonas, Pseudomonas, Burkholderia, Micrococcus, Chryseobacterium*, *Microbacterium,* and *Paenibacillus* – are well-known from plant studies (Haas and Défago, 2005; Ryan et al., 2009; Rybakova et al., submitted). This is an interesting finding because it shows that these bacteria have the same redundant function independent of the habitat. This observation is underlined by the greenhouse experiments, which have shown

that lichen-associated antagonists are active on plants. This also supports the hypothesis that natural ecosystems are interesting reservoirs for biotechnologically relevant bacteria. The present study depicts that cultivable bacterial taxa with lower occurrence on lichens are mainly responsible for the protection against biotic disturbance. A highly diversified bacterial microbiome enhances the available functional repertoire, which might play a crucial role for the stability and longevity of the lichen symbiosis. Comparison of isolate-derived 16S rRNA gene fragments and amplicon-based sequences of abundant antagonists has indicated that a high proportion of *Burkholderia* spp., *Pseudomonas* spp., and *Stenotrophomonas* spp. can be retrieved from lichen symbioses by cultivation experiments on conventional growth media. It was also demonstrated that several isolated antagonists, such as *Bacillus, Paenibacillus*, and *Micrococcus*, were not detectable in the amplicon library but partially in the metagenomic dataset. The most reasonable explanation is that these antagonistic species occur with low abundance within this lichen microbiome and therefore these species might be below the detection limit of the utilized 454 pyrosequencing approach. Other methods with higher coverage might be more suitable to uncover all present bacterial colonizers. Further studies that address this question should preferably subject less multiplexed samples to high-throughput sequencing platforms to obtain a higher read number per sample. This would allow more accurate characterizations of the rare microbial population.

The majority of the antagonistic isolates was assigned to the genus *Stenotrophomonas*. These bacteria have been reported to protect plants against unaffordable conditions like drought and elevated salinity by exudation of protective compounds like spermidine and different osmolytes (Berg et al., 2010; Alavi et al., 2013). Corresponding to this, polyamines which also include spermidine were shown to be involved in plant response to abiotic stress in prior studies (Alcazar et al., 2006; Liu et al., 2007). Environmental strains of *S. maltophilia* and *S. rhizophila* were reported to exert a certain degree of tolerance toward salinity of up to 9% (w/v) NaCl which was correlated with the ability to produce the osmolytes trehalose and glucosylglycerol (Ribbeck-Busch et al., 2005). Even though *Stenotrophomonas*-specific spermidine synthases were not present in the analyzed metagenome, it was evident that utilization of spermidine is widely distributed among various detected organisms. Since lichens presents a habitat that is frequently subjected to drought, the association with bacteria having protective properties appears favorable. However, lichens themselves have mechanisms to account for desiccation, which also includes osmolytes (Green et al., 2011). Moreover, protection mechanisms against oxidative stress-related damage act in a mutual manner among the eukaryotic partners (Kranner et al., 2005). Therefore, we consider stress-protective functions of lichen-associated bacteria to act as an enhancer, which might react more flexibly to local fluctuations of the conditions than their hosts.

Although synthesized and excreted in different amounts, for all selected *Stenotrophomonas* isolates, *in vitro* spermidine production was detected. According to the result, we assume that the function of lichen-associated bacteria includes assistance in the protection against pathogens as well as against damage caused by desiccation. *Stenotrophomonas* is a well-known antagonist of plant-associated origin (Ryan et al., 2009) and connected with a beneficial effect on plant hosts (Berg et al., 1996; Egamberdieva et al., 2011; Alavi et al., 2013). *Stenotrophomonas* strains might also have the same function independent of the habitat. However, in the last two decades, they have received additional attention for opportunistic infections in humans (Berg and Martinez, 2015). It is difficult to identify specific factors of pathogenicity of the virulent isolates but the ability of persistence, resistance and survival – also essential to colonize lichens – allowed a colonization of immunocompromised patients with predisposition. Similar to the plant rhizosphere (Berg et al., 2005), lichens may act as a reservoir for facultative human pathogenic bacteria, or close relatives thereof.

The host-microbiome balance as well as indigenous diversity is essential for functional stability in ecosystems. This balance also depends on the mutual effects among bacteria within the microbiome. In our co-occurrence analyses we found indications for positive and negative correlations among bacterial groups on samples of the same host lichen symbiosis in the same habitat, which is a strong indication of antagonistic and synergistic effect in the lichen habitat. Further analyses are required for clarifying the mechanisms responsible for these effects, as these might involve direct interactions or diffusible metabolites in the system or both. The present results have already shown that lichen symbioses are valuable bioresources to discover bacteria with antagonistic potential and we suggest that a systematic screening of a broader range of lichens may be useful for finding biocontrol solutions that are specifically tailored for ecologically different plant habitats.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00620

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