THE PLANT HOLOBIONT VOLUME II: IMPACTS OF THE RHIZOSPHERE ON PLANT HEALTH

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EDITORIAL
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Editorial: The Plant Holobiont Volume II: Impacts of the Rhizosphere on Plant Health

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Editorial on the Research Topic

The Plant Holobiont Volume II: Impacts of the Rhizosphere on Plant Health

The concept of "holobiont" introduced by Margulis (1991), as a simple biological unit involving a host and a single inherited symbiont, today assumes a more general meaning recognizing that microorganisms are universally present on and associated with multicellular eukaryotic organisms. The plant "holobiont" represents an assembly of the host with its symbiotic microorganisms in interaction, that may occur internally or be associated with the host tissue, acting as a biological hub able to replicate and transmit genetic information (Zilber-Rosenberg and Rosenberg, 2008). Thus, the soil community represents a reservoir of various microbes from which the plant can selectively retrieve a specific microbiome to meet its requirements. Numerous microorganisms are able to perform biological and physiological functions for the plant, such as nutrient cycling and water distribution, that significantly impact soil properties, plant development and health. Different microorganism groups represent key components in the soil-plant systems directly involved in the network of interactions occurring in the rhizosphere, inner plant tissues and phyllosphere (Barberán et al., 2012; Hassani et al., 2018). Soil and plant microbiomes have an important role in plant development and in soil health, providing a secondary genome that codes for the genetic machinery activated in biosynthetic processes regulating ecological and biological functions. Moreover, beneficial microbes can influence plant health and productivity by improving tolerance to stress through modulation of different functional traits, thus affecting quality and safety of plant products (Timmusk et al., 2017; Compant et al., 2019).

The composition of each microbiome is influenced by a myriad of factors, including environmental, soil physical properties, availability of nutrients, and associated plant species. Among the research papers included in this Research Topic, Seitz et al. studied the influence of climate changes connected with permafrost thaw on the composition of the soil microbiome. Significant differences were found in the taxonomy of the microbial species associated with the different active layers of soil in the permafrost thaw gradient. It was revealed that microbes associated with highly disturbed soils, negatively affected productivity of several boreal plant species. Di Lelio et al. demonstrated that varying temperatures (20 and 25°C) affected diversely the development of two *Trichoderma* species applied to tomato plants that subsequently resulted in a different induced defense response of the plants to the aphid *Macrosiphum euphorbiae* and the noctuid moth *Spodoptera littoralis*. Although the mechanisms activated in the plant by the microbiome are not yet fully understood, there is substantial evidence that many

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microbes provide beneficial effects to crops including disease control, improved nutrient acquisition, and stress tolerance. Zheng et al. revealed that the key species in suppressive soil and/or healthy tobacco plants were fundamental in the inhibition of soil-borne pathogens, such as *Ralstonia solanacearum*. The authors found that healthy plants have more complex bacterial networks than diseased plants, and that many potential beneficial bacteria, having inhibitory effects on the pathogen, included isolates related to Proteobacteria and Actinobacteria, were found in the disease-suppressive soil. Moreover, the high abundance of *Fusarium* in the conducive soil and infected roots was correlated to the occurrence of bacterial wilt disease.

Soil microorganisms establish close relationships with the host plant during mycorrhizal symbiosis. The review by Li et al. presented an updated perspective on the importance of the interaction between orchids and orchid mycorrhizal fungi (OMF). OMF involvement in key functions such as development, distribution and dynamics, environmental adaptability and protection against different soil pathogens was reported. The authors suggested that changes in the structure and quantity of OMF may have important consequences on orchid distribution and health.

The composition of the root microbiome changes according to the root compartment association. The work of Illescas et al. analyzed the effect of inorganic N (calcium nitrate-CAN) top dressing applications, *T. harzianum* T34 and their combination on wheat root microbial community composition. It was highlighted that there was an overall differentiated bacterial and fungal composition in bulk soil, rhizosphere and root endosphere. In all three compartments, the bacterial diversity was always higher than that recorded for fungi, and the microbial biodiversity decreased from bulk soil to root endosphere. Moreover, the combination of CAN and T34 affected to a greater extent the bulk soil bacterial levels in comparison to the individual applications, while the fungal composition was notably affected by each treatments assayed.

Anzalone et al. obtained 400 bacterial isolates from different tomato root-associated compartments (the soil close to the root surface, the root surface, and internal the root), and tested their ability to solubilise phosphates, produce siderophores, and act as antagonists. The authors highlighted that the sampling site influenced bacterial composition more than the root partition.

The development of the holobiont concept is now providing a new scientific basis for variation genetics, which is heritable and offered by the plant microbiome, in particular by the endophytic compartment (Nogales et al., 2016). The review by Harman et al. analyzed the beneficial effects of endophytes on plants, providing examples indicating improved nutritional status, photosynthetic capability and maintenance of an efficient internal cellular functioning. Moreover, it was explained that the mechanisms involved in the activation of plant resistance to diseases and abiotic stresses occur through the production of Symbiont-Associated Molecular Patterns, microbial elicitors that interact with receptors in plant cell membranes, thus resulting in signal transduction *via* MAP kinase and modification in plant gene expression and physiology.

Varga et al. utilized x-ray fluorescence spectromicroscopy and proteomics to demonstrate that two diazotrophic endophytes promoted organic and inorganic phosphorus uptake in poplar roots. Carro-Huerga et al. used scanning electron microscopy and confocal scanning laser microscopy analyses, to verify the presence of *Trichoderma* TI154, 6 weeks after inoculation, in parenchyma fibers and xylem vessels of grapevine wood, confirming the endophytic colonization by the beneficial fungus and its ability to limit the development of the pathogen *Phaeoacremonium aleophilum*, the causal agent of Grapevine Trunk Diseases.

The specific interactions that occur between plant species and the soil microbial community are also important due to the differential influence on plant growth and resistance to stress. There is a reciprocal exchange whereby the plants influence the microbiome composition in the surrounding soil, and in turn the microbial complex modulates the growth and protection of the plants. This interaction may involve different plant species that can recruit the microbes that can suppress plant pathogens, and consequently stimulate plant growth. In this context, there is a growing interest in applying and transplanting soil microbiomes in order to enhance plant performance during cultivation. Howard et al. tested four different crop species that were inoculated with diverse soil microbiomes, originating from actively-managed agricultural fields or native unplanted fields, and analyzed their growth and resistance to two insect pests. Soil microbiomes derived from late succession plant communities increased maize, cucumber, tomato, and lettuce resistance to *Trichoplusia ni*, in contrast to those crops treated with microbiomes from agricultural sources, although the same application failed to provide protection of tomato against *Spodoptera frugiperda*. In addition, plant growth promotion effects were found to be species-specific and depended upon the different microbiomes used. This evidence was also confirmed by Choi et al. who demonstrated that the upland soil microbial fraction increased the resistance to *R. solanacearum* in tomato cultivar Hawaii 7996, but not in the susceptible cultivar Moneymaker. In addition, the resistance of Hawaii 7996 to bacterial wilt was lost when the transplanted microbiota was heat-killed.

The MiSeq sequencing performed by Wang et al. provided new insights on transplantation as feasible approach for controlling soil-borne diseases in *Panax ginseng* plants over long growth periods. The authors found that the abundances of some pathogenic bacteria and fungi, as well as the functional richness associated with nutrient elements, decreased with increasing age of transplanted ginseng plants.

Plants can influence their microbiomes by producing and releasing various metabolites in their surrounding environment. In addition, when subjected to a stress condition the generated compounds can regulate the structure of the plant microbiome. The diverse manners that plant secondary metabolites may shape plant microbiome have been reviewed by Pang et al. They focused on the recent developments in analytical methodologies and multi-omics, providing an excellent overview of the networks between the co-operating partners which have implications on sustainable crop productions.

In their study, Wei et al. demonstrated that the total fungal biomass was significantly higher in the rhizosphere of *Verticillium* infected cotton plants, as compared to the rhizosphere of healthy plants, represented by an increase in different saprophytes and a decrease in mycorrhizal fungi. The diseased plants showed a ratio of total fungi to total bacteria much higher than in the healthy plants, suggesting a modification of the root microbial community due to the stress condition.

All the contributions collected in this Research Topic strengthen the already known relevance of microbiomes in the management of a sustainable agriculture. We hope this Research Topic of articles will encourage new researches on this subject of current increasing interest and importance.

AUTHOR CONTRIBUTIONS

NL wrote the first draft of the manuscript. RM, FV, DT, and SW revised and improved the manuscript. All authors defined the subject of this Research Topic, joined in the editing procedure, and approved this Editorial for publication.

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REFERENCES

Barberán, A., Bates, S. T., Casamayor, E. O., and Fierer, N. (2012). Using network analysis to explore cooccurrence patterns in soil microbial communities. *ISME J.* 6, 343–351. doi: 10.1038/ismej.2011.119

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004

Hassani, M. A., Durán, P., and Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome* 6:58. doi: 10.1186/s40168-018-0445-0

Margulis, L. (1991). "Symbiosis as a source of evolutionary innovation," in *Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis*, eds L. Margulis and R. Fester (Cambridge, MA: MIT press).

Nogales, A., Nobre, T., Valadas, V., Ragonezi, C., Döring, M., Polidoros, A., et al. (2016). Can functional hologenomics aid tackling current challenges in plant breeding? *Briefings Functional Genom.* 15, 288–297. doi: 10.1093/bfgp/elv030

Timmusk, S., Behers, L., Muthoni, J., Muraya, A., and Aronsson, A. C. (2017). Perspectives and challenges of microbial application for crop improvement. *Front. Plant Sci.* 8:49. doi: 10.3389/fpls.2017.00049

Zilber-Rosenberg, I., and Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol. Rev.* 32, 723–725. doi: 10.1111/j.1574-6976.2008.00123.x

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ORIGINAL RESEARCH
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Colonization of *Vitis vinifera* L. by the Endophyte *Trichoderma* sp. Strain T154: Biocontrol Activity Against *Phaeoacremonium minimum*

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Trichoderma strains used in biological control products usually exhibit high efficiency in the control of plant diseases. However, their behavior under field conditions is difficult to predict. In addition, the potential of indigenous strains has been poorly assayed as well as their possible behavior as endophytes. Hence, niche colonization is a key feature for an effective protection. In this study, we aimed to: (i) explore the possibility of using a new *Trichoderma* strain isolated from vine to control pathogens, (ii) study the *in planta* interaction with the pathogen *Phaeoacremonium minimum* W. Gams, Crous, M.J. Wingf. & L. Mugnai (formerly *Phaeoacremonium aleophilum*), a pioneer fungus involved in Grapevine Trunk Diseases (GTDs) such as esca. For this purpose, fluorescently tagged *Trichoderma* sp. T154 and a *P. minimum* strain were used for scanning electron microscopy and confocal scanning laser microscopy analyses. Data showed that the *Trichoderma* strain is able to colonize plants up to 12 weeks post inoculation and is located in xylem, fibers, as well as in parenchymatic tissues inside the wood. The beneficial fungus reduced colonization of the esca-related pathogen colonizing the same niches. The main observed mechanism involved in biocontrol of *Trichoderma* against the esca pathogen was spore adhesion, niche exclusion and only few typical hypha coiling was found between *Trichoderma* and the pathogen. These results suggest that the *Trichoderma* strain has potential for reducing the colonization of *Phaeoacremonium minimum* and thus, an inoculation of this biological control agent can protect the plant by limiting the development of GTD, and the strain can behave as an endophyte.

Keywords: *Trichoderma* colonization, vine, *Phaeoacremonium minimum*, indigenous strain, mycoparasitism

INTRODUCTION

During the last 50 years, intensive agriculture has led to a range of problems to human health, environment, flora, and fauna (Geiger et al., 2010). One of the crops requiring considerable amount of pesticides for increasing production is grapevine. *Vitis vinifera* L. (common grapevine) cultivars are among the most widely planted crops in the world and have a high commercial value (Gramaje et al., 2018). A significant amount of costs associated with these cultivars, however, come from intense pest and disease management programs (Cooper et al., 2012). Thus, important efforts are needed to search for alternative control strategies to reduce costs and dependence of chemicals. Currently, Grapevine Trunk Diseases GTDs (Bertsch et al., 2013; Roblin et al., 2019) are among the most important vine destructive diseases. In economic terms, in France, losses due to these diseases are estimated at around one thousand million € per year (Lorch, 2014) in yield production terms. In South Australia, losses were estimated in 1500 kg/ha (Wicks and Davies, 1999), and according to epidemiologic studies, an increasing incidence of GTDs has been found in the region of Castilla and León (Spain), increasing from 1.8% in 2001 to 7% in 2006 (Martín et al., 2007).

Since the ban of sodium arsenite in 2003 for controlling esca or Petri diseases, the use of Biological Control Agents (BCAs) has been described as an interesting, promising, and ecological strategy for controlling these diseases. Many BCAs have shown positive results against GTDs. For instance, bacteria such as a *Bacillus subtilis* isolate (ARC Infruitec-Nietvoorbij) have demonstrated reduction of symptoms (Ferreira et al., 1991; Fourie and Halleen, 2004). Also, indigenous strains of *Streptomyces* have been useful to reduce young grapevine decline caused by *Dactylonectria* sp., *Ilyonectria* sp., *Phaeomoniella chlamydospora*, and *Phaeoacremonium minimum* (Álvarez-Pérez et al., 2017) involved in GTDs.

Regarding fungi, *Trichoderma* strains have been also successfully tested in different conditions. Thus, under *in vitro* conditions, good results have been reported especially against causal agents of the trunk diseases such as *Phomopsis viticola, Eutypa lata, P. chlamydospora, Neofusicoccum australe, Neofusicoccum parvum, Diplodia seriata*, and *Lasidiplodia theobromae* (Kotze et al., 2011). In grapevine nurseries, *Trichoderma* strains have also shown the ability to control infections caused by *P. chlamydospora* and *P. minimum* during the grafting process (Pertot et al., 2016). However, in the field, positive and negative results have been obtained, and the grapevine trunk diseases continue to spread. The use of *Trichoderma* has been proved, also as an efficient protective method against *E. lata* in pruning wounds (Halleen et al., 2010) and can prevent black goo and necroses in the wood below the wound (Di Marco et al., 2004). The use of some *Trichoderma* strains or their metabolites was shown to control grapevine diseases (Mutawila et al., 2016b; Pascale et al., 2017). *Trichoderma* indigenous strains have been able to reduce the incidence of *Rhizoctonia solani* and inducing plant defense-related genes in bean plants (Mayo et al., 2015). However, little information is available on how *Trichoderma* indigenous strains could be protective and colonize endophytically the same niches as the pathogens. Preliminary studies suggest the possibility of using indigenous strains as promising agents for biocontrol in grapevines (Carro-Huerga et al., 2017; Carro-Huerga et al., 2019). Indeed, the use of *Trichoderma* to reduce symptoms and inoculum of pathogen in grapevine plants has been widely described (Di Marco et al., 2004; Fourie and Halleen, 2004; Halleen et al., 2010; Pertot et al., 2016). However, no studies are available regarding the niches, inside the plant, for *Trichoderma* colonization, nor to the kind of interaction established between *Trichoderma* spp. and pathogens belonging to the GTD complex inside grapevine plants.

In this study, we analyzed if an indigenous *Trichoderma* strain could colonize grapevine plants and protect vines against *P. minimum*. For that aim we analyzed: (i) the interaction between a *Trichoderma* sp. T154 isolated from vine and *P. minimum* under *in vitro* and *in planta* conditions, (ii) the niches of colonization of the *Trichoderma* sp. T154 and its behavior with the grapevine plant, and (iii) the persistence and mobility of pathogen and biocontrol agent in penetrating grapevine wood in the tripartite interaction grapevine-*Trichoderma*-*P. minimum*.

MATERIALS AND METHODS

Fungal Strains

Trichoderma sp. isolate T154 was isolated from wood of *Vitis vinifera* cv. Tempranillo (unpublished data). This strain was stored at the culture collection of Plant and Pest Diagnostic Laboratory under accession number ULET154 (University of León, Spain).

Phaeoacremonium minimum CBS 100398 (formerly *Phaeoacremonium aleophilum* CBS 100398) a GTD related pathogen was used in this study. A *P. minimum*::*gfp7* (formerly *P. aleophilum*::*gfp7*) strain was also used for colonization studies. The latter strain was transformed to express *gfp* and its colonization behavior on vine cuttings was reported by Pierron et al. (2015). Both were initially analyzed under normal light microscope.

Trichoderma Identification

After 3 days of growth on PDA medium, genomic DNA of the *Trichoderma* strain T154 was isolated from 100 mg of mycelia using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Extracts were eluted in 50 µl of sterile water and DNA concentration was estimated using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). PCR amplification was performed using 50 ng of template DNA in a final volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM for each dNTP, 400 nM for each primer, and 1.5 U of DreamTaq DNA polymerase (Thermo Scientific). ITS5-ITS4 were used to amplify nuclear rDNA-ITS regions (White et al., 1990). PCR products were first purified by the NucleoSpinExtract II kit (Machery-Nagel, Düren, Germany) and were then sequenced using primer ITS4 and the kit BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and

an automatic capilar sequencer ABI 3130xl (Applied Biosystems) according to the manufacturer's instructions. For fungal identification, sequences were then compared with NCBI Genbank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) databases using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Secondly, for a more accurate analysis of the *Trichoderma* isolate, sequences of six housekeeping genes [*act1* (encoding for the actin); *cal1* (calmodulin); *fas1* (fatty acid synthase alpha subunit); *lcb2* (sphinganine palmitoyl transferase subunit 2); *rpb2* (RNA polymerase 2nd largest subunit); and *tef1* (translation elongation factor 1-alpha)] were retrieved from the genome sequence of that isolate, and were used for a phylogenetic analysis against the same housekeeping sequences retrieved from GenBank genomic sequences of other 12 *Trichoderma* species belonging to four representative clades, i.e. clade Viride, Brevicopactum, Green Spored, and Longibrachiatum (**Supplementary Data S1: Figure S1**). The procedure used to retrieve all these sequences was as follows: First, sequences of these six genes were retrieved by BLASTn software from the genome of *T. harzianum* CBS 226.95, using as queries the sequences of fungal homologous genes, which were found at the GenBank database, against the genome sequence of that strain. Second, the six *T. harzianum* CBS 226.95 housekeeping genes were further used as queries to retrieve the homologous sequences from the genomes of the other 12 *Trichoderma* strains (including *Trichoderma* sp. T154), following the same procedure described above. Third, once the complete genomic sequences of the six housekeeping genes were available from the 13 *Trichoderma* strains used in this study, the intron regions were manually removed and the resulting coding sequences (cds) were used to generate the phylogenetic trees as indicated in the legend to (**Supplementary Data S1: Figure S1; Supplementary Data S3: Appendixes S1–S6**).

Sequences of the six housekeeping genes retrieved from the *Trichoderma* isolate identified in the present work were deposited in the GenBank under the accession numbers: MT701786 for *act1*; MT708552 (*cal1*); MT708557 (*fas1*); MT708564 (*lcb2*); MT708568 (*rpb2*); MT708571 (*tef1*).

Trichoderma Transformation

To transform the *Trichoderma* sp. strain T154, a hygromycin B test was performed to determine its sensitivity to different concentrations of hygromycin B (50, 100, 150, and 200 μg mL^{-1}) in PDA medium. It was obtained that 200 μg mL^{-1} was the optimum concentration for performing the experiment, no growth of *Trichoderma* was observed (**Supplementary Data S2: Figure S3**). The transformation of *Trichoderma* sp. T154 with plasmid pBHt2-tdTom (Caasi et al., 2010) was then carried out from a fresh *Trichoderma* sp. T154 spores suspension according to Cardoza et al. (2006). The binary vector pBHt2-tdTom contains Td tomato fluorescent protein under the control of the *tox*A promoter and the hygromycin resistance marker *hph*. Plates containing PPG [mashed-potato-glucose agar (Sousa, 2004)] medium were inoculated with 1 × 10^7 spores and incubated at 28°C for 3 days. The spores collected from the plate were then used to inoculate 50 ml of CM (5 g malt extract, 5 g yeast extract and 5 g glucose and distilled water up to 1 L) medium and incubated in an orbital shaker at 250 rpm and 28°C for 24 h. Then, 25 mL of that culture were filtered through Nytal® (30 μM pore diameter) (Maissa, Barcelona, Spain) and washed twice with 0.7 M NaCl. After that, the mycelium was re-suspended in 20 ml of NaCl 0.7M containing a mix of lytic enzymes (Lysing enzymes L-1412, Driselase D-8037, Chitinase C-6137, Sigma, USA) at concentrations of 5, 15, and 0.05 mg mL^{-1}, respectively. The mycelium was then incubated at 30°C on an orbital shaker at 80 rpm for 20 h. Protoplast formation was analyzed under the microscope at the end of the incubation period to verify the hydrolysis of the mycelium cell-walls. Once the protoplasts were released, they were collected by filtration through Nytal® filters (30 μm pore diameter) and centrifuged for 15 min at 4,000 rpm. The pellet was re-suspended in 0.5 ml STC buffer (10 mM Tris HCl; pH 7.5, 1.2 M sorbitol, and 50 mM $CaCl_2$), counted with a Thoma cells, and diluted with STC solution to a concentration of 1 × 10^8 protoplasts per ml. Finally, the protoplasts were mixed with solution 1 (v:v), to obtain a 5 × 10^7 protoplast mL^{-1} (solution 1 was prepared by mixing five volumes of STC with 1 volume of PEG [10 mM Tris HCl; pH 7.5, 50 mM $CaCl_2$, 30% polyethylene glycol 8000]). One-hundred microliters of this protoplast suspension containing 5 × 10^7 protoplasts mL^{-1}, were then mixed with 10 μg of linearized plasmid, pBHt2-tdTom, resuspended in 100 μl of STC, and 50 μl of PEG. This plasmid was previously linearized with the enzyme HindIII to facilitate the integration of the vector into the fungal genome. The plasmid and the protoplast suspension were then mixed and maintained at room temperature for 15 min, followed by another 15 min at 42°C. Then, 2 ml of PEG were added, and the mixture was incubated at room temperature for another 5 min. Finally, the mixture was diluted with 2 ml of STC and poured as an overlay on regeneration medium plates (27.4% sucrose; 0.1% yeast extract, 0.1% NZ-amine, and 1.2% Bacto-agar). The plates were maintained at room temperature for 5 to 10 min until the medium has solidified, and subsequently incubated at 28°C for 24 h to allow the regeneration of the protoplasts. Finally, a 1% agar overlay containing hygromycin B at a concentration of 200 μg mL^{-1} was added to the plates, and they were left in incubation, at 28°C, until the appearance of the transformants, for 3 to 5 days (**Supplementary Data S2: Figure S3**).

The transformants were analyzed and confirmed by PCR following the TERRA method (PCR Direct polymerase mix. Clontech, Mountain View, CA).

Pure Culture Interaction

In addition to the plant assays and for evaluating the *in vitro* mycoparasitic interaction between *Trichoderma* sp. T154:: *tdTom3* and *P. minimum*::*gfp7*, a confrontation dual assay was carried out as previously described (Kotze et al., 2011) with some modifications. The experiment was performed twice with three biological replicates each.

For analyzing both types of interaction and the mechanisms of biocontrol, confocal laser scanning microscopy (CSLM) and SEM were used. Thus, a mycelial plug of *P. minimum*::*gfp7* was placed on a 90 mm diameter Petri dish, and after 14 days (when

the pathogen reached an important grade of growth) another plug of *Trichoderma* sp. T154::*tdTom3*, collected from a 7-day-old culture grown on PDA, was placed at 5-cm distance of the pathogen´s plug. The plates were incubated at 25°C for 10 days in the dark (**Supplementary Data S2, Figure S4**).

Plant Interaction

One-year-old dormant grapevine grafted plants of Tempranillo/110 Richter combination from Vivai Cooperativi Rauscedo (Rauscedo, Italy) were potted up in June 2018. Plants were placed in a phytotronic chamber (photoperiod 16/8, 25°C; 45% humidity) and watered with autoclaved tap water during the experiment. Each plant was considered as a biological replicate and 12 biological replicates per treatment were performed. Budding took four to 6 weeks in 6-L pots containing a sterile mixture of vermiculite and turf (1:1 v/v). These plants (n= 72) were inoculated when at least six leaves were fully developed. In the upper part of the *Vitis vinifera* cv. Tempranillo, a wounding damage at the internode was made using a drilling device with a 3-mm drill head. For each sampling time (6 and 12 weeks) plants were inoculated with hyphae and spores of *Trichoderma* sp. T154::*tdTom3* (n=12), *Trichoderma* sp. T154 (n=12), *P. minimum*::*gfp7* (n=12), or *P. minimum* (n=12) and *Trichoderma* sp. T154::*tdTom3* + *P. minimum*::*gfp7* (n=12). For inoculation, all fungi were grown separately on three PDA plates. A cylindrical plug (3 mm long and 1 mm diameter) of *P. minimum*::*gfp7*, *Trichoderma* sp. T154::*tdTom3*, *P. minimum* or *Trichoderma* sp. T154 strains growing on PDA medium was applied to the wound. In the case of dual inoculation, *Trichoderma* sp. T154::*tdTom3* + *P. minimum*::*gfp7*, both of them were applied in the same hole simultaneously. Only hyphae in the periphery of the growing fungus were collected to avoid selecting fungal material at a different reproductive and metabolic stages at different locations on the same plate. Control plants were inoculated with a plug of uninoculated PDA medium. After inoculation, the wound was covered using a cellophane membrane. Then, plants were maintained in the phytotronic chamber with the same conditions described before and were watered once a week with autoclaved tap water. Plants were harvested after 6 and 12 weeks post-inoculation (**Supplementary Data S2, Figure S5**).

Plant Sampling and Preparation for Microscopy

At sampling, i.e. at 6 and 12 weeks post-inoculation, secateurs were cleaned with Incidin™ (Ecolab, UK) and used to cut plants up to 15 cm above the inoculation point. All samples with different treatments were then kept in sterile paper bags at 4°C to avoid fungal growth (Mukherjee et al., 2013; Domingues et al., 2016). Samples were then prepared for analyzing the inoculation point by dividing the samples in small parts (technical replicates) (**Supplementary Data S2, Figure S4**).

Confocal Laser Scanning Microscopy

Observations of fungi and plants were carried out using a confocal microscope (Olympus Fluoview FV1000 with multi-line laser FV5-LAMAR-2 and HeNe(G)laser FV10-LAHEG230-2, Japan). No additional treatment was applied to avoid destruction or reduction of the GFP and tdTom signals. Observations with the confocal microscope were done with objectives of 10×, 20×, and 40× and between 20 and 40 X, Y, Z pictures containing 20 to 60 scans were separately taken at wavelengths of 405, 488, 549 nm in blue/green/orange-red channels, respectively, with the same settings each time. The Imaris 9.3 software (Oxford Instruments) was then used at the confocal microscope to visualize 3D reconstructions. 3D modelling was further applied to the pictures to improve the fungal images. Each CSLM analyze of pure culture interaction consisted of three biological replicates that were analyzed containing three technical replicates. Plant interaction analyses consisted of 12 biological replicates that were analyzed containing four technical replicates.

Scanning Electron Microscopy (SEM)

Observations of hyphae and spores of pure cultures and vine plant samples were carried out using a scanning electron microscope Hitachi TM3030 device (Hitachi, Germany) to further describe niches of fungal colonization in addition to the confocal microscopy. This study was performed immediately after the analysis with CSLM microscope. For this purpose, pure cultures or plant samples were frozen and fixed with a cooling stage at −25 C. A 15 kV accelerating voltage was used. Each SEM analyze of pure culture interaction consisted of three biological replicates that were analyzed containing five technical replicates. Plant interaction analyze consisted of 12 biological samples with five technical replicates. Each technical replicate was taken at three different magnifications and at least five pictures of every zone were taken where an interaction or a typical fungal structure were visualized. Combined analyses (CSLM and SEM) performed over *Trichoderma*-pathogen-plant interaction consisted of 12 biological samples containing three technical replicates.

RESULTS

Identification of *Trichoderma* sp. T154 and *Trichoderma* sp. T154::*tdTom3* by CSLM and SEM Analysis

A preliminary identification of *Trichoderma* sp. T154, based on the analysis of ITS sequences by BLASTn software, indicates that it would correspond to a species close to *T. harzianum*. Thus, this fungal strain belongs to the clade Green Spored according to Jaklitsch and Voglmayr (2015). However, a more detailed phylogenetic analysis, based on the comparison of a concatenated sequence of six housekeeping genes (i.e. *act1, cal1, fas1, lcb2, rpb2*, and *tef1*) with the same sequences of 12 species belonging to the same or to other close *Trichoderma* clades (**Supplementary Data S1: Table S1**), indicated that the strain isolated in the present work, even when it is very close to some species of the Green Spored clade, could not be assigned to a species name (**Supplementary Data S1: Figures S1 and S2**). Pairwise distance values obtained from this analysis further

confirmed this conclusion (**Supplementary Data S1: Table S2**). As result, based on these data, the strain isolated in this work was named as *Trichoderma* sp. T154.

This strain exhibited a massive production of spores after 7 days of growth and the typical structures of *Trichoderma* were visualized under normal light microscope. Isolate T154 was further transformed with the *tdTom* gene. As result, six transformants of *Trichoderma* sp. 154 were transferred to selection medium amended with hygromycin and after two selection rounds, a monosporic transformant was chosen. Transformant number 3, named *Trichoderma* sp. T154::*tdTom3*, exhibited the highest fluorescence intensity under a confocal microscope and was selected for further experiments. Conidia and conidiophores of this transformant showed a high red fluorescent signal. The fluorescence was intense and sometimes can be also visualized with punctuated fluorescence along the mycelium.

Similarly, to *Trichoderma* sp. T154, the *tdTom3* transformant also showed the typical *Trichoderma* structures, i.e. conidiophores and phialides (**Supplementary Data S2: Figure S6**).

CSLM and SEM Analysis of *P. minimum* CBS 100398 and *P. minimum*::*gfp7*

P. minimum CBS 100398 was visualized for verifying that the fungus did not present any autofluorescence signal and was only visible under normal light. The *gfp* transformant of this strain showed a strong green fluorescence as expected (**Supplementary Data S2: Figure S7**) and shorts and unbranched conidiophores (**Supplementary Data S2: Figures S7A–C**). Also, typical warts of *P. minimum* on hyphae were observed (**Supplementary Data S2: Figure S8G**). This strain is identified as type II, phialides that are elongate-ampulliform and attenuated at the base tapering toward the apex (**Supplementary Data S2: Figures S8G, I**) (Crous et al., 1996; Mostert et al., 2005).

CSLM and SEM Analysis of the Interaction Between *Trichoderma* sp. T154::*tdTom3* and *P. minimum*::*gfp7* and Wild-Type Strains *In Vitro*

The use of the SEM microscope allowed to visualize the typical structures of both fungi in pure culture conditions with spores, mycelium, and phialides of transformed or wild-type strains, both for *Trichoderma* T154 wild-type, *Trichoderma* sp. T154::*tdTom3, P. minimum* wild type or *P. minimum::gfp7* (**Supplementary Data S2: Figure S8**).

The CSLM microscope was also used for evaluating the interaction between *Trichoderma* sp. T154::*tdTom3* and *P. minimum*::*gfp7*. During dual confrontation assay, *Trichoderma* sp. T154::*tdTom3* was able to colonize the whole Petri dish and exhibited a high ability to overgrow the pathogen (**Figure 1A**). In order to evaluate the overgrowth of *Trichoderma* that was completely covering the pathogen, each sample was evaluated three times over the same point using the tape touch method (Harris, 2000), and three different parts were observed (**Figure 1**): **1**) the upper part where *Trichoderma* was totally overgrowing the pathogen; **2**) a medium part in which there was a strong interaction between both fungi; and **3**) finally, the bottom part, where the pathogen was still resisting the attack of *Trichoderma* (**Figure 1**).

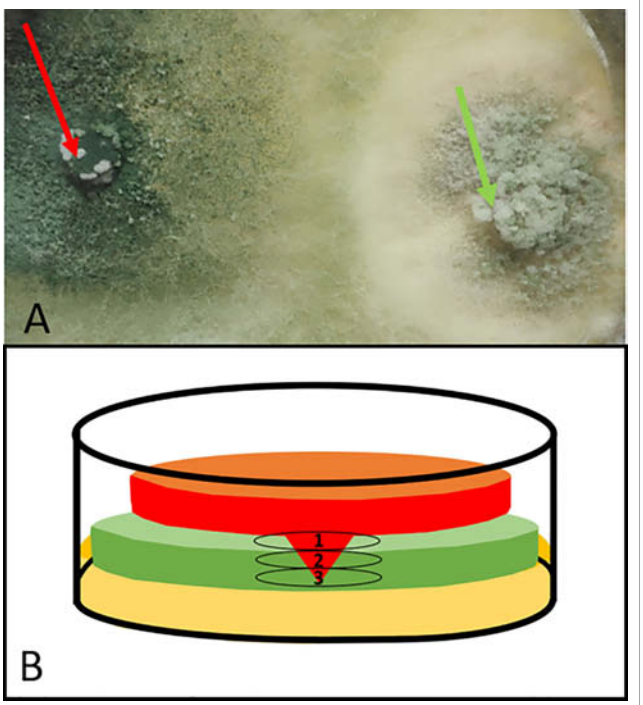

FIGURE 1 | Dual confrontation culture of *Trichoderma* sp. T154::*tdTom3* (left plug) (red arrow) and *P. minimum*::*gfp7* (right plug) (green arrow): **(A)** zoom of a dual culture after 10 days of incubation. Pathogen has been totally colonized. **(B)** Drawing of a transversal section of Petri dish over the interaction between *Trichoderma* sp. T154::*tdTom3* (red) *and P. minimum*::*gfp7* (green) where three different levels can be observed. From the upper part to the bottom. 1, Upper Part. 2, Medium Part. and 3, Bottom Part. Light brown color at the bottom part reflects media culture.

Each part was analyzed separately (**Figure 1B**). In the upper part, both fungi were confronted. Thus, a massive and strong presence of both fungi was detected under the microscope (green and red) (**Figures 2A, B**). Another situation was found in the upper part, where *P. minimum*::*gfp7* was trying to grow but *Trichoderma* sp. T154::*tdTom3* did not allow it due to a high amount of mycelium of the beneficial fungus (**Figures 2C, D**). In the medium part, the mycelia of both fungi were intimately linked (yellow color due to combination of green and red fluorescence) (**Figures 2E, F**). During this interaction, *Trichoderma* spores were detected over the *P. minimum*::*gfp7* and also mycelium of *Trichoderma* sp. T154::*tdTom3* was growing close to the mycelium of *P. minimum*::*gfp7* (**Figure 2F**). And finally, in the bottom part, green fluorescence color was predominant, indicating that most hyphae belong to *P. minimum*::*gfp7* (**Figure 2G**).

SEM analysis was further performed to visualize the interaction between both fungi in all interaction zones. The co-cultivation resulted in a mix of both mycelia (**Figure 3A**). Both fungi started to interact by growing in parallel (**Figures 3B, C**). *Trichoderma* spores were also observed, adhered to the

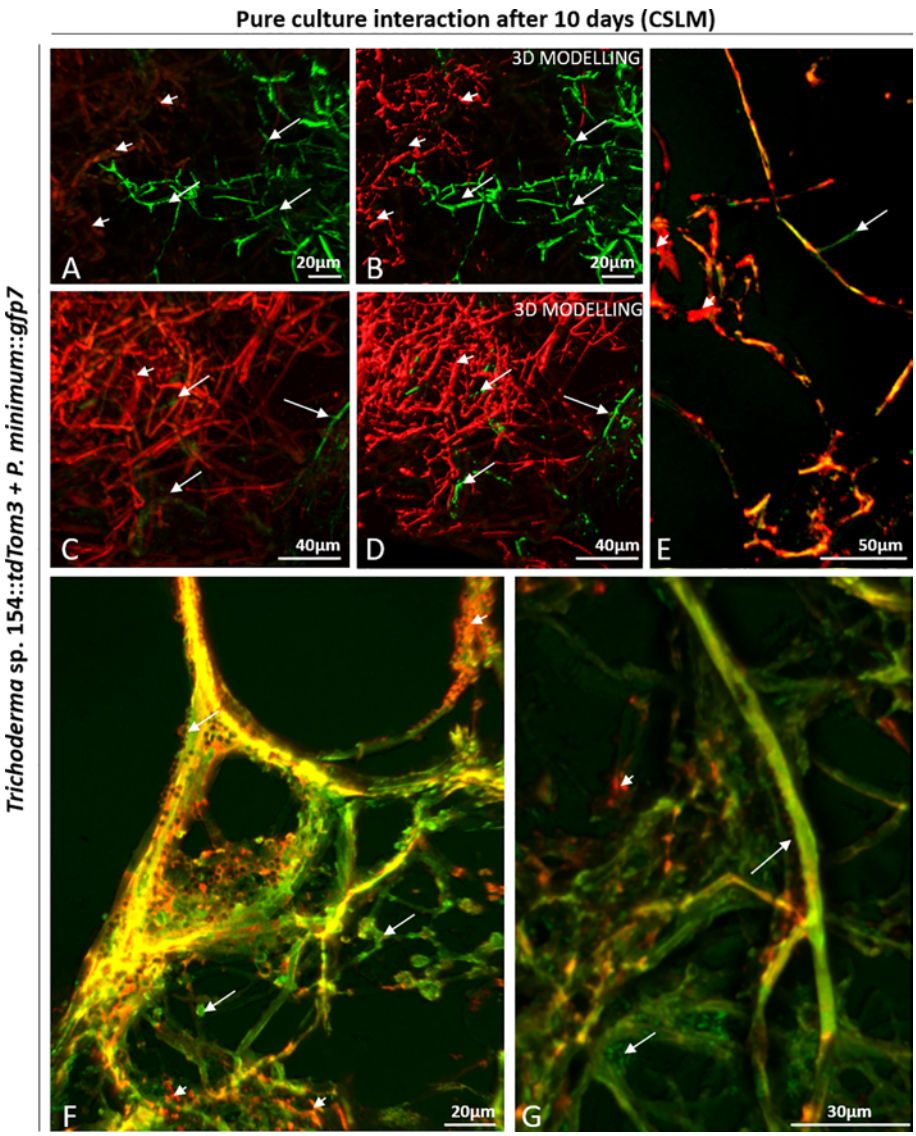

FIGURE 2 | CSLM observation of the interaction zone in a dual culture of *Trichoderma* sp. T154::*tdTom3* (red) (arrowheads) and *P. minimum*::*gfp7* (green) (arrows). **(A–D)** Upper part, confrontation zone between *Trichoderma* sp. T154::*tdTom3 and P. minimum*::*gfp7*. **(E, F)** Medium part, *Trichoderma* sp. T154::*tdTom3* is controlling *P. minimum*::*gfp7*, and a clear interaction between both fungi is observed. **(G)** Bottom part of the sample, zone where *P. minimum*::*gfp7* is established. Representative pictures of biological replicates (3) are presented in this figure.

pathogen´s hyphae (**Figures 3C, D**). Also, conidiophores of *Trichoderma* were detected, which were producing spores that were stuck to the mycelium of *P. minimum*::*gfp7* and where a hyphal coiling was observed (**Figures 3D, E**).

Evaluation of Plant Inner Tissues After 6 Weeks Post *In Planta* Inoculation Using CSLM Microscope

Plant samples were first evaluated after 6 weeks post inoculation. Mock controls were evaluated to confirm the lack of green or red fluorescence in any of the plant tissues after adjusting the settings to only detect GFP and red fluorescence from the fungi and to ensure that plants had not any type of structural alteration. First, regions close to the injury showed no presence of any red and green fluorescence in parenchymatic tissues (**Figure 4A**). Furthermore, no sign of transformed strains was found in dead tissues, where injury was performed with a PDA plug (**Figure 4B**). Other tissues close to the injury were also evaluated for confirming that mock controls did not show the presence of any kind of alteration and any green or red fluorescence (**Figure 4C**).

Colonization by *Trichoderma* sp. T154::*tdTom3* inoculated in plant vines was evaluated. Close to the injury, plant fibers were colonized by this fungus with small groups of spores (**Figures 4D, E**), but spores were also observed through different locations around the injury zone (**Figure 4F**). Fungal hyphae were also

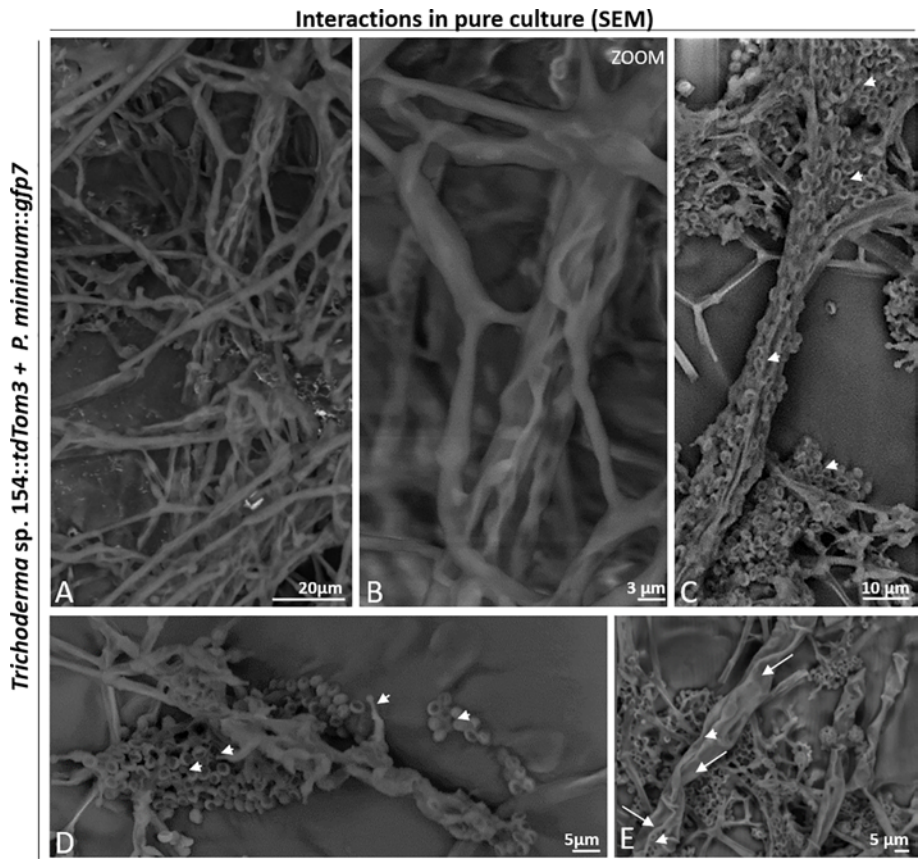

FIGURE 3 | Interaction between *Trichoderma* sp. T154::*tdTom3* (arrowhead) and *P. minimum*::*gfp7* (arrows) in pure culture. **(A)** General overview of the interaction of both fungi. **(B)** Interaction of different hyphae of *Trichoderma* sp. T154::*tdTom3* over a hyphae of *P. minimum*::*gfp7* showing mycoparasitism. **(C)** Spores of *Trichoderma* sp. T154::*tdTom3* colonizing *P. minimum*::*gfp7*. **(D)** Mycelium of both fungi in contact. Spores and structures of *Trichoderma* can be distinguished. **(E)** Different hyphae of *Trichoderma* sp. T154::*tdTom3* colonizing *P. minimum*::*gfp7*. Representative pictures of biological replicates (3) are presented in this figure.

observed in this area (**Figures 4G, H**). However, *Trichoderma* sp. T154::*tdTom3* was not found far from the injury zone, and most of its presence was restricted to spores (**Figures 4D, I**) and few mycelial structures (**Figures 4G, H**).

P. minimum::*gfp7* was mainly found, 6 weeks post inoculation, close to the inoculation point (**Figure 5A**) and full of hyphae were found around and inside the wood fibers or the parenchyma in this area (**Figure 5B**). This fungus was also present in alive tissues, but only in the xylem vessels (**Figures 5C–E**). Interestingly, no symptoms of esca or grapevine trunk diseases were detected.

Finally, the interaction between *P. minimum*::*gfp7* and *Trichoderma* sp. T154::*tdTom3* was evaluated *in planta*. Most images show the presence only of *Trichoderma* sp. T154::*tdTom3* (**Figures 5F–K**). In the injury zone, dead tissues were mostly colonized by hyphae of *Trichoderma* sp. T154::*tdTom3* (**Figure 5F**). In addition, 3D modelling demonstrated a medium degree of fungal colonization of the injury zone (**Figure 5G**). Also, fungal spores were found in this tissue (**Figures 5H, K**) as well as dead and alive fungal material exhibiting a very few blue fluorescence that was revealed by 3D modelling (**Figure 5K**) (corresponding to fungal cell-walls of either *Trichoderma*, *Phaeoacremonium* or natural endophytes). In dual inoculation, no fluorescence of *P. minimum*::*gfp7* was found in the injury zone (**Figures 5F–H, K**), and only in a few cases the mycelium of *P. minimum*::*gfp7* was observed in areas where *Trichoderma* sp. T154::*tdTom3* was present (**Figures 5I, J**).

Evaluation of Plant Inner Tissues After 6 Weeks Post *In Planta* Inoculation by Comparing CSLM and SEM Microscope

CSLM and SEM were used over same tissues for evaluating fungal-plant interaction. First, using a CSLM, transformed fungi were identified using this technique because of the color emitted by fluorescent green and red proteins, and secondly, a SEM microscopy was performed to define the type of interaction.

CSLM microscope images have shown that many different tissues were colonized by the two fungi but they were never found in the same position (**Figures 6A–E**).

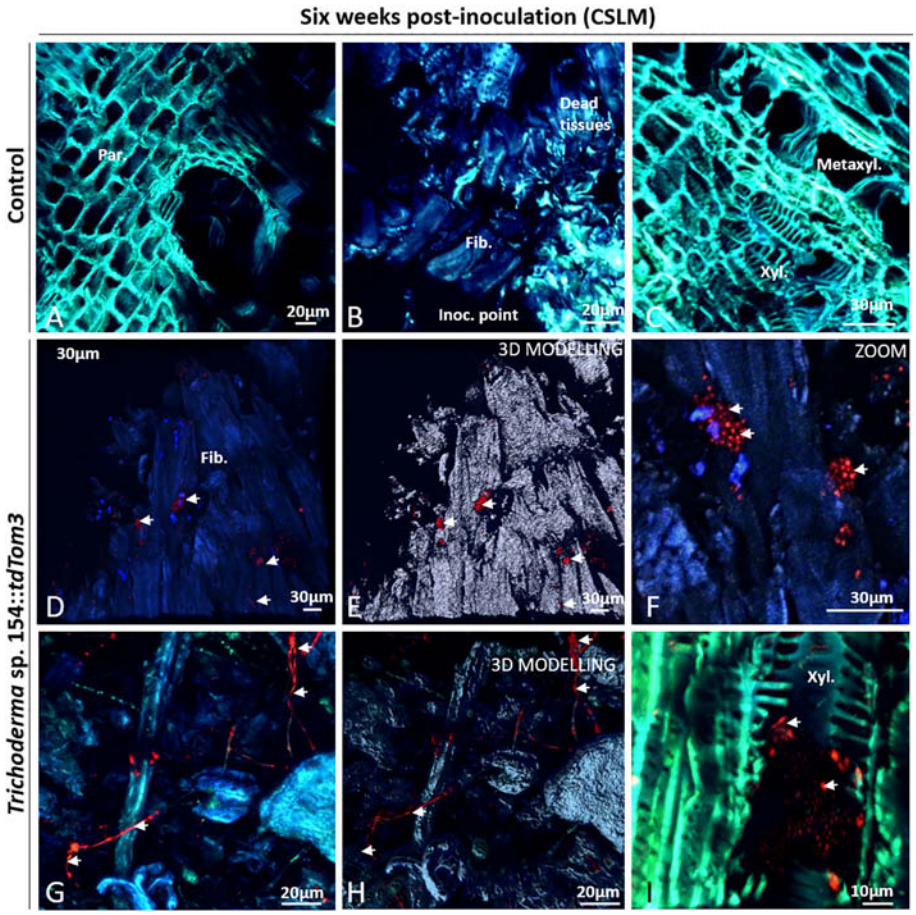

FIGURE 4 | CSLM Control plants and plants inoculated with *Trichoderma* sp. T154*::tdTom3* (arrowhead) after 6 weeks. **(A)** Parenchyma zone without any sign of fluorescence. **(B)** Inoculation point showing different tissues free of fungi. **(C)** Xylem zone and occlusion close to the injured zone without any sign of fluorescent fungi. **(D**, **E)** Inoculation point colonized by spores and its 3D modelling identifying clearly all spores. **(F)** Zoom of the previous picture for showing a big group of *Trichoderma* sp. T154*::tdTom3* alive spores dispersed randomly inside tissues. **(G**, **H)** Inside dead tissues is possible to identify *Trichoderma* sp. T154*::tdTom3 mycelium*. **(I)** Xylem section showing spores of *Trichoderma* sp. T154*::TdTom3* inside it. Fib.: fibers, Inoc. point: inoculation point, Metaxyl.: metaxylem, xyl.:xylem. Representative pictures of biological replicates (12) are presented in this figure.

In both cases, the green and red fluorescences were located in different places without showing clear interactions between the fungi (**Figure 6A**). In **Figure 6C**, both fungi were found colonizing parenchymatic tissues but in different locations without showing any kind of interaction. Hyphae of *Trichoderma* sp. T154::*tdTom3* were found in the xylem vessels but growing in parallel to them, showing the behavior of a beneficial endophyte without causing any damage to the plant (**Figure 6D**). Using CSLM microscope we observed that other tissues were colonized (**Figure 6E**). A high performance of colonization in some parts was visualized for *Trichoderma* sp. T154::*tdTom3* and a strong but rare colonization of *P. minimum*::*gfp7* was observed (**Figure 6E**). No hyphal coiling was proved but *Trichoderma* was found as colonizing fibers possibly with another fungus (**Figures 6F, G**) and *P. minimum*::*gfp7* was detected in other places (**Figure 6E**).

Evaluation of Plant Inner Tissues After 12 Weeks Post *In Planta* Inoculation Using CSLM Microscope

After 12 weeks post inoculation, no differences were visualized in any structure of vine plants in comparison to mock inoculation (**Figures 7A–C**).

In the case of *Trichoderma*, after 12 weeks, large groups of spores and very few hyphae of *Trichoderma* sp. T154::*tdTom3* were found over the inoculation point (**Figure 7D**). In comparison to 6 weeks post inoculation, mycelium was indeed poorly found and spores were mainly visualized. In the wood fibers, xylem or parenchyma, close to the injury, a great number of *Trichoderma* sp. T154::*tdTom3* spores were also observed (**Figures 7E, F**). Thus, a poor colonization by the *Trichoderma* strain was observed after 12 weeks post inoculation.

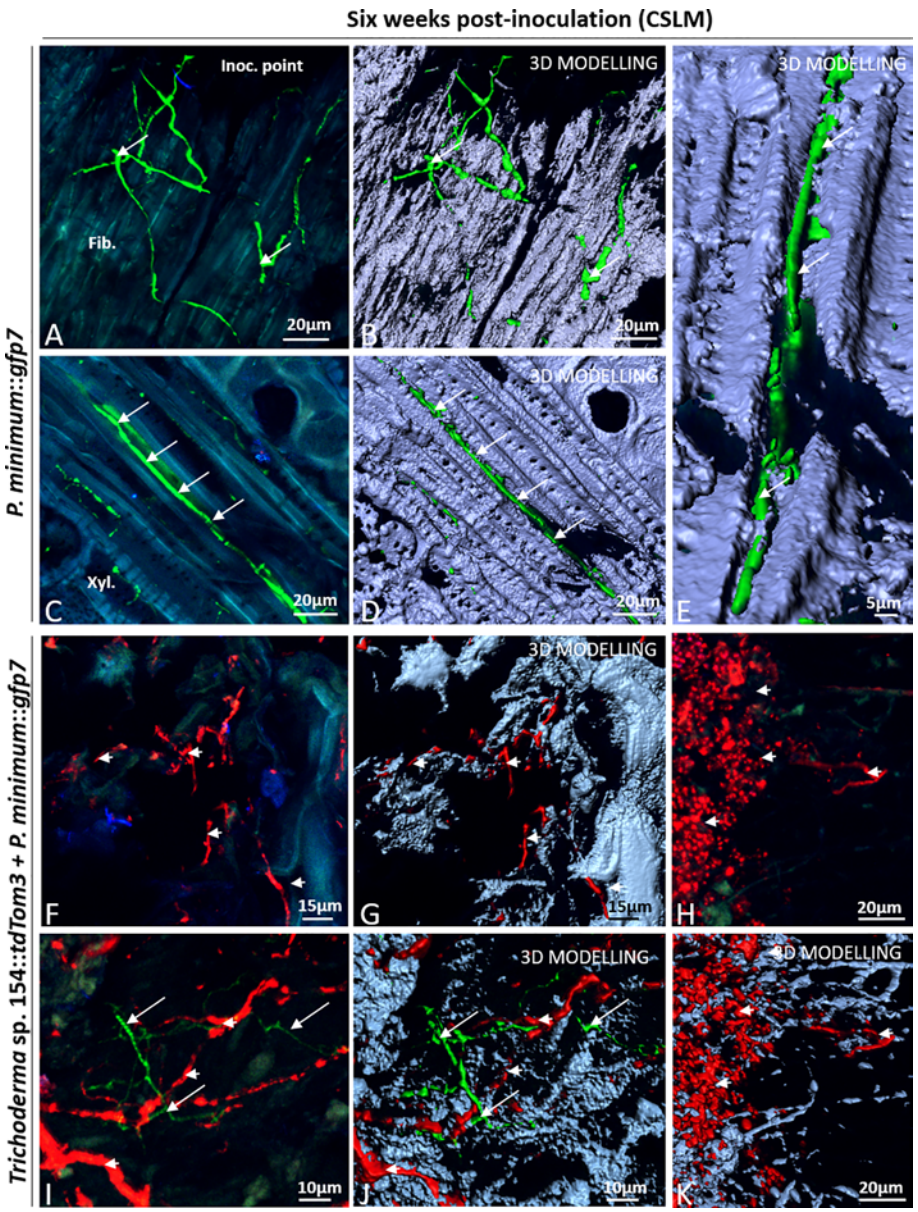

FIGURE 5 | CSLM in plants inoculated with *P. minimum*::*gfp7* (arrows) and plants inoculated with both fungi, *Trichoderma* sp. T154::*tdTom3* (arrowhead) and *P. minimum*::*gfp7* (arrows) after 6 weeks. **(A, B)** Mycelium of *P. minimum*::*gfp7* colonizing fiber tissues. **(C–E)** Hyphae of *P. minimum*::*gfp7* between xylem and phloem vessels following transversal section of tissues. **(F, G)** Dominance of *Trichoderma* sp. T154::*tdTom3* mycelium in dead tissues. **(H–K)** Presencce of spores and some hyphae of *Trichoderma* sp. T154::*tdTom3* can be identified. **(I, J)** Interaction into plant between *Trichoderma* sp. T154::*tdTom3 and P. minimum*::*gfp7* in different locations. Fib.: fibers, Metaxyl.: metaxylem, Phl: phloem, Xyl.:xylem. Representative pictures of biological replicates (12) are presented in this figure.

In the case of single inoculation of *P. minimum*::*gfp7*, mycelia were visualized mainly colonizing the xylem, wood fibers or parenchyma in dead tissues close to the injury (**Figures 8A–E**).

In dual inoculation, *Trichoderma* spores were found in most images (**Figures 8 F–I**). A very few hyphae of *P. minimum*::*gfp7* was observed over different locations but they were found as colonizing the same tissues as *Trichoderma* sp. T154::*tdTom3* (**Figures 8G, H, J, K**). *Trichoderma* sp. T154::*tdTom3* was found mainly at xylem tissues (**Figures 8I, J**). No *in planta* interaction was found after 12 weeks between *Trichoderma* and the pathogen, so that, no SEM was performed for this reason.

DISCUSSION

Previous studies have evaluated *Trichoderma* strains isolated from grapevine plants against *P. minimum* under *in vitro* conditions (Kotze et al., 2011; Carro-Huerga et al., 2017). Also,

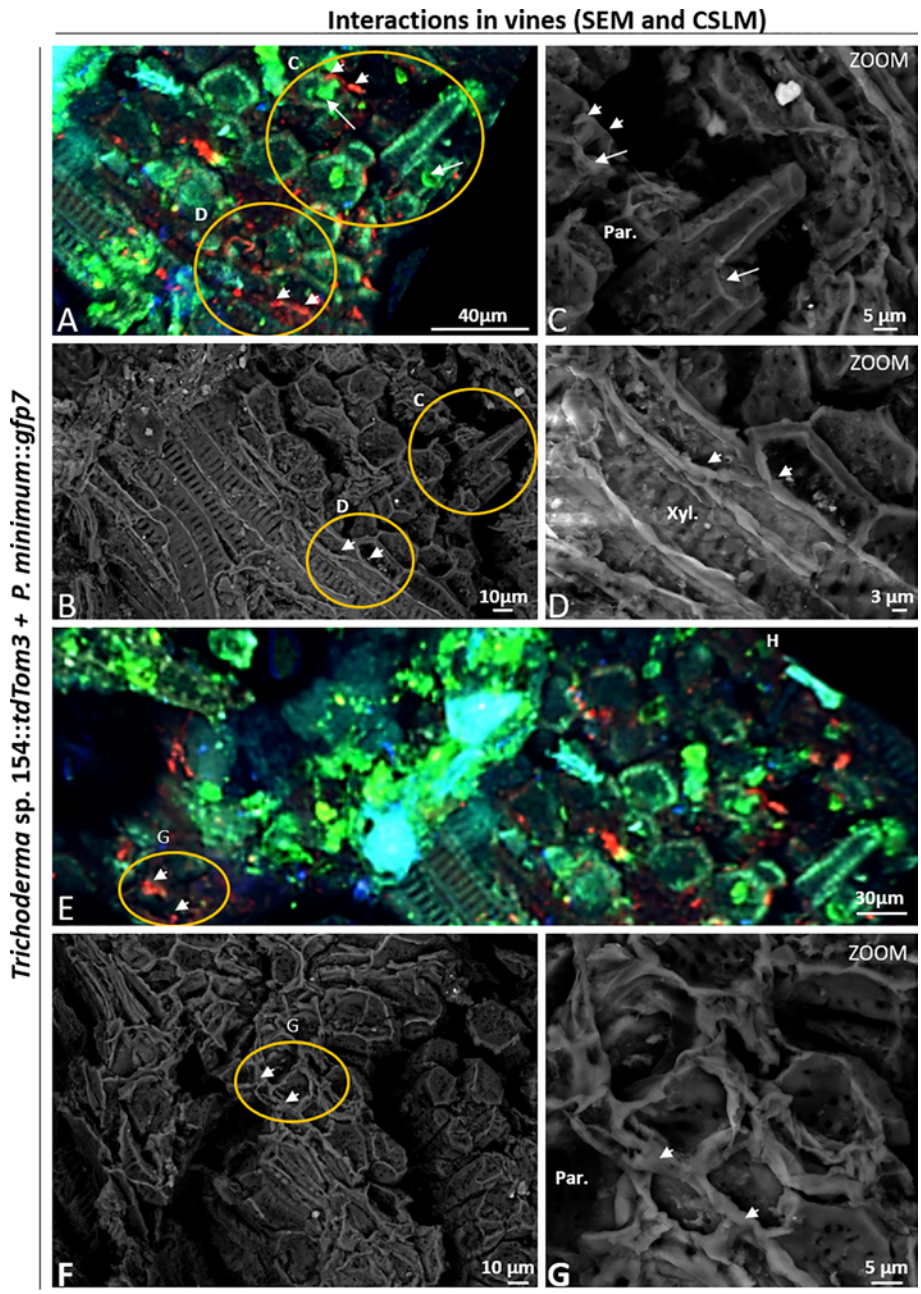

FIGURE 6 | CSLM and SEM in plants inoculated with *P. minimum*::*gfp7* (arrows) and *Trichoderma* sp. T154::*tdTom3* (arrowhead) *and P. minimum*::*gfp7* (arrows) after 6 weeks. **(A**, **B)** Detail of vine plant tissues that were analyzed by CSLM in the injury. **(C)** *P. minimum*::*gfp7* and *Trichoderma* sp. T154::*tdTom3* colonizing parenchyma tissues in different locations. **(D)** *Trichoderma* sp. T154::*tdTom3* over xylem vessels. **(E**, **F)** Another detail of vine plant tissues that were evaluated using CSLM in the injury**. (G)** *Trichoderma* sp. T154::*tdTom3* colonizing parenchymatic tissues. Par.: parenchyma, Xyl.: Xylem. Representative pictures of biological replicates (12) are presented in this figure.

the influence of *Trichoderma* species inoculated on grapevine cultivars has been previously described for protecting vine against GTD´s (Mutawila et al., 2011a). The optimum period for inoculating *Trichoderma* is 6 h after pruning in cultivars Chenin Blanc and Cabernet Sauvignon in field conditions (Mutawila et al., 2016a). Moreover, different experiments about colonization have described a good wound colonization after applying *Trichoderma* strains (John et al., 2005; John et al., 2008) Mutawila et al. (2011b) described grapevine woody tissue colonization by *Trichoderma harzianum* using strains carrying reporter genes, one expressing *gfp* (STE-U 6517) and the other *DsRed* (STE-U 6518). Mutawila et al. (2011c) described grapevine woody tissue colonization by *Trichoderma harzianum* using strains carrying reporter genes, one

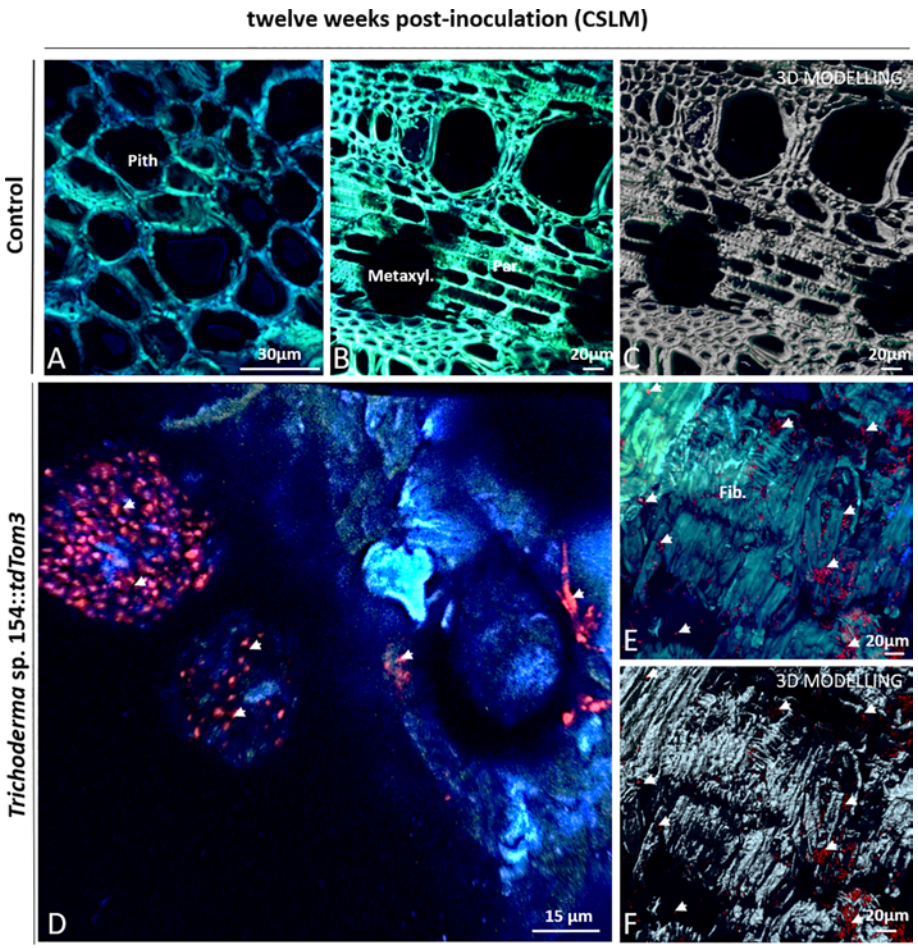

FIGURE 7 | CSLM Control plants and plants inoculated with *Trichoderma* sp. T154::*tdTom3* (arrowhead) after 12 weeks. **(A)** Pith zone without any sign of fluorescence. **(B, C)** Xylem zone and occlusion close to injured zone without any sign of fluorescent fungi. **(D)** Magnification of the inoculation point. *Trichoderma* sp. T154::*tdTom3* spores can be seen with some hyphae. **(E, F)** Tissue colonized with plenty of *Trichoderma* sp. T154::*tdTom3* spores distributed in fiber elements. Fib.: fibers, Metaxyl.: metaxylem, Par: parenchyma. Representative pictures of biological replicates (12) are presented in this figure.

expressing *gfp* (STE-U 6517) and the other *DsRed* (STE-U 6518) (Mutawila et al., 2011b). For pathogens such as *P. chlamydospora* and *E. lata*, over one-year-old canes were then used for evaluating the efficacy of the *Trichoderma* strain, and the authors concluded that *Trichoderma harzianum* reduced the growth of the inoculated pathogens, being re-isolated more frequently from the xylem than from the pith and reaching at maximum depths of 30 mm in dual-inoculated shoots. Albeit (GFP)-labeled *Trichoderma harzianum* strains were used, no *Trichoderma* strains were visualized in grapevines nor their behavior inside tissues, rather they were used for re-isolation from plant tissues. Only the pathogens were visualized *in planta*.

There was no evidence using biomarkers, such as GFP or tdTom, about the three-way interaction *Trichoderma*-plant-pathogen, which corresponds to an approach that would simulate the natural interactions occurring in agro-ecosystems (Lu et al., 2004; Vinale et al., 2008).

In our study, we deciphered the location of a *Trichoderma* indigenous strain inside the plant and its behavior as an endophyte in grapevine wood; also, its interaction with the pathogen *P. minimum*, related to esca and Petri diseases, was tested in *in vitro* assay and in grapevine plants by CSLM and SEM.

Some strains of *Trichoderma* spp. are endophytes (Jaklitsch et al., 2006; Gazis et al., 2011) and several strains are recognized as promising biological control agents against GTDs (González and Tello, 2011). For instance, strains of this genus have been tested during the last 15 years against Botryosphaeria dieback, Esca complex and Eutypa dieback with promising positive results (Mondello et al., 2018). *Trichoderma* genus is the second most abundant of the endophytic mycobiota in woody tissues (Bruez et al., 2014). The use of grapevine endophytes shows a significant protection against other grapevine diseases (Ferrigo et al., 2017). Thus, it can be an important source for searching for an efficient

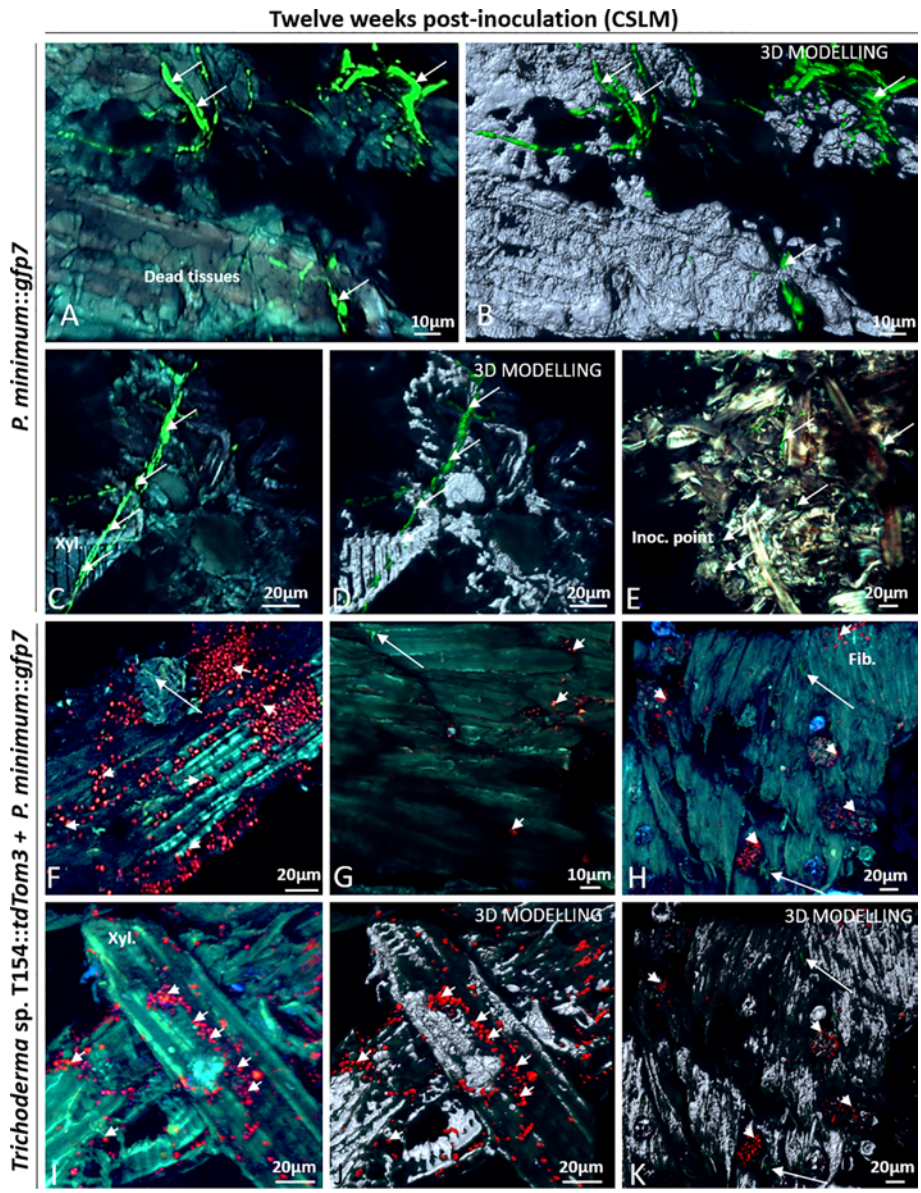

FIGURE 8 | CSLM over plants inoculated with *P. minimum*::*gfp7* (arrows) and plants inoculated with both fungi, *Trichoderma* sp. T154::*tdTom3* (arrowhead) *and P. minimum*::*gfp7* (arrows) after 12 weeks. **(A, B)** Fiber tissues colonized by hyphae of *P. minimum*::*gfp7*. **(C, D)** Xylem colonized by *P. minimum*::*gfp7* and expanding to other tissues. **(E)** Inoculation point colonized partially by *P. minimum*::*gfp7*. **(F)** Presence of spores of *Trichoderma* sp. T154::*tdTom3* colonizing fibers and some hyphae of *P. minimum*::*gfp7*. **(G–I)** Few colonization of hyphae of *P. minimum*::*gfp7* and spores of *Trichoderma* sp. T154::*tdTom3*. **(J, K)** Spores of *Trichoderma* sp. T154::*tdTom3* over xylem vessels. Fib.: fibers, Inoc. point: inoculation point, Xyl.:xylem. Representative pictures of biological replicates (12) are presented in this figure.

biological control strain. However, the location of these fungi inside the plant has been poorly studied, and we indeed did not know before this study where the *Trichoderma* strains could be present in vines as well as on how it can interact with some vine pathogens as *P. minimum*.

In this study, we evaluated the exact location of one strain of *Trichoderma* that was isolated from grapevine cv. Tempranillo and its behavior upon penetration into the same variety and into a 1-year-old injured tissue. Thus, transformants of *Trichoderma* sp. T154 with *tdTom* gene were selected, which allowed to visualize red fluorescence under fluorescence microscopy. The strain was identified as colonizing parenchyma, fibers, and xylem vessels inside wood mainly after 6 weeks post inoculation. Fluorescence of *P. minimum* (GFP) allowed to distinguish both fungi in co-inoculation. The *P. minimum* strain was located mainly in xylem vessels as an endophyte during times of experiments.

To ensure that interactions are the same between wild-type strains and transformed fungi, *in vitro* assays on plates were

performed (see **Supplementary Material**). Previously to analyzing any kind of interaction in grapevine plants, plates with *Trichoderma* sp. T154, *Trichoderma* sp. T154::*tdTom3*, *P. minimum*, *P. minimum*::*gfp7*, and interaction of both transformed fungi were visualized by CSLM and in SEM, which let to identify conidiophores, conidia, and hyphae. These fungi were also visualized under normal light (data not shown) to show that no macroscopic and phenotypic differences were found between the transformants and their wild-type strains.

Trichoderma sp. strain T154 was able to overgrow the pathogen. The most usual observation was adhesion of spores to the pathogen hyphae. Coiling of hyphae was also observed, albeit rarely. In some cases coiling interaction was indeed identified in agreement with previous descriptions (Lu et al., 2004), but only in a few cases.

The main mycoparasitism mechanisms exhibited by *Trichoderma* in this work were spore adhesion and parallel growth adhesion. These results are in agreement with previous reports, analyzing a *T. harzianum* strain against different wood decay fungi (Murmanis et al., 1988). Alignment of *Trichoderma* with the host hyphae and spore adhesion were identified as the main mechanisms of action. In this case, no typical coiling and hooks were visualized. Also a *T. harzianum* strain from the product Biotricho [Agro-Organics (PTY) Ltd., RSA] showed hyphal adhesion as a main mechanism of action against *P. minimum* as determined with a microscopic analysis. Furthermore, strains belonging to other *Trichoderma* species showed this kind of interaction against other pathogens such as *Diplodia seriata* or *Neofusicoccum parvum* (Kotze et al., 2011). Also, parallel growth was observed for the *T. harzianum* strains AG1, AG2, and AG3 (Agrimm Technologies Ltd, New Zealand) (John et al., 2004).

In our study the niches of colonization were analyzed. After 6 weeks, *P. minimum*::*gfp7* was only found close to the fibers next to the injury and mainly very close to the inoculation point, which is in agreement with previously described results (Fleurat-lessard et al., 2014; Pierron et al., 2015). *P. minimum*::*gfp7* was able to colonize xylem. In comparison to Pierron et al. (2015), a different scion and kind of tissue (one-year-old tissue) was assayed. No fungus was found outside of the inoculation points. In most of the sites tested, a strong fungal presence was shown mainly in all dead tissues in the injury and xylem vessels. Thus, a different adaptation to the cultivar could be a reason for the observed differences.

After 6 weeks post inoculation, *Trichoderma* sp. T154::*tdTom3* exhibited a good development in plant, which resulted in the finding of a high proportion of hyphae and spores. Plant fibers were colonized, with different groups of spores and hyphae inside this type of tissue. However, after 12 weeks, the strain of *Trichoderma* was found at a very low rate only in fibers. Most of *Trichoderma* hyphae started to colonize the injury but after 12 weeks most of them were not fluorescent, which would indicate a poor endophytic ability of this strain inside the grapevine plant or a lack of development due to not enough nutrients, pH, moisture. Thus, further studies are needed for evaluating the capability of growing under these conditions to optimize applications.

In this work, the environmental conditions in the growth chamber were 24°C and 45% of relative humidity, which were similar to those described previously (Pierron et al., 2015). Good results were obtained at a combination of high relative humidity and high temperature (24°C × 95°C and 100%RH and 28°C and 95%RH) with BCAs (Hannusch and Boland, 1996), being a very important factor for a successful colonization. This refers that different climatic events such as raining could improve *Trichoderma* colonization.

Colonization of grapevine plants by *Trichoderma* sp. T154::*tdTom3* and *P. minimum*::*gfp7* (after 6 and 12 weeks) revealed less quantity of *P. minimum* in most of the sites investigated. After the evaluation of 6 weeks post inoculation was done, most of *P. minimum* mycelium had disappeared and only spores have been able to survive in comparison to *Trichoderma* sp. T154::*tdTom3*. Spores of *Trichoderma* sp. T154 were found in xylem vessels and fibers. Inoculation was done inside the plant, so no bark colonization was analyzed. No movement of fungal mycelium and spores through the plant was detected. This finding suggests that both fungi did not show a spread of colonization during this short period of time. But, according to the reduction of pathogen inoculum, if any of these fungi arrives and colonizes the injury, it will not allow the other one to grow, hence prevention is the most important thing for avoiding GTD´s in vines. In conclusion, applying a biocontrol agent after pruning or planting could protect plant for the most critical period when the plant is very vulnerable to be infected.

Both fungi colonize parenchymatic cells and were located around plant cells enabling normal development of the plant. Xylem vessels and parenchymatic tissues were other types of tissues colonized by the *Trichoderma* strain. In addition, no interaction between the two fungi was found in grapevine plants where fungi where able to establish different niches of colonization inside the plant. However, the pathogen colonization was reduced. This reinforces the idea that the first fungus that arrives and is established outcompetes the other one. Mechanisms are likely related to secretion of antifungal substances by the *Trichoderma* strain, induction of the plant systemic resistance, and niche exclusion. This is in agreement with previous reports, indicating that competitive exclusion would be a key factor for persisting in the wood (Mutawila et al., 2011a).

CONCLUSION

The results indicate that an indigenous *Trichoderma* strain can reduce *P. minimum* plant colonization during their endophytic colonization and can also exclude the pathogen from plant niches. Both fungi colonize different plant tissues, such as xylem vessels and parenchymatic cells. Albeit hyphal coiling from *Trichoderma* around pathogen is a well-known mechanism of action described for *Trichoderma* spp., our study shows that rather spore adhesion and niche exclusion constitute the main mechanism of action for biocontrol of the pathogen as analyzed by microscopic studies. Indigenous *Trichoderma* spp. have

potential for reducing the colonization of *P. minimum*, a pioneer agent causing GTD. Thus, an inoculation of one of these BCAs can protect the vine plant by limiting the development of the disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

GC-H, SC, MG, RC, SG, and PC designed the experiments. GC-H, RC, SG, and MG made the fungal transformation. GC-H and SC made the observation. GC-H, SC, MG, RC, MS, SG, and PC performed the data interpretation and manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01170/full#supplementary-material

REFERENCES

Álvarez-Pérez, J. M., González-García, S., Cobos, R., Olego, M.Á., Ibañez, A., Díez-Galán, A., et al. (2017). Use of endophytic and rhizosphere actinobacteria from grapevine plants to reduce nursery fungal graft infections that lead to young grapevine decline. *Appl. Environ. Microbiol.* 83, 1–16. doi: 10.1128/AEM.01564-17

Bertsch, C., Ramírez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., et al. (2013). Grapevine trunk diseases:complex and still poorly understood. *Plant Pathol.* 62, 243–265. doi: 10.1126/science.324

Bruez, E., Vallance, J., Gerbore, J., Lecomte, P., Da Costa, J. P., Guerin-Dubrana, L., et al. (2014). Analyses of the temporal dynamics of fungal communities colonizing the healthy wood tissues of esca leaf-symptomatic and asymptomatic vines. *PloS One* 9, 1-15. doi: 10.1371/journal.pone.0095928

Caasi, O. C., Walker, N. R., Marek, S. M., Enis, J. N., and Mitchell, T. K. (2010). Infection and colonization of turf-type bermudagrass by *Ophiosphaerella herpotricha* expressing green or red fluorescent proteins. *Phytopathology* 100, 415–423. doi: 10.1094/PHYTO-100-5-0415

Cardoza, R. E., Vizcaino, J. A., Hermosa, M. R., Monte, E., and Gutiérrez, S. (2006). A comparison of the phenotypic and genetic stability of recombinant *Trichoderma* spp. generated by protoplast- and *Agrobacterium*-mediated transformation. *J. Microbiol.* 44, 383–395.

Carro-Huerga, G., Mayo-Prieto, S., Rodriguez-Gonzalez, A., Alvarez-García, S., Gutiérrez, S., and Casquero, P. A. (2017). "*Trichoderma* strains isolated from vineyards in Castile-Leon (Spain) against *Phaeoacremonium aleophilum* in 'in vitro' assays," in *Phytopathologia Mediterranea (Abstract retrieved from Abstracts of oral and poster presentations given at the 10th International Workshop on Grapevine Trunk Diseases, Reims, France, 4–7 July 2017.)*, 513–588. doi: 10.14601/Phytopathol_Mediterr-21865

Carro-Huerga, G., Mayo-Prieto, S., Rodriguez-Gonzalez, A., Gonzalez-Lopez, O., Saiz-Gadea, J., Alvarez-García, S., et al. (2019). "Pruning wound colonization of *Trichoderma* native strains from Castilla y León (Spain) in semi-field conditions in winter," in *Phytopathologia Mediterranea (Abstract retrieved from Abstracts of oral and poster presentations given at the 11th International Workshop on Grapevine Trunk Diseases, Penticton, British Columbia, Canada, July 7–12, 2019)*, 395–449. doi: 10.14601/Phytopathol

Cooper, M., Klonsky, K. M., and De Moura, R. L. (2012). Sample cost to establish a vineyard and produce winegrapes (Cabernet Sauvignon) in the North Coast Region (Napa County). *Univ. Calif. Coop. Ext.* 19.

Crous, P. W., Gams, W., Wingfield, M. J., and van Wyk, P. S. (1996). *Phaeoacremonium* gen. nov. associated with wilt and decline diseases of woody hosts and human infections. *Mycologia* 88, 786. doi: 10.2307/3760973

Di Marco, S., Osti, F., and Cesari, A. (2004). Experiments on the control of esca by *Trichoderma*. *Phytopathol. Mediterr.* 43, 108–115. doi: 10.14601/Phytopathol_Mediterr-1730

Domingues, M. V. P. F., De Moura, K. E., Salomão, D., Elias, L. M., and Patricio, F. R. A. (2016). Effect of temperature on mycelial growth of *Trichoderma, Sclerotinia minor* and *S. sclerotiorum*, as well as on mycoparasitism. *Summa Phytopathol.* 42, 222–227. doi: 10.1590/0100-5405/2146

Ferreira, J. H. S., Matthee, F. N., and Thomas, A. C. (1991). Biological control of *Eutypa lata* on grapevine by an antagonistic strain of *Bacillus subtilis*. *Am. Phytopathol. Soc* 81, 283–287. doi: 10.1094/Phyto-81-283

Ferrigo, D., Causin, R., and Raiola, A. (2017). Effect of potential biocontrol agents selected among grapevine endophytes and commercial products on crown gall disease. *BioControl* 62, 821–833. doi: 10.1007/s10526-017-9847-3

Fleurat-lessard, P., Luini, E., Berjeaud, J., and Roblin, G. (2014). Immunological detection of *Phaeoacremonium aleophilum*, a fungal pathogen found in esca disease. *Eur. J. Plant Pathol.* 139, 137–150. doi: 10.1007/s10658-013-0372-7

Fourie, P. H., and Halleen, F. (2004). Proactive control of Petri disease of grapevine through treatment of propagation material. *Am. Phytopathol. Soc* 88, 1241–1245. doi: 10.1094/PDIS.2004.88.11.1241

Gazis, R., Rehner, S., and Chaverri, P. (2011). Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences. *Mol. Ecol.* 20, 3001–3013. doi: 10.1111/j.1365-294X.2011.05110.x

Geiger, F., Bengtsson, J., Berendse, F., Weisser, W. W., Emmerson, M., Morales, M. B., et al. (2010). Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic Appl. Ecol.* 11, 97–105. doi: 10.1016/j.baae.2009.12.001

González, V., and Tello, M. L. (2011). The endophytic mycota associated with *Vitis vinifera* in central Spain. *Fungal Divers* 47, 29–42. doi: 10.1007/s13225-010-0073-x

Gramaje, D., Urbez-Torres, J. R., and Sosnowski, M. R. (2018). Managing grapevine trunk diseases with respect to etiology and epidemiology: current strategies and future prospects. *Plant Dis.* 102, PDIS–04-17-0512-FE. doi: 10.1094/PDIS-04-17-0512-FE

Halleen, F., Fourie, P. H., and Lombard, P. J. (2010). Protection of grapevine pruning wounds against *Eutypa lata* by biological and chemical methods. *South Afr. J. Enol. Vitic.* 31, 125–132. doi: 10.21548/31-2-1409

Hannusch, D. J., and Boland, G. J. (1996). Interactions of air temperature, relative humidity and biological control agents on grey mold of bean. *Eur. J. Plant Pathol.* 102, 133–142. doi: 10.1007/BF01877100

Harris, J. L. (2000). Letter to the editor: Safe, low-distortion tape touch method for fungal slide mounts. *J. Clin. Microbiol.* 38, 4683–4684. doi: 10.1128/jcm.38.12.4683-4684.2000

Jaklitsch, W. M., Samuels, G. J., Dodd, S. L., Lu, B. S., and Druzhinina, I. S. (2006). *Hypocrea rufa/Trichoderma viride*: A reassessment, and description of five closely related species with and without warted conidia. *Stud. Mycol.* 55, 135–177. doi: 10.3114/sim.2006.56.04

Jaklitsch, W. M., and Voglmayr, H. (2015). Biodiversity of *Trichoderma* (*Hypocreacea*) in southern Europe and Macaronesia. *Stud. Mycol.* 80, 1–87. doi: 10.1016/j.simyco.2014.11.001

John, S., Scott, E. S., Wicks, T. J., and Hunt, J. S. (2004). Interactions between *Eutypa lata* and *Trichoderma harzianum*. *Phytopathol. Mediterr.* 43, 95–104. doi: 10.1400/14578

John, S., Wicks, T. J., Hunt, J. S., Lorimer, M. F., Oakey, H., and Scott, E. S. (2005). Protection of grapevine pruning wounds from infection by *Eutypa lata*. *Aust. J. Grape Wine Res.* 14, 134–142. doi: 10.1111/j.1755-0238.2008.00015.x

John, S., Wicks, T. J., Hunt, J. S., and Scott, E. S. (2008). Colonisation of grapevine wood by *Trichoderma harzianum* and *Eutypa lata*. *Aust. J. Grape Wine Res.* 14, 18–24. doi: 10.1111/j.1755-0238.2008.00003.x

Kotze, C., Van Niekerk, J., Mostert, L., Halleen, F., Fourie, P., Niekerk, J. V. A. N., et al. (2011). Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. *Phytopathol. Mediterr.* 50, 247–263. doi: 10.14601/Phytopathol_Mediterr-8960

Lorch, W. (2014). *Fatal wood disease affects 12 percent of French vineyards* (Accessed November 29, 2019). Available at: https://www.wine-searcher.com/m/2014/10/fatal-wood-diseases-affect-12-percent-of-french-vineyards.

Lu, Z., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M., and Jansson, J. K. (2004). In Vivo study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl. Environ. Microbiol.* 70, 3073–3081. doi: 10.1128/AEM.70.5.3073-3081.2004

Martín, M. T., Cobos, R., Martin, M. T., and Cobos, R. (2007). Identification of fungi associated with grapevine decline in Castilla y León (Spain). *Phytopathol. Mediterr.* 46, 18–25. doi: 10.1017/CBO9781107415324.004

Mayo, S., Gutiérrez, S., Malmierca, M. G., Lorenzana, A., Campelo, M. P., Hermosa, R., et al. (2015). Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes. *Front. Plant Sci.* 6:685. doi: 10.3389/fpls.2015.00685

Mondello, V., Songy, A., Battiston, E., Pinto, C., Coppin, C., Trotel-Aziz, P., et al. (2018). Grapevine trunk diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Dis.* 102, 1189–1217. doi: 10.1094/PDIS-08-17-1181-FE

Mostert, L., Groenewald, J. Z., Summerbell, R. C., Robert, V., Sutton, D. A., Padhye, A. A., et al. (2005). Species of *Phaeoacremonium* associated with infections in humans and environmental reservoirs in infected woody plants. *J. Clin. Microbiol.* 43, 1752–1767. doi: 10.1128/JCM.43.4.1752-1767.2005

Mukherjee, P. K., Horwitz, B. A., Herrera-Estrella, A., Schmoll, M., and Kenerley, C. M. (2013). *Trichoderma* research in the genome era. *Annu. Rev. Phytopathol.* 51, 105–129. doi: 10.1146/annurev-phyto-082712-102353

Murmanis, L. L., Highley, T. L., and Ricard, J. (1988). *Hyphal Interaction of* Trichoderma harzianum *and* Trichoderma polysporum *with wood decay fungi*. ed. U. of California Riverside: Library government publication departament U.S. depository.

Mutawila, C., Fourie, P. H., Halleen, F., and Mostert, L. (2011a). Grapevine cultivar variation to pruning wound protection by *Trichoderma* species against trunk pathogens. *Phytopathol. Mediterr.* 50, 264–276. doi: 10.14601/Phytopathol_Mediterr-8981

Mutawila, C., Fourie, P. H., Halleen, F., and Mostert, L. (2011b). Histo-pathology study of the growth of *Trichoderma harzianum*, *Phaeomoniella chlamydospora* and *Eutypa lata* on grapevine pruning wounds. *Phytopathol. Mediterr.* 50, 46–60. doi: 10.2307/26458710

Mutawila, C., Halleen, F., and Mostert, L. (2016a). Optimisation of time of application of *Trichoderma* biocontrol agents for protection of grapevine pruning wounds. *Aust. J. Grape Wine Res.* 22, 279–287. doi: 10.1111/ajgw.12218

Mutawila, C., Vinale, F., Halleen, F., Lorito, M., and Mostert, L. (2016b). Isolation, production and in vitro effects of the major secondary metabolite produced by *Trichoderma* species used for the control of grapevine trunk diseases. *Plant Pathol.* 65, 104–113. doi: 10.1111/ppa.12385

Pascale, A., Vinale, F., Manganiello, G., Nigro, M., Lanzuise, S., Ruocco, M., et al. (2017). *Trichoderma* and its secondary metabolites improve yield and quality of grapes. *Crop Prot.* 92, 176–181. doi: 10.1016/j.cropro.2016.11.010

Pertot, I., Prodorutti, D., Colombini, A., and Pasini, L. (2016). *Trichoderma atroviride* SC1 prevents *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* infection of grapevine plants during the grafting process in nurseries. *BioControl* 61, 257–267. doi: 10.1007/s10526-016-9723-6

Pierron, R., Gorfer, M., Berger, H., Jacques, A., Sessitsch, A., Strauss, J., et al. (2015). Deciphering the niches of colonisation of *Vitis vinifera* L. by the Esca-associated fungus *Phaeoacremonium aleophilum* using a gfp marked strain and cutting systems. *PloS One* 10, 1–22. doi: 10.1371/journal.pone.0126851

Roblin, G., Luini, E., Fleurat-Lessard, P., Larignon, P., and Berjeaud, J. M. (2019). Towards a preventive and/or curative treatment of esca in grapevine trunk disease: General basis in the elaboration of treatments to control plant pathogen attacks. *Crop Prot.* 116, 156–169. doi: 10.1016/j.cropro.2018.10.016

Sousa, S. (2004). *Mejora del sistema de expresión en Trichoderma harzianum CECT 2413 para la producción de proteínas de interés biotecnológico. PhD thesis* (Sevilla: Universidad de Sevilla), 256p.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., and Lorito, M. (2008). *Trichoderma*–plant–pathogen interactions. *Soil Biol. Biochem.* 40, 1–10. doi: 10.1016/j.soilbio.2007.07.002

White, T. J., Bruns, T., Lee, S., and Taylor, J. W. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics" in *PCR protocols. A guide to methods and applications* (San Diego, CA: Academic Press), 315–322.

Wicks, T., and Davies, K. (1999). The effect of *Eutypa* on grapevine yield. *Aust. Grapegrow Winemak* 406a, 15–16.

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Soil Microbiomes From Fallow Fields Have Species-Specific Effects on Crop Growth and Pest Resistance

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Communities of microorganisms in the soil can affect plants' growth and interactions with aboveground herbivores. Thus, there is growing interest in utilizing soil microbiomes to improve plant performance in agriculture (*e.g.*, for pest control), but little is known about the phenotypic responses of various crop species to different microbiomes. In this study, we inoculated four crop species from different botanical families, maize (*Zea mays*, Poaceae), cucumber (*Cucumis sativus*, Cucurbitaceae), tomato (*Solanum lycopersicum*, Solanaceae), and lettuce (*Lactuca sativa*, Asteraceae), with diverse soil microbiomes originating from actively-managed agricultural fields or fallow fields under varying stages of succession (1, 3, and 16-years post-agriculture) sourced from a large-scale field experiment. We compared the crops' responses to these different microbiomes by assessing their growth and resistance to two generalist insect pests, cabbage looper (*Trichoplusia ni*) and fall armyworm (*Spodoptera frugiperda*). These different microbiomes affected both plant growth and resistance, but the effects were species-specific. For instance, lettuce produced the largest leaves when inoculated with a 3-year fallow microbiome, the microbiome in which cucumber performed worst. Plants were generally more resistant to *T. ni* when inoculated with the later succession microbiomes, particularly in contrast to those treated with agricultural microbiomes. However, for tomato plants, the opposite pattern was observed with regard to *S. frugiperda* resistance. Collectively, these results indicate that plant responses to microbiomes are species-specific and emphasize the need to characterize the responses of taxonomically diverse plant species to different microbiomes.

Keywords: aboveground-belowground interactions, agricultural microbiome, herbivore resistance, old-field succession, plant-soil feedbacks, rhizosphere, *Spodoptera frugiperda*, *Trichoplusia ni*

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INTRODUCTION

Some microorganisms in the soil can improve the performance of plants, and hence, there is growing interest in manipulating soil microbiomes to improve yield and pest control in agriculture (Bell et al., 2019). Soil microbiomes can promote plant growth by enhancing host tolerance to abiotic stresses, such as drought (Lau and Lennon, 2012), as well as to biotic stresses, such as

pathogens and herbivores. Microorganisms can affect plants' resistance to pathogens and herbivores through altering secondary metabolite production, as well as inducing plant defense responses (Ludwig-Müller, 2015; Harun-Or-Rashid and Chung, 2017). One recent study suggests that the rhizosphere microbiome may even serve as a stronger driver than plant genetics in determining plant resistance to insect herbivores (Hubbard et al., 2019). Thus, the soil microbiome is frequently proposed as a target for improving pest management in agriculture (Pineda et al., 2017).

Agricultural cultivation alters soil conditions, which can have long-term negative consequences for plant performance. These disturbances can be caused by multiple conventional practices such as tillage and fertilizer application (reviewed in Howard et al., 2019) and greater agricultural intensification (*i.e.*, conventional versus organic farming) has been associated with lower levels of beneficial soil microbes, such as arbuscular mycorrhizal fungi, and decreased complexity of fungal networks (Banerjee et al., 2019). In some cases, continuous cultivation of crop monocultures can result in the build-up of pathogens in the soil, which is often implicated as a causal agent of "replant disease", reducing yields of a broad range of crops from annuals, such as maize, to tree fruits (Traquair, 1984; Bennett et al., 2012). This condition is typically avoided by reducing the abundance of suitable plant hosts through crop rotation or polyculture (Bennett et al., 2012). With regard to insect pests, one recent study suggests that the lower insect resistance of plants in conventionally versus organically managed fields is at least partially due to differences in soil microbial communities (Blundell et al., 2020). Recent work has also shown that soil microbiomes conditioned by non-crop plants, such as grassland species, can reduce the susceptibility of chrysanthemums to both pathogens and insect herbivores (Ma et al., 2017; Ma et al., 2019; Pineda et al., 2020), suggesting that soil microbiomes from natural systems could be used to improve crop performance.

Leaving fields fallow and promoting the establishment of biota unimpeded by tillage and other disruptive management practices not only results in drastic changes in plant communities, but also in soil quality and soil microbiomes. Levels of soil organic matter, nutrients, and microbial biomass increase in cultivated fields that are left fallow for extended lengths of time (Post and Kwon, 2000; Howard et al., 2020) and these successional changes in soils may affect plant growth and resistance to herbivores (Howard et al., 2018). The composition of soil microbiomes is also widely known to shift over ecological succession (Maharning et al., 2009), which is likely to affect the performance of the plants with which they interact. For example, the abundance of pathogenic fungi has been found to decrease over successional time in abandoned agricultural fields (Hannula et al., 2017), suggesting that these shifts are functional and may benefit plants over succession. Our recent work also suggests that these successional shifts in microbial communities may improve plants' resistance to herbivores (Howard et al., 2020).

We previously found that the rhizosphere microbial communities of a native plant, tall goldenrod, *Solidago altissima* (Asteraceae), shift over oldfield succession in fallow maize fields, with functional effects on their resistance to herbivores. When we inoculated *S. altissima* with soil microbiomes from a plant community that had been left fallow for 15 years, these plants were more resistant to the specialist goldenrod leaf beetle, *Trirhabda virgata*, than their counterparts inoculated with early succession microbiomes, paralleling the pattern of greater herbivore resistance observed among goldenrods in late succession communities (Howard et al., 2020). However, it is not known whether this microbiome-mediated resistance associated with the later oldfield succession soils is specific to the community-dominating goldenrods or could more broadly enhance the insect resistance of other plant species as well.

Little is known about the consistency of phenotypic responses of diverse plant species to whole soil microbial communities in terms of plant performance and especially herbivore resistance. The broad effectiveness of individual growth-promoting microbes across plant species has been demonstrated for several "beneficial" microbes, such as mycorrhizae and other fungal endophytes, which can promote the growth of annual crops and trees alike, albeit to different degrees (Munyanziza et al., 1997; Khan et al., 2012; Van Geel et al., 2016). Yet, while diverse plant species assemble different microbiomes—likely influenced by their phylogenetic relatedness (Fitzpatrick et al., 2018), interspecific comparisons of phenotypic responses to whole soil microbiomes are limited and have shown that responses can vary by plant species (Fitzpatrick et al., 2018; Hahl et al., 2019). However, Fitzpatrick et al. (2018) found that plants grew larger in soil microbial communities conditioned by plant species with microbiomes that were more dissimilar to their own, suggesting that plant species responses may be predictable.

Understanding the predictability of plant responses to microbiomes will be important for assessing the potential and general applicability of microbiome-manipulations in agriculture, including the applications of findings based on native plants and non-crop models to agronomically important species. Panke-Buisse et al. (2015) found that complex soil microbiomes that were artificially selected to promote earlier flowering time in *Arabidopsis thaliana* effectively decreased the latency of flowering in the confamilial crop *Brassica rapa*. Yet, whether these same microbiomes would similarly manipulate the phenology of more distantly related species is unknown. In addition to variation driven by phylogenetic distance, there may be differences in plant-microbe interactions in crop plants versus wild plants due to domestication, particularly breeding under high-input conventional agricultural conditions in which forming symbioses with mutualist microorganisms may not be as crucial for plants as in natural systems (Pérez-Jaramillo et al., 2016; Porter and Sachs, 2020).

In this study, we sought to examine whether the microbiome-mediated trend in herbivore resistance that we discovered in an ecologically important native plant, *S. altissima*, could be applied to manipulate the pest resistance of crop species. We selected four crop species from different families: maize (*Zea mays*, Poaceae), cucumber (*Cucumis sativus*, Cucurbitaceae), tomato (*Solanum lycopersicum*, Solanaceae), and lettuce (*Lactuca sativa*,

Asteraceae) and inoculated them in a glasshouse with soil microbiomes collected from experimental field plots that were currently under cultivation (conventional maize agriculture) or had been fallow for 1, 3, or 16 years. We assessed their early-season growth and resistance to two agricultural pests, *Spodoptera frugiperda* (fall armyworm) and *Trichoplusia ni* (cabbage looper). Based on our finding that late succession soil microbiomes conferred the greatest herbivore resistance to *S. altissima* (Howard et al., 2020), we predicted that the crop plants would be most resistant to the insect pests when inoculated with the 16-year fallow microbiome.

MATERIALS AND METHODS

Plant Materials

We obtained crop seeds (one cultivar per species) from Burpee Co. (Warminster, PA, USA): Sweet Sunshine Hybrid sweet corn (maize), organic Roma tomato, Pick-a-Bushel Hybrid cucumber, and Parris Island Cos lettuce.

Successional Inoculants

We obtained soil microbiome inoculants from fields that had undergone three consecutive years of conventional maize cultivation (year "0") and plant communities in the 1st, 3rd, and 16th years of fallow (oldfield) succession from a large-scale successional field experiment at Dunlop Meadow in Brooktondale, NY, USA (42°23'13"N, 76°24'00"W) (described in Howard et al., 2020). Briefly, this field experiment consisted of duplicated 30 x 30 m plots in which maize is grown conventionally for 3 years and then left fallow. The plantings are staggered chronologically so that plots in different years of fallow succession can be sampled simultaneously and there are two plots representing each successional year. On May 22, 2019, we collected soils for use as inoculants from the top 10 cm of each plot. To generate an inoculant that was representative of the plot, we sampled soil from 5 locations within each plot, homogenized these subsamples in a plastic bag, and sieved them to 4.75 mm. We stored these soils at 4°C for one day before using them to inoculate sterilized soil. Based on our previous surveys of these plots (Howard et al., 2020), we expect these inoculants to vary substantially in the composition of their bacterial and fungal communities.

Plant Inoculation and Growth

We transferred the successional soil microbiomes to sterilized potting media by directly inoculating a mix of triple autoclaved (with 24 h rest periods in between cycles) commercial sphagnum moss potting media (75% (v/v)) (Lambert's All Purpose, Quebec, Canada) and topsoil (20% (v/v)) with each field soil inoculant at a rate of 5% (v/v), an inoculation method which we previously optimized for this recipient soil type (Howard et al., 2017). As our objective was to compare plant responses to microbiomes from agricultural fields with those from communities in different stages of fallow succession, rather than the effect of inoculation more generally, we did not include a sterile or mock inoculation treatment. We prepared six replicate pots of inoculated soil per inoculant, for a total of 12 pots per successional inoculant year, watered them with filter-sterilized deionized water (0.1 μm pore size, Sawyer Products, Inc., Florida, USA) to a moisture level of approximately 10% (v/v) and allowed the pots to incubate at ambient temperature (~ 27°C) in a glasshouse at Cornell University (Ithaca, NY, USA) for 24 h prior to planting. We surface-sterilized seeds in an aqueous solution containing 1% (w/v) NaOCl (diluted from household bleach) and 0.0042% (v/v) Tween20 for 10 min prior to planting on 24-May-2019 to minimize the effect of the existing microbes colonizing them. We planted three seeds in each 10 cm diameter pot (ultimately thinned to one plant—the largest seedling—per pot) to a depth of 25 mm for maize and cucumber and 6 mm for tomato and lettuce and positioned the pots in a randomized block design in a glasshouse with a 16 h photoperiod. We irrigated the pots with filter-sterilized water as needed and removed any weeds that germinated from the seedbank of the inoculant soil.

Plant Growth and Measurements

As measures of plant size, we recorded the number of leaves and length of the longest leaf of each maize and cucumber plant 20 days after planting (DAP), and for each tomato and lettuce plant 23 and 31 DAP, respectively. To obtain an approximate final biomass (roots and shoots, minus the tissues collected for bioassays and analysis) of each plant, we harvested the maize, cucumber, tomato, and lettuce plants 32, 32, 33, and 39 DAP, respectively, washed the soil off of their roots, and weighed them after drying in an oven at 60°C for 7 days. At the time of harvest, two of the largest leaves were already removed for use in herbivore resistance bioassays (see below). We also measured specific leaf area (SLA) at harvest by punching two 10 mm diameter leaf discs from the youngest fully expanded leaves of maize, cucumber, and tomato plants and the second fully-expanded leaves of lettuce, and dividing the area of these discs by their dry weight.

Herbivore Resistance Bioassays

We obtained *Trichoplusia ni* (cabbage looper) and *Spodoptera frugiperda* (fall armyworm) as eggs from Benzon Research, Inc. (Carlisle, PA, USA) and reared them on cabbage looper diet (Southland Projects, Inc., Lake Village, AR, USA) prepared according to the manufacturer's instructions.

Choice Bioassays

To assess the feeding preference of herbivores for plants grown with agricultural or fallow microbiomes, we conducted two-way cafeteria choice tests with *T. ni* and *S. frugiperda* (illustrated in **Figures 3A** and **5A**). In each test, we simultaneously presented a neonate larva (*c.* 2 days post-hatching) with 7 mm diameter leaf discs (punched from the youngest fully-expanded leaf, or the first collared leaf for maize) from a plant inoculated with an agricultural (year 0) and a fallow (year 1, 3, or 16) microbiome, in a 118 mL soufflé cup (Solo Cup Co., Urbana, IL, USA) with a thin layer of agar (12.5 g/L) at the bottom to prevent desiccation. We recorded the first disc the larvae were observed to eat and if they did not feed within 2 h, we excluded the test from the analysis.

We randomly paired each agricultural microbiome-inoculated plant with a plant from each of the three successional year treatments and tested each of these pairings twice with individual, naïve larvae of each herbivore species (N = 20-24 tests per plant species × fallow inoculant age level × herbivore species combination). We performed the choice tests 21, 21, 23, and 33 DAP for maize, cucumber, tomato, and lettuce, respectively.

No-Choice Bioassays

As another measure of herbivore resistance, we performed no-choice feeding assays in which we offered *T. ni* and *S. frugiperda* larvae (*c.* 6 days post-hatching) leaf tissue from a single plant in individual agar cups (as in the choice assay). This piece of tissue was excised from the second youngest collared leaf for maize (mean ± SE: 9.76 ± 0.2 cm^2), half of the youngest uncurled leaf for cucumber cut down the midvein (16.1 ± 0.5 cm^2), one of the side leaflets from the second youngest fully-expanded leaf for tomato (10.6 ± 0.3 cm^2), or half of the second youngest fully-expanded leaf for lettuce cut down the midvein (18.8 ± 0.5 cm^2). We performed the no-choice assays 26 DAP for the maize, cucumber, and tomato plants and 35 DAP for the lettuce plants. We measured the amount of weight gained and leaf tissue eaten by the larvae after 3 d for maize, cucumber, and tomato, and after 4 d for lettuce. We quantified the area of leaf tissue eaten using Adobe Photoshop. As an integrated measure of herbivore performance, we calculated biomass accumulation efficiency by dividing the amount of weight gained by the area of leaf tissue consumed.

Statistical Analyses

We performed all statistical analyses using R version 3.6.2 (R Core Team, 2019). We analyzed the plant size and no-choice bioassay data using linear mixed effects models using the function *lmer* in the package *lme4* (Bates et al., 2015) with fixed effects of inoculant age, plant species, their interaction, and inoculant source (spatial block in the field) and a random effect of greenhouse block (position in the greenhouse experiment). In addition to these cross-species analyses, we separately analyzed the plant size and no-choice bioassay data for each crop, using the same model design minus the plant species fixed effect (and interaction term). We omitted one maize and one tomato plant that died from the 1- and 3-year microbiome treatment groups, respectively. We also removed insects that died, lost weight, or did not consume any tissue in the no-choice bioassays. If necessary, we used logarithmic or square-root transformations to meet the assumptions of normality and homoscedasticity of residuals. We assessed the significance of the fixed effects using F-tests with Kenward-Rogers approximated degrees of freedom *via* the *anova* function in the package *lmerTest* (Kuznetsova et al., 2017) and used the *emmeans* function in the package *emmeans* (Lenth, 2020) to examine pairwise contrasts between the different inoculant age levels. We analyzed the choice bioassay data using generalized linear mixed-effects models (family = binomial) with choice as a binary variable (agricultural microbiome vs. fallow microbiome), inoculant successional age, crop species, and their interaction as fixed effects, and individual plants (both agricultural and fallow microbiome-inoculated) as random effects using *glmer* in the package *lme4*. Additionally, we separately analyzed choice data for each crop species, using the same model design minus the crop species fixed effect (and the interaction term). We calculated 95% confidence intervals for the probabilities of eating a disc from each inoculant treatment level using *emmeans* and then determined whether or not insects showed a significant preference based on whether this interval included 0.5 (where the probabilities of choosing the agricultural microbiome-inoculated plant vs. the fallow microbiome-inoculated plant are equal).

RESULTS

Plant growth was a function of inoculant successional age treatment for some crops, but the effects varied both in pattern and by growth measurement (**Figure 1**, **Table 1**). Plants generally produced the most biomass when inoculated with the oldest soil microbiome, but there were significant crop species by inoculant interactions for both leaf size and total plant biomass (**Table 1**). While inoculant successional age had a significant effect on the leaf sizes of both cucumber and lettuce plants, cucumber produced the largest leaves when inoculated with agricultural soil microbiomes and the smallest leaves when inoculated with soil from fields that had been fallow for 3 years, the opposite of the pattern observed for lettuce (**Figure 1A**; cucumber: inoculant age: $F_{3,41}$ = 2.8500, P = 0.0491, inoculant source: $F_{1,41}$ = 1.0906, P = 0.3025; lettuce: inoculant age: $F_{3,41}$ = 3.5328, P = 0.0229, inoculant source: $F_{1,41}$ = 1.7969, P = 0.1875). The dry weight biomass of cucumber and lettuce paralleled these leaf length trends, but did not differ significantly with regard to inoculant age for these species (**Figure 1B**). Maize biomass varied with inoculant successional age, growing largest when inoculated with the oldest, 16-year fallow microbiome (**Figure 1B**; inoculant age: $F_{3,40}$ = 5.5873, P = 0.0027, inoculant source: $F_{1,40}$ = 0.5276, P = 0.4718). Maize plants in this inoculation treatment also tended to have the largest leaves, and there was also an effect of inoculant source on leaf size (**Figure 1A**, inoculant age: $F_{3,40}$ = 2.4187, P = 0.0803, inoculant source: $F_{1,40}$ = 4.8069, P = 0.0342). Moreover, maize SLA varied with inoculant age, whereby larger, late-succession microbiome plants had the lowest SLA (**Figure 1C**, inoculant age: $F_{3,40}$ = 3.8493, P = 0.0164, inoculant source: $F_{1,40}$ = 0.8477, P = 0.3627). SLA did not differ significantly by inoculant age for any other crop species. In contrast to the other species, none of the growth measurements of tomato plants were affected by inoculant age (**Figure 1**).

Overall, the resistance of plants to *T. ni* varied by both species and inoculant age based on the no-choice feeding experiments (**Figure 2**, **Table 2**). The agricultural microbiomes conferred the least resistance to the plants, with *T. ni* larvae gaining the most weight, and consuming the greatest amount, while feeding on the plants inoculated with agricultural soil in the no-choice experiments (**Figure 2**, **Table 2**). However, when looking at the crop species individually, we only observed a significant effect of inoculant age on the resistance of cucumber plants. *Trichoplusia ni* consumed less leaf tissue from cucumber plants inoculated with the late succession (16-year) soil compared to

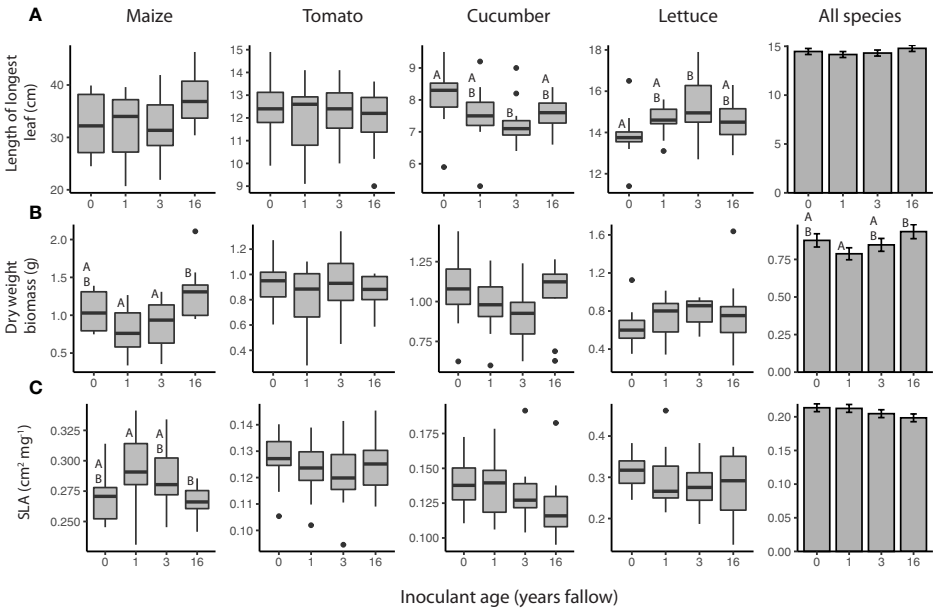

FIGURE 1 | Sizes of maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) plants inoculated with agricultural (0 year) or fallow microbiomes of different ages (1, 3, or 16 years post-agriculture) measured as **(A)** the length of the longest leaf, **(B)** dry weight biomass (roots and shoots), and **(C)** specific leaf area (SLA). All plants (N = 11-12 per species × inoculant age treatment) were grown in a glasshouse common garden experiment. Note that 2 leaves had been harvested prior to the dry weight measurement for use in the herbivore resistance bioassays. Boxes enclose the middle 50% of values, with the 50th percentile indicated by the midline and error bars spanning 1.5 times the interquartile range in both directions; values outside this range are indicated as black dots. Bar plots show estimated marginal means (± SE) averaged across species, and inoculant and greenhouse spatial blocks (see model outputs in **Table 1**). Letters above the boxes/bars indicate significant differences between inoculant age levels within plant species for the boxplots and across all species for the bar plots ($\alpha = 0.05$).

TABLE 1 | Results of ANOVAs assessing the overall effects of soil microbial inoculants on plant growth across crop species.

Growth measurement	Variable	*F*	*P*
Leaf length	Inoculant age	$F_{3,165} = 1.0429$	0.3752
	Crop species	$F_{3,8} = 442.9242$	<0.0001***
	Inoculant source	$F_{1,165} = 2.1440$	0.1450
	Inoculant age × crop species	$F_{9,165} = 2.3019$	0.0184*
Dry weight biomass	Inoculant age	$F_{3,165} = 2.9407$	0.0348*
	Crop species	$F_{3,8} = 5.0985$	0.0291*
	Inoculant source	$F_{1,165} = 1.7916$	0.1826
	Inoculant age × crop species	$F_{9,165} = 2.3807$	0.0147*
SLA	Inoculant age	$F_{3,165} = 1.8785$	0.1352
	Crop species	$F_{3,8} = 152.0031$	<0.0001***
	Inoculant source	$F_{1,165} = 0.0601$	0.8067
	Inoculant age × crop species	$F_{9,165} = 1.1778$	0.3123

*Maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) plants were inoculated with soil microbiomes from agricultural (0 years) or fallow plots of different ages (1, 3, or 16 years post-agriculture), with 2 plots representing each age (inoculant source). Plant growth was measured as the length of the longest leaf, dry weight biomass (roots and shoots), and specific leaf area (SLA). N = 11-12 per species × inoculant age treatment. Note that 2 leaves had been harvested prior to the dry weight measurement for use in the herbivore resistance bioassays. *p < 0.05, ***p < 0.001.*

those treated with the agricultural microbiome (**Figure 2B**; inoculant age: $F_{3,29} = 3.0218$, $P = 0.0456$, inoculant source: $F_{1,28} = 3.9438$, $P = 0.0568$). Consistent with this consumption trend, the larvae also tended to gain less weight feeding on these later succession-inoculated cucumber plants, though there was not a statistically significant effect of inoculant age (**Figure 2A**; inoculant age: $F_{3,29} = 2.1902$, $P = 0.1105$, inoculant source: $F_{1,28} = 3.0123$, $P = 0.0935$) and there was no difference in biomass accumulation efficiency (**Figure 2C**, inoculant age: $F_{3,29} = 0.7550$, $P = 0.5285$, inoculant source: $F_{1,28} = 0.0101$, $P = 0.9206$).

Trichoplusia ni larvae generally did not exhibit a preference for feeding on crops inoculated with agricultural versus fallow soil microbiomes, regardless of plant species or the successional age of the fallow inoculant (**Figure 3**). However, *T. ni* exhibited a marginal preference for crops with 3-year fallow microbiomes over those inoculated with agricultural soil (**Figure 3B**, probability of selecting the agricultural microbiome plant: 0.3627 with an upper 95% confidence limit of 0.5020). Yet, when looking at individual crop species, the only significant preference observed was for 1-year fallow inoculated cucumber plants; larvae selected the agricultural microbiome cucumber with a probability of only 0.2 (95% confidence interval: 0.0659 to 0.470) when tested against their 1-year fallow-inoculated counterparts (**Figure 3C**).

The inoculation treatments generally did not affect resistance to *S. frugiperda* in the no-choice experiments (**Figure 4**, **Table 3**), except for one species: tomato. The larvae gained more weight feeding on tomato plants inoculated with microbiomes from fields that had been fallow for 3 and 16 years compared to those treated with agricultural microbiomes (**Figure 4A**; inoculant age: $F_{3,36} = 3.8566$, $P = 0.0173$, inoculant source: $F_{1,36} = 0.1336$, $P =$

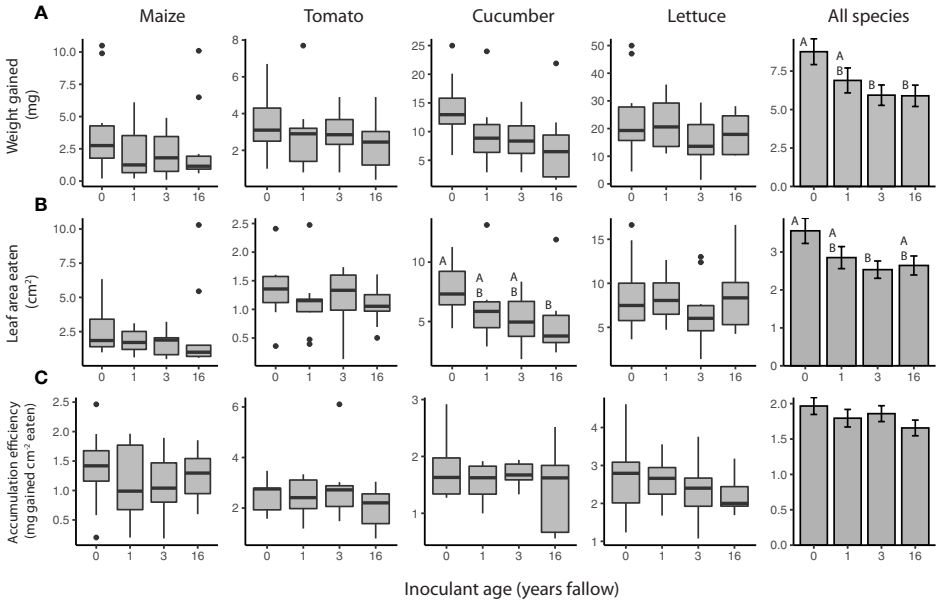

FIGURE 2 | *Trichoplusia ni* resistance of maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) plants inoculated with agricultural (0 year) or fallow microbiomes of different ages (1, 3, or 16 years post-agriculture) in a no-choice assay. Resistance measured as **(A)** weight gained **(B)** amount of tissue eaten by *T. ni* larvae (*c.* 6 d old at start), as well as their **(C)** biomass accumulation efficiency after feeding for 3 (maize, tomato, and cucumber assays) or 4 d (lettuce assays). N = 8-12 replicates per species × inoculant age treatment after omitting insects that died, lost weight, or did not eat. Boxes enclose the middle 50% of values, with the 50th percentile indicated by the midline and error bars spanning 1.5 times the interquartile range in both directions; values outside this range are indicated as black dots. Bar plots show estimated marginal means (± SE) averaged across species, and inoculant and greenhouse spatial blocks (see model outputs in **Table 2**). Letters above the boxes/bars indicate significant differences between inoculant age levels within plant species for the boxplots and across all species for the bar plots (α = 0.05).

TABLE 2 | Results of ANOVAs assessing the overall effects of soil microbial inoculants on resistance to T. ni in a no-choice test across crop species.

Resistance measurement	Variable	*F*	*P*
Weight gain	Inoculant age	$F_{3,131}$ = 3.1318	0.0279*
	Crop species	$F_{3,8}$ = 86.9621	<0.0001***
	Inoculant source	$F_{1,131}$ = 2.4207	0.1222
	Inoculant age × crop species	$F_{9,132}$ = 0.6468	0.7552
Leaf area eaten	Inoculant age	$F_{3,131}$ = 2.7717	0.0441*
	Crop species	$F_{3,8}$ = 83.2206	<0.0001***
	Inoculant source	$F_{1,130}$ = 1.9539	0.1645
	Inoculant age × crop species	$F_{9,131}$ = 0.4388	0.9118
Accumulation efficiency	Inoculant age	$F_{3,131}$ = 1.3854	0.2501
	Crop species	$F_{3,8}$ = 23.7509	0.0003***
	Inoculant source	$F_{1,130}$ = 0.2690	0.6049
	Inoculant age × crop species	$F_{9,131}$ = 0.7149	0.6945

*Maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) plants were inoculated with soil microbiomes from agricultural (0 years) or fallow plots of different ages (1, 3, or 16 years post-agriculture), with 2 plots representing each age (inoculant source). Herbivore resistance was measured in no-choice bioassays with c. 6 d old T. ni larvae, in which weight gain, leaf area eaten, and biomass accumulation efficiency were measured after feeding for 3 (maize, tomato, and cucumber assays) or 4 d (lettuce assays). N = 8-12 replicates per species × inoculant age treatment after omitting insects that died, lost weight, or did not eat. *p < 0.05, ***p < 0.001.*

0.7169). While the larvae did not consume significantly greater amounts of leaf tissue from these later succession microbiome-inoculated plants (**Figure 4B**), they gained weight more efficiently feeding on the 16-year fallow microbiome plants compared to their agricultural counterparts (**Figure 4C**; inoculant age: $F_{3,36}$ = 4.3989, P = 0.0099, inoculant source: $F_{1,36}$ = 0.1320, P = 0.7185). The biomass accumulation efficiency of *S. frugiperda* varied by inoculant age, overall, though the response differed by plant species (**Table 3**, **Figure 4**).

Overall, the *S. frugiperda* larvae preferred to feed on plants inoculated with 16-year fallow versus agricultural soil microbiomes, but did not exhibit a significant preference for plants inoculated with the other fallow microbiomes (**Figure 5B**). Considering the species individually, the significant preference for late succession-treated plants was only observed for cucumbers, with the larvae choosing the agricultural microbiome plants with a probability of only 0.263 (95% CI: 0.1140 to 0.498) versus 16-year microbiome cucumber leaves (**Figure 5C**).

DISCUSSION

Overall, we found that soil microbiomes from varying stages of fallow succession can differentially affect both the pest resistance

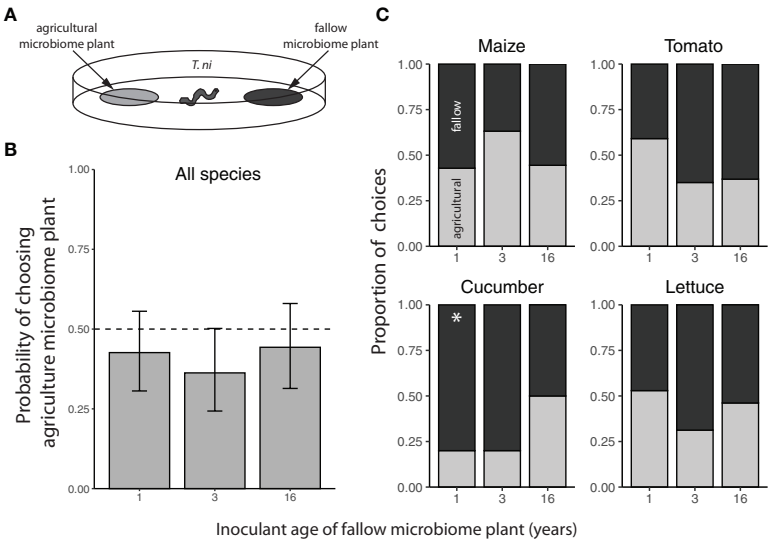

FIGURE 3 | Feeding preference of *Trichoplusia ni* for maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) inoculated with agricultural or fallow microbiomes of different ages. **(A)** Neonatal *T. ni* larvae were simultaneously presented with a disc of leaf tissue from a plant inoculated with an agricultural soil microbiome and a disc of leaf tissue from a plant inoculated with a microbiome from a fallow field (1, 3, or 16 years post-agriculture) in an arena. All choice tests were performed within plant species. Choices represent the microbiome treatment of the disc each larva was first observed to eat. **(B)** Probabilities (with 95% confidence intervals) of selecting the agriculture microbiome-inoculated plant averaged across plant species; the dashed line indicates an equal preference for agriculture- and fallow-microbiome treated plants. **(C)** Choices by crop species; light grey bars indicate the proportion of larvae that chose the plant treated with the agricultural soil microbiome whereas the dark grey bars indicate instances in which the fallow microbiome plant was chosen. N = 9-22 tests per plant species × fallow inoculant age level after omitting larvae that did not feed within the 2 h trial. An asterisk (*) indicates a significant preference.

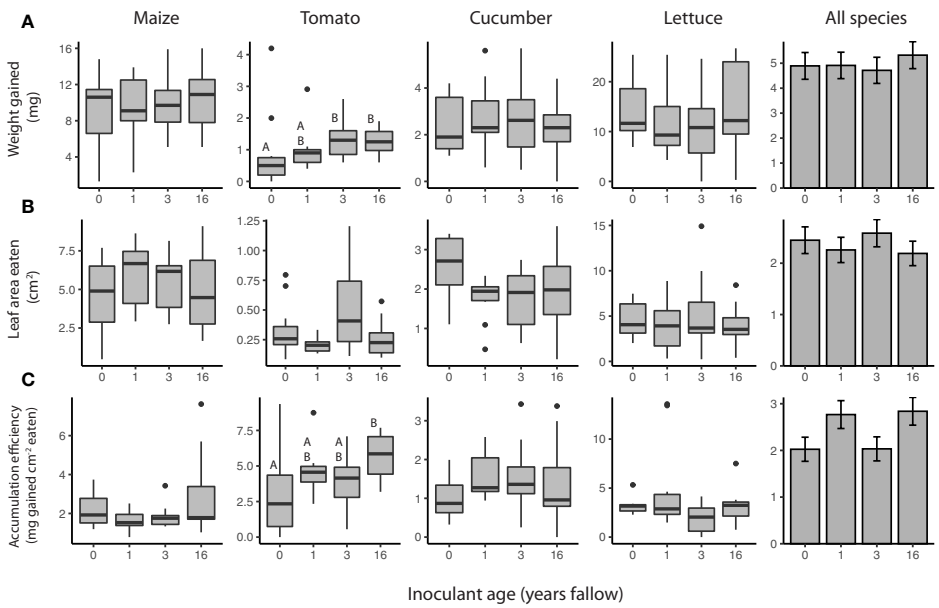

FIGURE 4 | *Spodoptera frugiperda* resistance of maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) plants inoculated with agricultural (0 year) or fallow microbiomes of different ages (1, 3, or 16 years post-agriculture) in a no-choice assay. Resistance measured as **(A)** weight gained **(B)** amount of tissue eaten by *S. frugiperda* larvae (*c.* 6 d old at start), as well as their **(C)** biomass accumulation efficiency after feeding for 3 (maize, tomato, and cucumber assays) or 4 d (lettuce assays). N = 8-12 replicates per species × inoculant age treatment after omitting insects that died. Boxes enclose the middle 50% of values, with the 50th percentile indicated by the midline and error bars spanning 1.5 times the interquartile range in both directions; values outside this range are indicated as black dots. Bar plots show estimated marginal means (± SE) averaged across species, and inoculant and greenhouse spatial blocks (see model outputs in **Table 3**). Letters above the boxes indicate significant differences between inoculant age levels within plant species ($\alpha = 0.05$).

TABLE 3 | Results of ANOVAs assessing the overall effects of soil microbial inoculants on resistance to S. frugiperda in a no-choice test across crop species.

Resistance measurement	Variable	*F*	*P*
Weight gain	Inoculant age	$F_{3,140}$ = 0.2579	0.8556
	Crop species	$F_{3,8}$ = 72.5039	<0.0001***
	Inoculant source	$F_{1,141}$ = 0.3924	0.5320
	Inoculant age × crop species	$F_{9,140}$ = 0.8693	0.5543
Leaf area eaten	Inoculant age	$F_{3,140}$ = 0.6133	0.6075
	Crop species	$F_{3,8}$ = 56.0374	<0.0001***
	Inoculant source	$F_{1,141}$ = 0.3841	0.5364
	Inoculant age × crop species	$F_{9,140}$ = 0.9578	0.4777
Accumulation efficiency	Inoculant age	$F_{3,140}$ = 3.6408	0.0144*
	Crop species	$F_{3,8}$ = 8.5635	0.0071**
	Inoculant source	$F_{1,140}$ = 0.0109	0.9171
	Inoculant age × crop species	$F_{9,140}$ = 3.0900	0.0021**

*Maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) plants were inoculated with soil microbiomes from agricultural (0 years) or fallow plots of different ages (1, 3, or 16 years post-agriculture), with 2 plots representing each age (inoculant source). Herbivore resistance was measured in no-choice bioassays with* c. *6 d old S. frugiperda larvae, in which weight gain, leaf area eaten, and biomass accumulation efficiency were measured after feeding for 3 (maize, tomato, and cucumber assays) or 4 d (lettuce assays). N = 8-12 replicates per species × inoculant age treatment after omitting insects that died, lost weight, or did not eat. *p < 0.05, ***p < 0.001.*

and growth of different crop species, but that the effects are species-specific, and often contrasting. In line with the pattern we previously observed in the *S. altissima* study that motivated this experiment (Howard et al., 2020), later succession microbiomes conferred greater *T. ni* resistance to the crop species, particularly cucumber (**Figure 2**). In contrast, tomato plants were least resistant to *S. frugiperda* when inoculated with late succession (16 year fallow) microbiomes (**Figure 4**). These species-specific responses to the various microbiomes were further illustrated through differences in plant growth. For example, lettuce produced the largest leaves when inoculated with a 3-year fallow microbiome, the microbiome in which cucumber performed worst, while tomato growth was overall unaffected by inoculant successional age. Collectively, these results indicate that various plant species have different phenotypic responses to different microbiomes and point to the need to study microbiome-influenced phenotypes in a broad and taxon-replicated range of plant species, as well as the underlying mechanisms of how microbiomes assemble and affect these plant traits.

These differential phenotypic responses of plant species to soil microbial communities of varying successional age could be due to differences in microbiome assembly, as well as the degree to which different plants rely on microorganisms for different

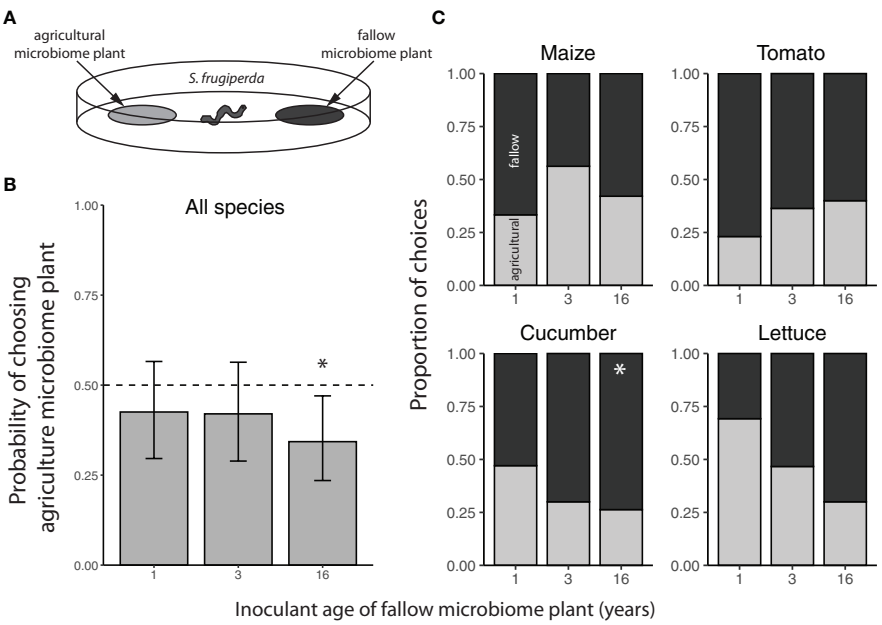

FIGURE 5 | Feeding preference of *Spodoptera frugiperda* for maize (*Z. mays*), tomato (*S. lycopersicum*), cucumber (*C. sativus*), and lettuce (*L. sativa*) inoculated with agricultural or fallow microbiomes of different ages. **(A)** Neonatal *S. frugiperda* larvae were simultaneously presented with a disc of leaf tissue from a plant inoculated with an agricultural soil microbiome and a disc of leaf tissue from a plant inoculated with a microbiome from a fallow field (1, 3, or 16 years post-agriculture) in an arena. All choice tests were performed within plant species. Choices represent the microbiome treatment of the disc each larva was first observed to eat. **(B)** Probabilities (with 95% confidence intervals) of selecting the agriculture microbiome-inoculated plant averaged across plant species; the dashed line indicates an equal preference for agriculture- and fallow-microbiome treated plants. **(C)** Choices by crop species; light grey bars indicate the proportion of larvae that chose the plant treated with the agricultural soil microbiome whereas the dark grey bars indicate instances in which the fallow microbiome plant was chosen. N = 10-20 tests per plant species × fallow inoculant age level after omitting larvae that did not feed within the 2 h trial. An asterisk (*) indicates a significant preference.

functions. It is well known that different species, and even different cultivars and genotypes, of plants assemble distinct microbiomes (Lundberg et al., 2013; Peiffer et al., 2013; Cardinale et al., 2015; Fitzpatrick et al., 2018; Matthews et al., 2019). While few studies have directly compared microbiome assembly on different crop species, Matthews et al. (2019) recently found that maize, tomato, pea, and onion plants not only assembled microbiomes with distinct community structures, but also differed in the variability of their rhizosphere microbiome composition when grown in grassland versus woodland soils. In their study, tomato and maize showed the greatest variation in rhizosphere assembly at the individual taxon (OTU) level by soil type. Yet, even with differences in nutrients between the soils, they found that tomato and maize growth were unaffected, indicating that the performance of these species is robust and might not easily be altered by shifts in microbiomes. However, as most of the differentially abundant OTUs between the two soil types in their study came from the same taxonomic families and were potentially functionally redundant, it is difficult to assess whether these plant species differed from the other crops in their capacity to discriminate or actively influence their microbiome assembly (Matthews et al., 2019). On the other hand, we saw that maize, cucumber, and lettuce growth was affected by microbial community treatments, suggesting that these species might assemble even more divergent microbial communities under the different inoculation treatments and perhaps are not as able to actively select a microbiome. Similar to Matthews et al. (2019), we found that tomato growth was relatively robust to our microbiome treatments. This may have to do with its physiology, as tomato growth and reproduction are particularly resilient, even when defoliated (Slack, 1986). Thus, some species may be less likely than others to be affected by changes in their microbiomes due to their physiology. Plants also vary in their reliance on microbes for different functions related to growth and defense, some patterns of which may be phylogenetically generalizable (*e.g.*, Brassicaceae not forming symbioses with arbuscular mycorrhizal fungi for resource acquisition (Tester et al., 1987), many grasses hosting alkaloid-producing endophytes for herbivore defense (Clay, 1988). Thus, studying a diverse range of plant species, especially in a taxon-replicated manner, may help us understand and better predict plant responses to microbiomes.

While the differences between the patterns of herbivore resistance we saw here and in our previous study of *S. altissima* may be driven by the phylogenetic diversity of the plant hosts, some differences might also be due to comparing domesticated crops to wild plants. While *S. altissima* may be ecologically similar to a crop in that it grows in agriculturally-altered habitats, including fallow fields, its abilities to form microbial symbioses may not have been under the same selective pressures as plants that have been bred for agricultural performance. Comparisons between crops and their wild relatives have indicated that domestication has altered their interactions with microorganisms (Pérez-Jaramillo et al., 2018), sometimes resulting in plants that form symbioses with resource mutualists, such as arbuscular mycorrhizal fungi, less readily (Martín-Robles et al., 2018). Breeding under high-input agricultural regimes (*e.g.*, ample fertilizer) may make roots less conducive to forming microbial symbioses and relax selection on the ability to form symbioses—or even select against forming symbioses due to the costs of maintaining them when resources are abundant (Pérez-Jaramillo et al., 2016; Porter and Sachs, 2020). Furthermore, breeding crops for improved pathogen resistance may also inadvertently reduce the ability of plants to form symbioses with mutualistic microbes due to common pathways of colonization (Porter and Sachs, 2020). Wild sunflower accessions are slightly more readily colonized by mycorrhizae (Turrini et al., 2016) and also less resistant to pathogens than domesticated lines (Leff et al., 2016). With regard to the crops studied here, previous studies have indicated that cultivated lettuce and maize plants assemble different microbiomes than their wild relatives (Cardinale et al., 2015; Szoboszlay et al., 2015), and that cultivated tomato plants respond differently to soil conditioning compared to their wild counterparts (Carrillo et al., 2019). It is also important to note that these studies found substantial cultivar-level variation in the microbiomes of these crop species, which emphasizes the need to not only characterize the responses of different plant species to microbiomes, but also a diverse range of genotypes within species. In our study, we only examined one cultivar per crop species to maximize the number of species tested. This, however, limits our ability to make generalizations for specific species and assess the intraspecific variation among cultivars. For a general application of microbiome-enhanced plant resistance, studies examining crop responses to microbiomes should aim to capture inter-cultivar variation.

While we observed effects of soil microbiomes on plant resistance to insect pests, we did not investigate the basis of this herbivore resistance, limiting our ability to understand how these microbiomes are affecting the plants. For instance, the differences in weight gained by *S. frugiperda* feeding on plants inoculated with different microbiomes appears to be due to greater biomass accumulation efficiency rather than amount of tissue eaten, but we cannot tell if this is driven by differences in defense compounds (*e.g.*, digestibility reducers) or the nutritional value of the leaves. Interestingly, while tomato's resistance to *S. frugiperda* was affected by the different microbiomes, its resistance to the other generalist noctuid pest we tested, *T. ni*, was not (**Figure 4**). Similarly, the microbiome treatments only affected cucumber's *T. ni* resistance (**Figure 2**), indicating that not only are these microbiome-mediated resistance phenotypes plant species-specific, but also herbivore-specific, even as generalist (and here also confamilial) herbivores are typically expected to be similarly affected by chemical defenses (Ali and Agrawal, 2012). The data presented here suggest that different plant defense traits or trait combinations expressed by the same plant can contribute to the resistance to different herbivore species, even when the herbivore species are closely related. Consequently, the findings expand the interaction diversity hypothesis (Berenbaum and Zangerl, 1996; Iason et al., 2011) to include defense-related plant secondary metabolism that is mediated by the microbial community. This further suggests microbe-mediated changes in plant secondary metabolism as one of the drivers of functional chemical diversity in plants (Kessler and Kalske, 2018). Investigating the leaf chemistry of these microbiome-

treated plants may help us understand whether these patterns of resistance are driven by different mechanisms. Additionally, we observed some discrepancies between our two measures of herbivore resistance that are difficult to resolve without understanding the underlying mechanisms of resistance. For example, plants treated with 3-year fallow microbiomes were overall more resistant than agricultural-microbiome plants to *T. ni* based on the no-choice assays (**Figure 2**), but the larvae exhibited a preference (albeit marginally significant) for them over the supposedly less resistant agricultural-microbiome plants in the choice experiments (**Figure 3B**). This discrepancy could be an artifact of using excised leaf discs in our assay, wherein defenses induced *via* the mechanical damage from cutting leaf tissue could result in the more defended leaves being more apparent, and thus more attractive to larval herbivores, than the less conspicuous, but actually less chemically-defended plants (Carroll et al., 2006).

Moreover, we do not know how, mechanistically, the microbiomes are contributing to the plants' resistance phenotype. In addition to directly altering plants' secondary metabolism, it is possible that pathogenic microbes in the soil (or non-pathogenic microbes perceived by the plants as pathogens) could be altering insect resistance by inducing salicylic acid-mediated responses and simultaneously suppressing the oft-reciprocally antagonistic jasmonic acid pathway that mediates defenses to chewing herbivores (Thaler et al., 2012). One recent study demonstrated that a rhizosphere-dwelling strain of *Pseudomonas* sp. that induced systemic pathogen susceptibility increased tomato plants' resistance to *T. ni*, further indicating that the salicylic acid-jasmonic acid trade-off may play an important role in mediating rhizosphere microbial influence on insect herbivory (Haney et al., 2018). Another recent study (Blundell et al., 2020) implicated salicylic acid as an important mediator of soil microbe-influenced resistance to a hemipteran herbivore (which generally tend to be more strongly affected by salicylic acid- versus jasmonic acid-mediated defenses), further emphasizing the potential importance of rhizosphere microbes affecting plant-insect interactions through altering phytohormonal signaling. Thus, changes in the functional composition of soil microbial communities, including the abundance of pathogens—which are expected to shift over fallow succession (Hannula et al., 2017)—could affect herbivore resistance through altering plant defense responses. It is important to note, however, that since we performed our herbivore resistance assays with excised leaf tissue, we are likely masking potential differences in herbivore-induced resistance responses between our microbial treatments. While our study shows that soil microbiomes can differentially affect plants' resistance to herbivores, the underlying mechanisms for these phenotypic shifts warrant further study.

With agricultural losses to pest damage expected to increase under a warming climate (Deutsch et al., 2018), investigating novel tools such as soil microbiome manipulations to improve the herbivore resistance of crop plants is becoming increasingly relevant (Pineda et al., 2017; Bell et al., 2019). In this study, we found that fallow agricultural fields may harbor soil microbiomes that can promote the growth and pest resistance of some crop plants. We found that, in comparison to agricultural microbiomes, the late succession soil microbiomes most notably improved cucumber's resistance to *T. ni*, a pest which is especially damaging to cucumbers in greenhouses, where microbial inoculants would be relatively easy to apply (Sarfraz et al., 2011). This finding is in line with our previous work, which has suggested that successional shifts in soil microbiomes are an important factor driving increases in the herbivore resistance of a wild plant (Howard et al., 2020), yet the underlying mechanisms for this microbe-mediated resistance are still unresolved. However, it is becoming clearer that various early successional forbs and grasses (Pineda et al., 2020), as well as organic management practices (Blundell et al., 2020), can condition soil to promote herbivore resistance. Thus, in addition to providing related ecosystem services as habitat for natural enemies of pests (Denys and Tscharntke, 2002), fallow land may be worth investigating as sources of beneficial soil microbiomes that are adapted to local edaphic conditions, not only potentially improving their establishment and efficacy (Hawkes and Connor, 2017), but also reducing the non-target risks of introducing non-native microbes (Hart et al., 2018). Yet, sources aside, our study indicates that observing beneficial effects of an inoculant on one plant species may not be predictive of its capacity to improve the performance of another.

DATA AVAILABILITY STATEMENT

The data that support this paper are available in the Cornell eCommons repository (https://ecommons.cornell.edu/) under the title of this publication.

AUTHOR CONTRIBUTIONS

MH, JK-K and AK designed the study. MH and CM conducted the experiments and analyzed the data. MH drafted the manuscript with input from AK and JK-K.

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REFERENCES

Ali, J. G., and Agrawal, A. A. (2012). Specialist versus generalist insect herbivores and plant defense. *Trends Plant Sci.* 17, 293–302. doi: 10.1016/j.tplants.2012.02.006

Banerjee, S., Walder, F., Büchi, L., Meyer, M., Held, A. Y., Gattinger, A., et al. (2019). Agricultural intensification reduces microbial network complexity and the abundance of keystone taxa in roots. *ISME J.* 13, 1722–1736. doi: 10.1038/s41396-019-0383-2

Bates, D., Mächler, M., Bolker, B. M., and Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Software* 67, 1–48. doi: 10.18637/jss.v067.i01

Bell, T. H., Hockett, K. L., Alcalá-Briseño, R.II, Barbercheck, M., Beattie, G. A., Bruns, M. A., et al. (2019). Manipulating wild and tamed phytobiomes: Challenges and opportunities. *Phytobiomes J.* 3, 3–21. doi: 10.1094/PBIOMES-01-19-0006-W

Bennett, A. J., Bending, G. D., Chandler, D., Hilton, S., and Mills, P. (2012). Meeting the demand for crop production: The challenge of yield decline in crops grown in short rotations. *Biol. Rev.* 87, 52–71. doi: 10.1111/j.1469-185X.2011.00184.x

Berenbaum, M. R., and Zangerl, A. R. (1996). "Phytochemical diversity: Adaptation or random variation?," in *Phytochemical Diversity and Redundancy in Ecological Interactions*. Eds. J. T. Romeo, J. A. Saunders and P. Barbosa (New York: Plenum Press), 1–24.

Blundell, R., Schmidt, J. E., Igwe, A., Cheung, A. L., Vannette, R. L., Gaudin, A. C. M., et al. (2020). Organic management promotes natural pest control through altered plant resistance to insects. *Nat. Plants* 6, 483–491. doi: 10.1038/s41477-020-0656-9

Cardinale, M., Grube, M., Erlacher, A., Quehenberger, J., and Berg, G. (2015). Bacterial networks and co-occurrence relationships in the lettuce root microbiota. *Environ. Microbiol.* 17, 239–252. doi: 10.1111/1462-2920.12686

Carrillo, J., Ingwell, L. L., Li, X., and Kaplan, I. (2019). Domesticated tomatoes are more vulnerable to negative plant–soil feedbacks than their wild relatives. *J. Ecol.* 107, 1753–1766. doi: 10.1111/1365-2745.13157

Carroll, M. J., Schmelz, E. A., Meagher, R. L., and Teal, P. E. A. (2006). Attraction of *Spodoptera frugiperda* larvae to volatiles from herbivore-damaged maize seedlings. *J. Chem. Ecol.* 32, 1911–1924. doi: 10.1007/s10886-006-9117-9

Clay, K. (1988). Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology* 69, 10–16. doi: 10.2307/1943155

Denys, C., and Tscharntke, T. (2002). Plant-insect communities and predator-prey ratios in field margin strips, adjacent crop fields, and fallows. *Oecologia* 130, 315–324. doi: 10.1007/s004420100796

Deutsch, C. A., Tewksbury, J. J., Tigchelaar, M., Battisti, D. S., Merrill, S. C., Huey, R. B., et al. (2018). Increase in crop losses to insect pests in a warming climate. *Science* 361, 916–919. doi: 10.1126/science.aat3466

Fitzpatrick, C. R., Copeland, J., Wang, P. W., Guttman, D. S., Kotanen, P. M., and Johnson, M. T. J. (2018). Assembly and ecological function of the root microbiome across angiosperm plant species. *Proc. Natl. Acad. Sci.* 115, E1157–E1165. doi: 10.1073/pnas.1717617115

Hahl, T., Moorsel, S. J., Van, Schmid, M. W., Schmid, B., Wagg, C., and Zuppinger-dingley, D. (2019). Plant responses to diversity-driven selection and associated rhizosphere microbial communities. *Funct. Ecol.* 34, 707–722. doi: 10.1111/1365-2435.13511

Haney, C. H., Wiesmann, C. L., Shapiro, L. R., Melnyk, R. A., O'Sullivan, L. R., Khorasani, S., et al. (2018). Rhizosphere-associated Pseudomonas induce systemic resistance to herbivores at the cost of susceptibility to bacterial pathogens. *Mol. Ecol.* 27, 1833–1847. doi: 10.1111/mec.14400

Hannula, S. E., Morriën, E., de Hollander, M., van der Putten, W. H., van Veen, J. A., and de Boer, W. (2017). Shifts in rhizosphere fungal community during secondary succession following abandonment from agriculture. *ISME J.* 11, 2294–2304. doi: 10.1038/ismej.2017.90

Hart, M. M., Antunes, P. M., Chaudhary, V. B., and Abbott, L. K. (2018). Fungal inoculants in the field: Is the reward greater than the risk? *Funct. Ecol.* 32, 126–135. doi: 10.1111/1365-2435.12976

Harun-Or-Rashid, M., and Chung, Y. R. (2017). Induction of systemic resistance against insect herbivores in plants by beneficial soil microbes. *Front. Plant Sci.* 8:1816. doi: 10.3389/fpls.2017.01816

Hawkes, C. V., and Connor, E. W. (2017). Translating phytobiomes from theory to practice: ecological and evolutionary considerations. *Phytobiomes* 1, 57–69. doi: 10.1094/PBIOMES-05-17-0019-RVW

Howard, M. M., Bell, T. H., and Kao-Kniffin, J. (2017). Soil microbiome transfer method affects microbiome composition, including dominant microorganisms, in a novel environment. *FEMS Microbiol. Lett.* 364, 1–8. doi: 10.1093/femsle/fnx092

Howard, M. M., Kalske, A., and Kessler, A. (2018). Eco-evolutionary processes affecting plant-herbivore interactions during early community succession. *Oecologia* 187, 547–559. doi: 10.1007/s00442-018-4088-4

Howard, M. M., Kaminsky, L. M., Kessler, A., and Bell, T. H. (2019). "Merging microbial and plant profiling to understand the impact of human-generated extreme environments in natural and agricultural systems," in *Advanced Techniques for Studying Microorganisms in Extreme Environments*. Ed. E. Yergeau (Berlin: de Gruyter), 57–91.

Howard, M. M., Kao-Kniffin, J., and Kessler, A. (2020). Shifts in plant-microbe interactions over community succession and their effects on plant resistance to herbivores. *New Phytol.* 226, 1144–1157. doi: 10.1016/s0740-5472(96)90021-5

Howard, M. M. (2020). *Eco-evolutionary shifts in plant-microbe-insect interactions over community succession. Dissertation* (Ithaca (NY): Cornell University).

Hubbard, C. J., Li, B., McMinn, R., Brock, M. T., Maignien, L., Ewers, B. E., et al. (2019). The effect of rhizosphere microbes outweighs host plant genetics in reducing insect herbivory. *Mol. Ecol.* 28, 1801–1811. doi: 10.1111/mec.14989

Iason, G. R., O'Reilly-Wapstra, J. M., Brewer, M. J., Summers, R. W., and Moore, B. D. (2011). Do multiple herbivores maintain chemical diversity of Scots pine monoterpenes? *Philos. Trans. R. Soc B. Biol. Sci.* 366, 1337–1345. doi: 10.1098/rstb.2010.0236

Kessler, A., and Kalske, A. (2018). Plant secondary metabolite diversity and species interactions. *Annu. Rev. Ecol. Evol. Syst.* 49, 115–138. doi: 10.1146/annurev-ecolsys-110617-062406

Khan, Z., Guelich, G., Phan, H., Redman, R., and Doty, S. (2012). Bacterial and yeast Endophytes from Poplar and Willow promote growth in crop plants and grasses. *ISRN Agron.* 2012, 1–11. doi: 10.5402/2012/890280

Kuznetsova, A., Brockhoff, P. B., and Christensen, R. H. B. (2017). lmerTest package: tests in linear mixed effects models. *J. Stat. Software* 82, 1–25. doi: 10.18637/jss.v082.i13

Lau, J. A., and Lennon, J. T. (2012). Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proc. Natl. Acad. Sci.* 109, 14058–14062. doi: 10.1073/pnas.1202319109

Leff, J. W., Lynch, R. C., Kane, N. C., and Fierer, N. (2016). Plant domestication and the assembly of bacterial and fungal communities associated with strains of the common sunflower, Helianthus annuus. *New Phytol.* 214, 412–423. doi: 10.1111/NPH.14323

Lenth, R. (2020). *emmeans: Estimated Marginal Means, aka Least-Squares Means*, R package version 1.4.4.

Ludwig-Müller, J. (2015). Plants and endophytes: equal partners in secondary metabolite production? *Biotechnol. Lett.* 37, 1325–1334. doi: 10.1007/s10529-015-1814-4

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2013). Defining the core Arabidopsis thaliana root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Ma, H., Pineda, A., Wurff, A. W. G., and Van Der Raaijmakers, C. (2017). Plant – soil feedback effects on growth, defense and susceptibility to a soil-borne disease in a cut flower crop: species and functional group effects. *Front. Plant Sci.* 8:2127. doi: 10.3389/fpls.2017.02127

Ma, H., Pineda, A., Hannula, S. E., Kielak, A. M., and Nindya, S. (2019). Steering root microbiomes of a commercial horticultural crop with plant-soil feedbacks. *Appl. Soil Ecol.* 150, 103468. doi: 10.1016/j.apsoil.2019.103468

Maharning, A. R., Mills, A. A. S., and Adl, S. M. (2009). Soil community changes during secondary succession to naturalized grasslands. *Appl. Soil Ecol.* 41, 137–147. doi: 10.1016/j.apsoil.2008.11.003

Martín-Robles, N., Lehmann, A., Seco, E., Aroca, R., Rillig, M. C., and Milla, R. (2018). Impacts of domestication on the arbuscular mycorrhizal symbiosis of 27 crop species. *New Phytol.* 218, 322–334. doi: 10.1111/nph.14962

Matthews, A., Pierce, S., Hipperson, H., and Raymond, B. (2019). Rhizobacterial community assembly patterns vary between crop species. *Front. Microbiol.* 10:581. doi: 10.3389/fmicb.2019.00581

Munyanziza, E., Kehri, H. K., and Bagyaraj, D. J. (1997). Agricultural intensification,soil biodiversity and agro-ecosystem function in the tropics: the role of mycorrhiza in crops and trees. *Appl. Soil Ecol.* 6, 77–85. doi: 10.1016/S0929-1393(96)00152-7

Panke-Buisse, K., Poole, A. C., Goodrich, J. K., Ley, R. E., and Kao-Kniffin, J. (2015). Selection on soil microbiomes reveals reproducible impacts on plant function. *ISME J.* 9, 980–989. doi: 10.1038/ismej.2014.196

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Pérez-Jaramillo, J. E., Mendes, R., and Raaijmakers, J. M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Mol. Biol.* 90, 635–644. doi: 10.1007/s11103-015-0337-7

Pérez-Jaramillo, J. E., Carrión, V. J., de Hollander, M., and Raaijmakers, J. M. (2018). The wild side of plant microbiomes. *Microbiome* 6, 4–9. doi: 10.1186/s40168-018-0519-z

Pineda, A., Kaplan, I., and Bezemer, T. M. (2017). Steering soil microbiomes to suppress aboveground insect pests. *Trends Plant Sci.* 22, 770–778. doi: 10.1016/j.tplants.2017.07.002

Pineda, A., Kaplan, I., Hannula, S. E., Ghanem, W., and Bezemer, T. M. (2020). Conditioning the soil microbiome through plant-soil feedbacks suppresses an aboveground insect pest. *New Phytol.* 22, 595–608. doi: 10.1111/nph.16385

Porter, S. S., and Sachs, J. L. (2020). Agriculture and the disruption of plant-microbial symbiosis. *Trends Ecol. Evol.* 35, 426–439. doi: 10.1016/j.tree.2020.01.006

Post, W. M., and Kwon, K. C. (2000). Soil carbon sequestration and land-use change: Processes and potential. *Glob. Change Biol.* 6, 317–327. doi: 10.1046/j.1365-2486.2000.00308.x

R Core Team (2019). *R: A language and environment for statistical computing. R Foundation for Statistical Computing* (Vienna, Austria).

Sarfraz, R. M., Cervantes, V., and Myers, J. H. (2011). The effect of host plant species on performance and movement behaviour of the cabbage looper *Trichoplusia ni* and their potential influences on infection by *Autographa californica* multiple nucleopolyhedrovirus. *Agric. For. Entomol.* 13, 157–164. doi: 10.1111/j.1461-9563.2010.00504.x

Slack, G. (1986). The effects of leaf removal on the development and yield of glasshouse tomatoes. *J. Hortic. Sci.* 61, 353–360. doi: 10.1080/14620316.1986.11515713

Szoboszlay, M., Lambers, J., Chappell, J., Kupper, J. V., Moe, L. A., and McNear, D. H. (2015). Comparison of root system architecture and rhizosphere microbial communities of Balsas teosinte and domesticated corn cultivars. *Soil Biol. Biochem.* 80, 34–44. doi: 10.1016/j.soilbio.2014.09.001

Tester, M., Smith, S. E., and Smith, F. A. (1987). The phenomenon of "nonmycorrhizal" plants. *Can. J. Bot.* 65, 419–431. doi: 10.1139/b87-051

Thaler, J. S., Humphrey, P. T., and Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci.* 17, 260–270. doi: 10.1016/j.tplants.2012.02.010

Traquair, J. A. (1984). Etiology and control of orchard replant problems: a review. *Can. J. Plant Pathol.* 6, 54–62. doi: 10.1080/07060668409501591

Turrini, A., Giordani, T., Avio, L., Natali, L., Giovannetti, M., and Cavallini, A. (2016). Large variation in mycorrhizal colonization among wild accessions, cultivars, and inbreds of sunflower (Helianthus annuus L.). *Euphytica* 207, 331–342. doi: 10.1007/s10681-015-1546-5

Van Geel, M., De Beenhouwer, M., Lievens, B., and Honnay, O. (2016). Crop-specific and single-species mycorrhizal inoculation is the best approach to improve crop growth in controlled environments. *Agron. Sustain. Dev.* 36:37. doi: 10.1007/s13593-016-0373-y

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Alteration of Bacterial Wilt Resistance in Tomato Plant by Microbiota Transplant

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Plant-associated microbiota plays an important role in plant disease resistance. Bacterial wilt resistance of tomato is a function of the quantitative trait of tomato plants; however, the mechanism underlying quantitative resistance is unexplored. In this study, we hypothesized that rhizosphere microbiota affects the resistance of tomato plants against soil-borne bacterial wilt caused by *Ralstonia solanacearum*. This hypothesis was tested using a tomato cultivar grown in a defined soil with various microbiota transplants. The bacterial wilt-resistant Hawaii 7996 tomato cultivar exhibited marked suppression and induction of disease severity after treatment with upland soil-derived and forest soil-derived microbiotas, respectively, whereas the transplants did not affect the disease severity in the susceptible tomato cultivar Moneymaker. The differential resistance of Hawaii 7996 to bacterial wilt was abolished by diluted or heat-killed microbiota transplantation. Microbial community analysis revealed the transplant-specific distinct community structure in the tomato rhizosphere and the significant enrichment of specific microbial operational taxonomic units (OTUs) in the rhizosphere of the upland soil microbiota-treated Hawaii 7996. These results suggest that the specific transplanted microbiota alters the bacterial wilt resistance in the resistant cultivar potentially through a priority effect.

Keywords: rhizosphere microbiome, tomato plant, microbiota transplant, bacterial wilt, *Ralstonia solanacearum*

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INTRODUCTION

The lethal bacterial wilt (BW) disease is caused by *Ralstonia solanacearum*, and the bacterial pathogen infects more than 400 plant species, especially plants belonging to the *Solanaceae* family (Hayward, 1991; Scott et al., 2005). *R. solanacearum* is a soil-borne pathogen that enters the plant through wounds or elongation zones and subsequently resides in the xylem vessels to block water transport (Vasse et al., 1995). The pathogenesis of BW, including bacterial invasion and pathogen colonization of the xylem, is regulated by a highly complex and sophisticated signaling mechanism (Hikichi et al., 2017). As there are no effective chemical agents for managing BW, the disease is generally managed by crop rotation and disease-resistant plants (Hanson et al., 1998;

Wang et al., 2000). There are several BW-resistant cultivars of tomato (Wang et al., 1998), pepper (Du et al., 2016), and eggplant (Salgon et al., 2017). One of the well-known BW-resistant tomato cultivars is Hawaii 7996, which exerts the most stable resistance against *R. solanacearum* infection by several major and minor quantitative trait loci (QTL) (Thoquet et al., 1996; Wang et al., 1998). However, the quantitative resistance to BW is not completely understood, and the genes and functions of QTL have not been characterized in Hawaii 7996 and other major crops. It is known that the performance of quantitative resistance in Hawaii 7996 is frequently influenced by environmental conditions such as the pathogen strain, temperature, and soil conditions (Wang et al., 2013).

The plant rhizosphere is the dynamic and complex interface between the plant root and soil. The plant rhizosphere serves as a niche where the soil microbiota derives nutrition from the plant (Dennis et al., 2010; Berendsen et al., 2012). Recent studies have demonstrated that diverse microorganisms are associated with the plant in the rhizosphere (Mendes et al., 2013) and form plant-specific microbial communities (Hassani et al., 2018). Various factors in the rhizosphere affect the composition of the microbial community (Marschner et al., 1986; Dennis et al., 2010). Plant functions, such as growth, development, and stress tolerance, are influenced by the rhizosphere microbiota (Lau and Lennon, 2012; Panke-Buisse et al., 2015; Robbins et al., 2018). The soil microbiota in this niche can affect plant health negatively or positively. The agronomic goal is to positively promote plant functions including plant growth and health (Mendes et al., 2011; Lundberg et al., 2012; Bulgarelli et al., 2013). The plants shape the bacterial community structure of their rhizosphere using the microbial reservoir of the soil. Both biotic and abiotic factors are reported to shape the structural and functional diversities of microbial communities in the rhizosphere (Berg and Smalla, 2009; Bulgarelli et al., 2012; Lundberg et al., 2012).

Recently, soil microbiota was reported to protect plants against various diseases, such as potato scab disease caused by *Streptomyces* species (Meng et al., 2012), *Fusarium* wilt of various plants (Chialva et al., 2018), damping-off disease of sugar beet caused by *Rhizoctonia solani* (Mendes et al., 2011), and take-all decline of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Weller et al., 1988). There is growing evidence that suggests the role of the rhizosphere microbiome in protecting the plant against soil-borne diseases (Kyselková et al., 2009; Chialva et al., 2018; Kwak et al., 2018). The soil microbiome can directly protect plants against disease or can modulate the plant's defense mechanism against disease (Millet et al., 2010; Mendes et al., 2011). Additionally, plant defense hormones, such as salicylic acid, can modulate the soil microbial communities (Lebeis et al., 2015).

Soil is a highly heterogeneous matrix that supports plant growth. The physicochemical properties and microbial diversity of soil vary with each soil type. Although soil properties also contribute to plant growth and health, the physicochemical properties frequently mask the microbial function that regulates plant traits. Therefore, it is necessary to investigate the microbial function in the rhizosphere under defined soil conditions to understand the role of microbiota in regulating plant traits (Vorholt et al., 2017; Kwak et al., 2018).

In this study, we used soil microbiota transplant in tomato plants under defined soil condition to investigate the disease progress of BW in tomato. We have previously shown that microbial community structure of BW-resistant Hawaii 7996 is distinct from that of BW-susceptible cultivar Moneymaker, and specific microbiota is recruited by host plant to protect themselves (Kwak et al., 2018). However, in this study, we focused on the microbiota associated with the resistant cultivar Hawaii 7996 to investigate the role of tomato rhizosphere microbiota in influencing BW resistance. Our hypothesis is that soil microbiota transplant contributes to the formation of distinct rhizosphere microbial communities and to subsequently affect plant traits, especially BW resistance in tomato. To our knowledge, this is the first to show that plant quantitative trait can be affected by plant-associated microbiota.

MATERIALS AND METHODS

Soil Sampling

In this study, we established a protocol to use soil microbial fraction (MF) for microbiota transplant from various soils. The initial soil samples comprised 18 different soils and included natural soils from different types of vegetation, such as various crop cultivated fields, forest, and alluvial soils from river estuarine and pasture areas, where there was no crop cultivation (**Supplementary Table S1**). The soil MFs were subjected to preliminary screening to determine the influence of soil microbiota on tomato BW progress. Based on these results, four different soils showing distinct and differential BW resistance by microbiota transplant were selected for further investigation: upland, paddy, forest, and alluvial soils. The topsoil (3–5 cm) and organic debris were removed, and the soil layer between 5 and 10 cm was collected using a shovel. The harvested soil was sieved through a 5 mm mesh to exclude the remaining organic debris. The sieved soil samples were stored in zipper bags at ambient temperature. For long-term storage, the soil samples were stored at −80°C in zipper bags under dark conditions until further use. The physicochemical properties of the soil samples were analyzed at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University, Seoul, Korea. The physicochemical properties of each soil are listed in **Supplementary Table S2**.

Preparation of the Soil MF

The soil MF was isolated from the soil samples using 170 g of soil. The soil sample was incubated with 250 ml of 2.5 mM MES buffer (pH 5.7) on a shaker at 200 rpm for 30 min. The mixture of soil and MES buffer was centrifuged at 500 rpm for 5 min to remove most of the soil particles. The supernatant was subsequently centrifuged again at 8,000 rpm for 15 min to collect the bacterial cell pellet. The bacterial cell pellet was resuspended in 220 ml of 2.5 mM MES buffer (pH 5.7)

(**Supplementary Figure S1A**). This final bacterial suspension derived from 170 g of soil was used as the soil MF for treating 10 tomato seedlings and for the comparison of bacterial community between bulk soil and soil MF (**Supplementary Figure S2**).

The Analysis System for Plant–Microbiome Interaction (ASPMI)

Tomato seeds (*Solanum lycopersicum* cv. Hawaii 7996 and cv. Moneymaker) were subjected to serial surface sterilization with 70% ethanol containing 0.1% TritonX-100 by vigorous vortexing for 1 min and 0.5% NaOCl containing 0.1% Triton X-100 for 15 min. The seeds were thoroughly washed with sterilized distilled water (SDW) and dried in a laminar flow hood before germination. The germination and plant growth conditions were 14 h/10 h of a light/dark regime at 28°C for all experiments. The seeds were germinated on sterilized filter paper in Petri dishes containing 5 mL SDW (ADVANTEC, Tokyo, Japan) for 7 days until planting. The germinated seedlings were planted in a 10 hole cell seedling tray that was surface-cleaned with 70% ethanol, and each hole contained 17 g of sterilized commercial horticultural nursery soil (Punong Co., Ltd, Korea). The horticultural nursery soils were autoclaved twice (121°C for 40 min) with an interval to allow the soil to reach ambient temperature before the second round of autoclaving. The planted tomato seedlings were treated with 20 ml of soil MF and were grown for 3 weeks before *R. solanacearum* inoculation. For the control, the seedlings were treated with an equal volume of 2.5 mM MES buffer (pH 5.7) (**Supplementary Figure S1B**). When necessary, the soil MF was diluted 10- or 100-fold with 2.5 mM MES buffer or was autoclaved at 121°C for 20 min before treatment to tomato seedlings.

BW Disease Incidence Assays and Quantification of *R. solanacearum* SL341

All strains of *R. solanacearum* (**Supplementary Table S3**) were cultured in CPG medium plates containing 2,3,4-triphenyl tetrazolium chloride (TZC) for 36 h at 30°C. Except for the virulence comparison of Hawaii 7996 among *R. solanacearum* strains, the strain SL341 (race 1, phylotype I, *i.e.*, *R. pseudosolanacearum*) (Safni et al., 2014) was used for most BW progress assays (**Supplementary Table S3**). The cultured bacterial cells were suspended in SDW, and the cell density was adjusted to 2×10^8 CFU/ml. The final bacterial suspension was poured onto the soil in the pot containing soil MF-treated plants (grown for 3 weeks after soil MF treatment) at a final concentration of 1×10^7 CFU/g of soil (**Supplementary Figure S1B**).

To investigate the population of *R. solanacearum* SL341 in the tomato rhizosphere and endosphere, Hawaii 7996 grown in sterilized nursery soil were treated with upland soil MF or forest soil MF, and then after 3 weeks, SL341 strain was inoculated. SL341 cell density was measured at 2 h, 5 and 14 days post inoculation (dpi); the population of the cells was determine 2 h post inoculation in rhizosphere, and the cell density in the roots and stems of Hawaii 7996 cultivar was quantified at 5 and 14 dpi. BW disease incidence was scored until 14 dpi using the following formula: (number of wilted leaves/total number of leaves) × 100 (%). For disease scoring, three replications were used, each containing 10 plants for the soil MF treatment and control.

Evaluation of the Antimicrobial Effect of the Rhizosphere Microbiome

In order to investigate whether the differential bacterial wilt resistance in Hawaii 7996 treated with soil MFs was due to direct antagonism to *R. solanacearum*, the antimicrobial activity of the rhizosphere soil of Hawaii 7996 treated with either upland soil MF or forest soil MF was tested. The soil MF-treated Hawaii 7996 cultivars were allowed to grow for 3 weeks. The rhizosphere soil was collected from the tomato plants. *R. solanacearum* SL341 at an OD_{600} of 0.3 was mixed with 32.5 ml of collected rhizosphere soil suspension in 2.5 mM MES buffer. This mixture was applied to 30 g sterilized nursery soil that was subsequently incubated at 30°C in a stationary incubator. The inoculated soil (1 g) from three replicates was collected to measure the colony forming units (CFUs) of *R. solanacearum* every 2.5 h until 10 h on semiselective SMSA medium (Engelbrecht, 1994).

DNA Extraction From Bulk Soil, MF, and Rhizosphere Soil

Metagenomic DNA was extracted from 500 mg of bulk soil, plant rhizosphere and MF to amplify 16S rRNA genes. The tomato plants were manually harvested from the pots to collect the rhizosphere soil. The large soil aggregates loosely attached to the roots were removed by gentle tapping, leaving only the firmly adhered soil particles. The plant roots were immersed in 5 ml of 2.5 mM MES buffer (pH 5.7) in 50 ml falcon tubes and sonicated at 135 W for 5 min using a sonicator (Branson 5500DTH, Danbury, USA). Next, 5 ml of the soil suspension was centrifuged at 13,000 rpm. The soil pellet was weighed and processed for DNA extraction using the FastDNA™ SPIN for soil kit (MP Biomedicals, Solon, USA) following the manufacturer's instructions.

Amplicon Library Preparation and Sequencing

The 16S rRNA gene was subjected to GS-FLX amplicon sequencing and Illumina sequencing. Sequencing of samples (comparison of the bacterial community between bulk soil and MF in **Supplementary Figure S2**) was conducted as follows: For 454 pyrosequencing of the 16S rRNA gene amplicon, a PCR amplicon library was generated using the 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) (Mizrahi-Man et al., 2013) primers. These primers amplify a region spanning approximately 400 bp of the hypervariable region (V3–V4 region) of the bacterial 16S rRNA gene with the addition of 33 and 34 mer adaptors (underlined). Polymerase chain reaction (PCR) was performed in a 25-μl reaction volume containing 2.5 μl of 5 ng/μl template DNA, 12.5 μl of 2× KAPA HiFi HotStart Ready Mix (KAPA Biosystems), and 5 μl (1 μM) of each primer. The PCR conditions were as follows: initial

denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min.

Sequencing of the majority samples except for the data shown in **Figure S2** was conducted using Illumina (MiSeq) paired-end sequencing. For Illumina sequencing of the 16S rRNA gene amplicon, amplicon libraries were developed using the PCR primers 341F and 805R (Herlemann et al., 2011). These primers were used to amplify a region spanning approximately 400 bp of the hypervariable region (V3–V4 region). PCR was performed in a thermal cycler (Gene Atlas, Astec—Japan) in a 25-μl reaction volume containing 2.5 μl of 5 ng/μl template DNA, 12.5 μl of 2× KAPA HiFi HotStart Ready Mix (KAPA Biosystems), and 5 μl (1 μM) of each primer. The PCR conditions were as follows: an initial template denaturation step at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. To remove traces of PCR primers and primer dimers, PCR amplicons were purified using the Agencourt AMPure XP PCR Purification system (Beckman Coulter, Brea, USA), following the manufacturer's instructions. The quality of amplicons (including the negative control) was evaluated by agarose gel electrophoresis using 1% gel. The DNA concentration was measured using a NanoDrop instrument (Thermo Scientific, Wilmington, MA). The libraries for paired-end sequencing and 454 pyrosequencing were prepared, and sequencing was performed at NICEM. The 16S rRNA gene amplicon sequences were analyzed for the microbial community structure as described in the supporting information. Microbiome network was analyzed using the Molecular Ecological Network Analysis (MENA) pipeline (Zhou et al., 2010) as described in the supporting information.

Statistical Analysis

All statistical analyses were performed with R software (version 3.2.2) (http://www.r-project.org/). The suitability of the alpha-diversity indices was examined using the Shapiro–Wilk normality test followed by one-way univariate analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) *post hoc* test in R. As the data were not normally distributed, statistically significant differences in alpha-diversity indices were examined by nonparametric Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparisons *post hoc* test. To identify taxa that were significantly different between the Hawaii 7996 rhizosphere microbiota under the ASPMI treated with two different soil MFs, we used DESeq2 package. DESeq2 was run under a negative binomial fit, and Wald test and q-values were calculated with the Benjamini–Hochberg procedure to correct *p*-values and control for false discovery rates. Differences in the abundance were considered significant when FDR adjusted *p*-values were lower than 0.0001. The significant differences among the four different bulk soil and MF treatment groups (alluvial, forest, paddy, and upland) were evaluated by multivariate analysis of variance using distance matrices (ADONIS), which calculates squared deviations and determines statistical significance by F-tests on sequential sums of squares from permutations of data.

RESULTS

Establishment of ASPMI

Soil MFs from the various natural bulk soils were collected from several places and added to the sterilized soil to grow tomato seedlings with the isolated soil MFs (**Supplementary Figures S1A, B**). This ASPMI method enables the investigation of plant traits relevant to the treated microbiota in sterilized soils with similar physicochemical properties containing various microbiota. To verify the utility of ASPMI, thirteen physiochemical properties were comparatively evaluated between the four different field soils and sterilized commercial nursery soils treated with the MFs isolated from the corresponding four field soils. The four field soils exhibited differential physicochemical properties. However, the physicochemical properties were similar between the sterilized nursery soils that were treated with the four soil MFs (**Supplementary Table S2** and **Supplementary Figure S3**). These results suggest that the ASPMI method successfully eliminated the differential effect of physicochemical properties among the field soil samples, which enabled the evaluation of plant–microbiota interactions under controlled soil conditions using various soil MFs.

The bacterial community structure of both soil MFs and bulk soils was compared to determine whether the isolated soil MF represented the microbiota of the respective bulk soil. Principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity measure was performed to determine the beta-diversity (community comparison among microbial community) of the microbiome in the bulk soils and the corresponding MFs. The PCoA revealed that each resultant microbiota separated across the first and second principal coordinates (22.6 and 17.8% of variation, respectively), whereas only limited separation was observed between bulk soil and its respective MF (**Supplementary Figure S2**). Therefore, this indicated that the isolated soil MFs represented the bacterial community of the respective bulk soils.

Microbiota Transplant Influences BW Resistance

The ASPMI method was used to test the differential effect of various soil MFs against BW disease in the BW-resistant tomato cultivar (Hawaii 7996) and BW-susceptible cultivar (Moneymaker). In this study, 18 different soil MFs from various natural ecosystems in Korea were used to test BW resistance by inoculation of *R. solanacearum* strain SL341. Each microbiota transplant exhibited differential BW progress in the resistant cultivar Hawaii 7996 (**Figure 1A** and **Supplementary Figure S4**). To further investigate the effect of microbiota on BW resistance on Hawaii 7996, four different soils from various natural ecosystems in Korea were selected based on the distinct and differential quantitative resistance of tomato BW and the reproducibility of the results after several repetitive

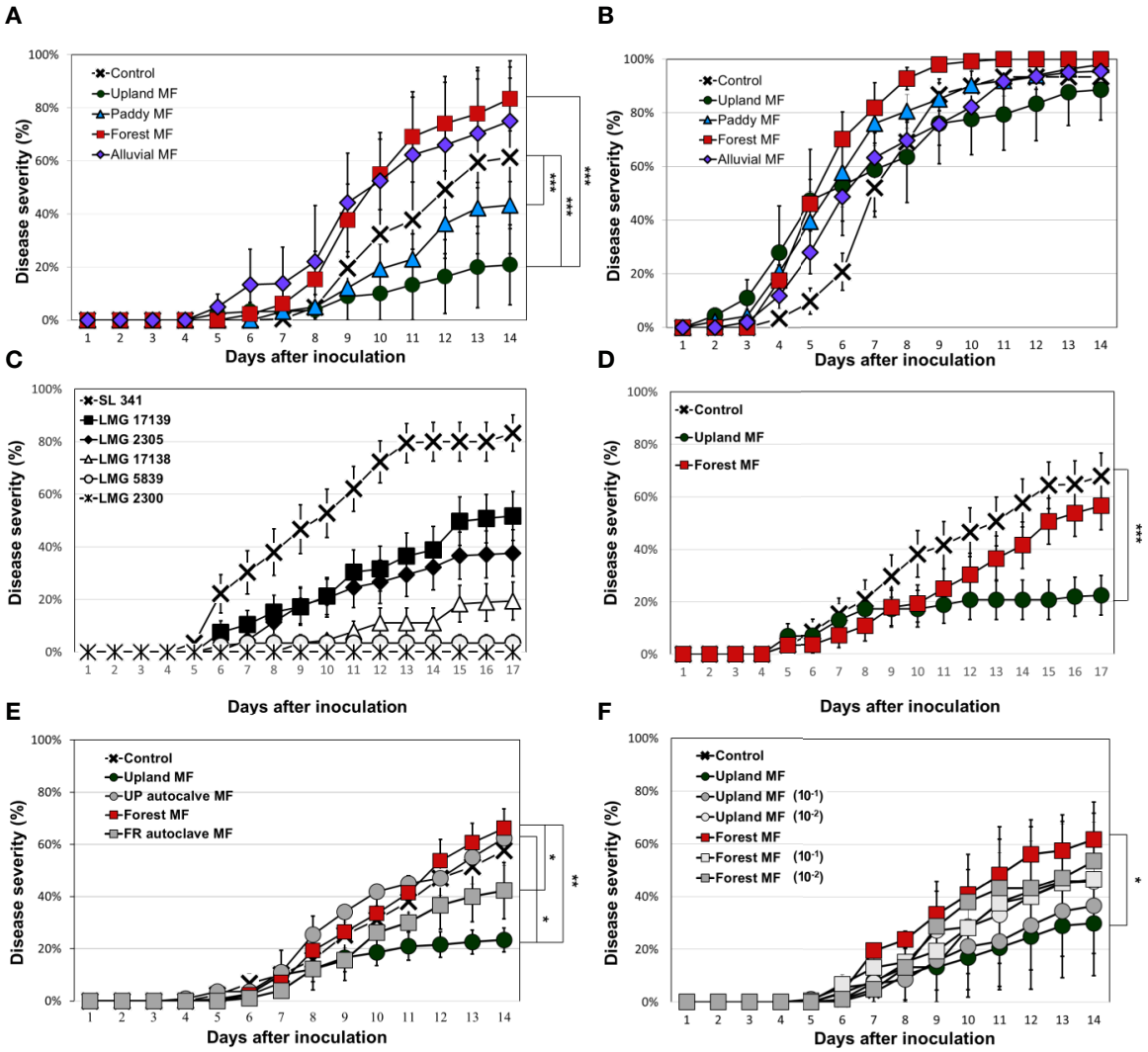

FIGURE 1 | Bacterial wilt (BW) disease progression in tomato cultivars treated with soil microbial fractions (MFs) evaluated by ASPMI method. BW disease progression by *R. solanacearum* SL341 in the tomato cultivar Hawaii 7996 treated with four different soil MFs **(A)** and in the susceptible cultivar Moneymaker treated with soils MFs **(B)**. BW disease progression in the Hawaii 7996 cultivar inoculated with six different *R. solanacearum* strains **(C)**. Evaluation of BW disease progression by *R. solanacearum* LMG 17139 strain in Hawaii 7996 treated with upland soil MF or forest soil MF **(D)**. Effect of heat-killed soil MFs (autoclave MF) **(E)** on BW disease progression in the Hawaii 7996 cultivar. Effect of diluted soil MF **(F)** on BW disease progression in the Hawaii 7996 cultivar (10^{-1}, ten-fold diluted MF, 10^{-2}, hundred-fold diluted MF). Control was treated with 2.5 mM MES buffer (pH 5.7). Each data point represents the mean disease incidence from three independent experiments. In total, 30 plants were analyzed for each treatment. Each vertical bar represents the standard error of the mean from three replicates (each replicate with 10 plants, $n = 30$). Significant difference was evaluated by repeated measure analysis of variance (ANOVA) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

experiments. The BW disease progression was significantly different among the soil MF-treated Hawaii 7966 plants. Interestingly, the progression of BW in the upland soil MF and paddy soil MF-treated plants was significantly delayed (repeated measures ANOVA, $p < 0.001$) compared to that in the control (**Figure 1A**). Treatment of upland soil MF was more effective to suppress disease progress in the Hawaii 7996 cultivar The forest soil MF-treated Hawaii 7996 plants exhibited the highest susceptibility to BW disease. However, the progression of BW in Moneymaker plants was similar among the plants treated with different soil MFs (**Figure 1B**).

Bacterial population of SL341 was estimated at three different time points in rhizosphere and endosphere of tomato plant (*cv.* Hawaii 7996). Initial inoculum densities of SL341 at 2 h post inoculation were not significantly different among treatments (**Figure 2A**), which is equivalent to 10^7 CFU/g of soil. Bacterial population of SL341 in the tomato roots was not significantly different at 5 dpi between upland soil MF transplant and forest soil MF transplant, while that with forest soil MF transplant was significantly higher than that with upland soil MF transplant at 14 dpi (**Figure 2B**). The population of SL341 was significantly increased by forest soil MF transplant from 5 dpi to 14 dpi.

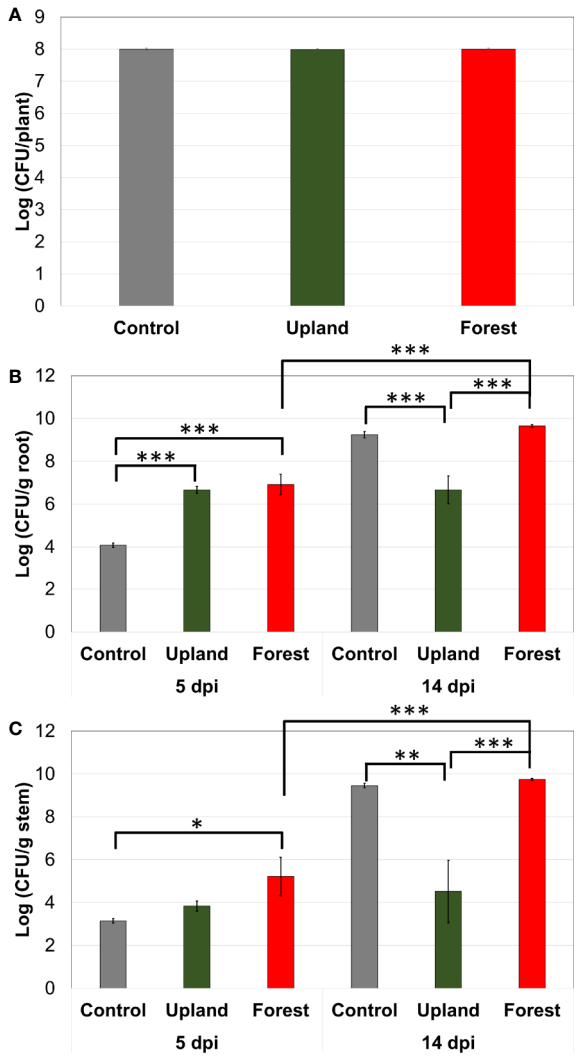

FIGURE 2 | Population dynamics of *R. solanacearum* SL341 in the tomato rhizosphere **(A)**, roots **(B)**, stems **(C)** of resistant cultivar treated by upland soil MF and forest soil MF over time. Control was treated with 2.5 mM MES buffer (pH 5.7). Each vertical bar represents the standard error of the mean from three replicates (each replicate with three plants, n = 3) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

However, the population of SL341 was maintained steady from 5 dpi to 14 dpi by upland soil MF transplant (**Figure 2B**). Similarly, bacterial population of SL341 in the tomato stems was not significantly different at 5 dpi between upland soil MF transplant and forest soil MF transplant. The bacterial population was significantly increased from 5 dpi to 14 dpi by forest soil MF transplant whereas that transplanted by upland soil MF was maintained steady (**Figure 2C**). Overall, the bacterial population of SL341 in tomato roots and stems treated with different soil MFs was coincident to the bacterial wilt progress in Hawaii 7996 (**Figure 1A**).

Hawaii 7996 is highly resistant to *R. solanacearum* strains; however, this cultivar was susceptible to strain SL341 in sterile soil without microbiota transplantation. Therefore, we tested whether other strains of *R. solanacearum* could affect BW resistance under the same conditions. Six strains of *R. solanacearum* were selected for the virulence test (**Supplementary Table S3**) based on the stable production of exopolysaccharide (EPS) in TZC medium. Among these six strains, the LMG17139 strain exhibited the highest virulence except SL341 strain in the Hawaii 7996 cultivar (**Figure 1C**). Furthermore, the upland soil MF-treated Hawaii 7996 cultivar inoculated with the LMG17139 strain exhibited significantly (repeated measures ANOVA, $p < 0.001$) delayed BW disease progression compared to the control and forest soil MF (**Figure 1D**). No significant difference of disease progress was observed between control and forest soil MF. Although it is speculative, the vulnerability of BW resistance in Hawaii 7996 to highly virulent strains of *R. solanacearum* could be altered by soil microbiota transplant.

Next, the effect of soil MF on BW disease progression was evaluated by treating the Hawaii 7996 cultivar with autoclaved (heat-killed) soil MFs. The heat-killed soil MFs completely diminished the suppression or induction of disease progression observed in the plants treated with live soil MFs (**Figure 1E**). No significant difference was noticed among treatments of heat-killed soil MFs. Moreover, the treatment with diluted (10^{-1} and 10^{-2}) soil MF decreased the positive or negative effect on the BW disease progression and equalized the disease severity (**Figure 1F**). Although each soil MF affected BW disease progression in the BW-resistant Hawaii 7996 cultivar but not in the BW-susceptible cultivar, we needed to test whether the rhizosphere microbiota of Hawaii 7996 treated by upland soil MF would inhibit the growth of bacterial pathogens by direct antagonistic effects. To test this, we incubated *R. solanacearum* SL341 in the rhizosphere soils from Hawaii 7996 cultivars treated with an upland soil MF or a forest soil MF. However, direct growth inhibition of *R. solanacearum* was not observed in the rhizosphere soils (**Supplementary Figure S5**).

Effect of Transplanted Soil MFs on the Microbiota Structure in the Tomato Rhizosphere

The effect of soil MF or MES buffer treatment (control) on the bacterial community structure was investigated in the tomato rhizosphere. Comparative analysis of alpha-diversity indices [Shannon diversity index (H')] revealed that there was a significant difference in the alpha diversity of the tomato rhizosphere microbiota (ANOVA with HSD *post hoc* test, $p < 0.05$), except between the rhizosphere microbiota of control and alluvial soil MF-treated plants and between the rhizosphere microbiota of paddy soil MF-treated and forest soil MF-treated plants. The alpha diversity of the rhizosphere microbiota of control plants exhibited the lowest H', whereas that of upland soil MF-treated plants exhibited the highest H' (**Figure 3A**). Bray–Curtis dissimilarity multivariate analysis was performed for tomato rhizosphere microbiota post soil MF treatment. Nonmetric multidimensional scaling (NMDS) was used to visualize the microbial community structure. Distinct separation of bacterial communities in the rhizosphere was apparent in the tomato plants treated with different soil MFs

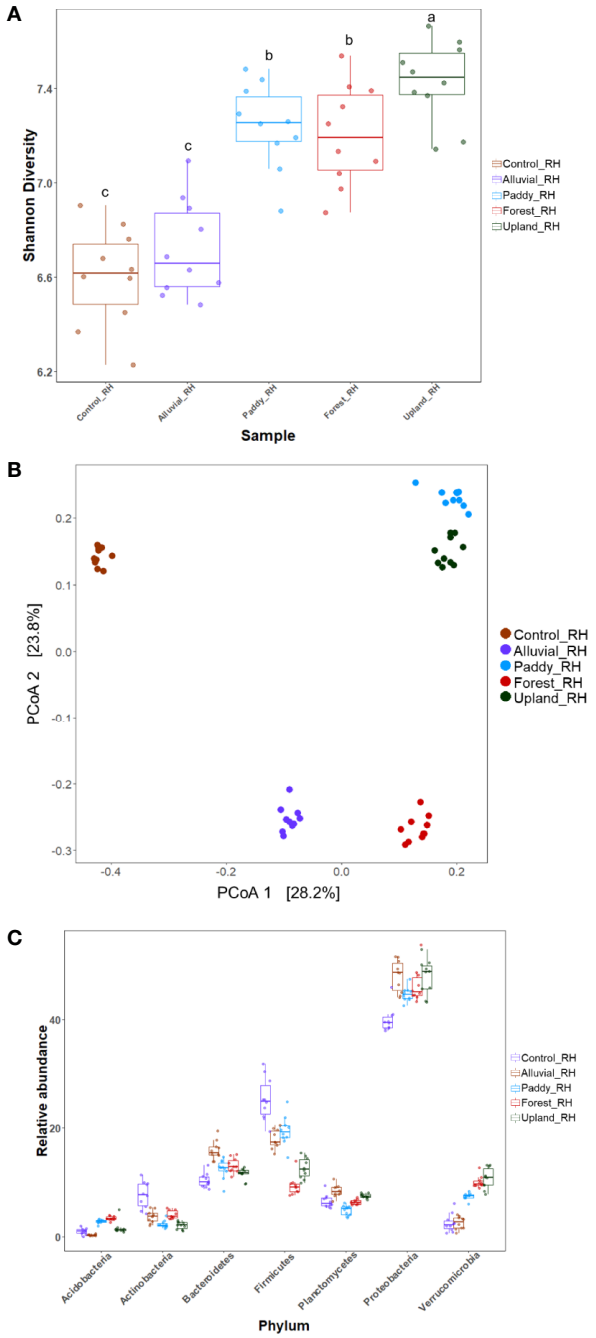

FIGURE 3 | Microbial community analysis of rhizosphere in the Hawaii 7996 cultivar treated with different soil microbial fractions (MFs). **(A)** Comparative alpha-diversity analysis of bacterial community among the bacterial wilt (BW)-resistant Hawaii 7996 cultivars treated with four different MFs. Shannon diversity index (*H'*) is calculated after subsampling ($p < 0.05$, ANOVA with HSD *post hoc* test). Different letters indicate significant differences. **(B)** Comparative beta diversity analysis of bacterial community among the BW-resistant Hawaii 7996 cultivars treated with four different MFs. The distribution pattern of four rhizosphere microbiomes in the BW-resistant cultivars evaluated using nonmetric multidimensional scaling based on the Bray–Curtis dissimilarity measures. **(C)** Relative abundance (%) of the major bacterial phyla in the rhizosphere microbiota of the BW-resistant cultivar treated with four different soil MFs.

(**Figure 3B**). ADONIS revealed that the rhizosphere microbiota of Hawaii 7996 exhibited significant differences in the microbial community structure between the groups ($R^2 = 0.37946$, $p < 0.001$).

Additionally, the rhizosphere microbiota of tomato plants was distinct from the microbiota of bulk soils in the same pot, which contained the initially treated MFs. There was no significant difference in species richness and evenness between the rhizosphere and bulk soils treated with upland soil MF and forest soil MF (**Supplementary Figure S6A**). Bray–Curtis dissimilarity measures revealed that there was a significant difference ($R^2 = 0.37838$, $p < 0.001$) in the microbial communities among bulk soils and rhizosphere soils of tomato plants treated with the upland soil MF or forest soil MF (**Supplementary Figure S6B**).

These results suggest that the rhizosphere microbiota in the tomato plants was sculpted to have a unique community structure from the respective soil MF input with a distinct community composition.

Microbial Taxa Distribution in the Rhizosphere of Soil MF-Treated Tomato

The microbial community composition of the tomato rhizosphere was comparatively evaluated between the upland soil MF-treated and forest soil MF-treated plants. The rhizosphere microbiota of the upland soil MF-treated tomato plants was different from that of forest soil MF-treated tomato plants (**Figures 3A, B**). The rhizosphere of upland soil MF-treated and forest soil MF-treated tomato plants exhibited significant differences in the relative abundance (RA) of the following bacterial phyla: *Acidobacteria* (1.60 and 3.34%, respectively), *Actinomycetes* (2.05 and 4.00%, respectively), *Planctomycetes* (7.49 and 6.33%, respectively), *Firmicutes* (12.6 and 9.32%, respectively) (**Figure 3C**). Further, individual taxa displaying differential abundance are listed in **Supplementary Table S4** (DESeq2, log2 fold change); plus log2 fold change indicates the individual taxa belongs to *Opitutaceae*, *Burkholderiaceae*, *Oxalobacteriaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Chitinophagaceae*, and *Planctomycetes* which are more abundant in upland soil MF-treated tomato plants. The minus log2 fold change indicates the individual taxa belongs to *Enterobacteriaceae*, *Calulobacteriaceae*, *Chthoniobacteriaceae*, *Chitinophagaceae*, *Isosphaeraceae*, *Cytophagaceae, and Sporolactobacillaceae* which are more abundant in forest soil MF-treated tomato plants (**Supplementary Table S4**). Particularly, 118 OTUs were enriched in upland soil MF-treated tomato plants, and 88 OTUs were enriched in forest soil MF-treated tomato plants (**Supplementary Table S4**). These data illustrate that certain rhizosphere bacterial OTUs or a combination of OTUs may be responsible for the differential disease progress of BW in Hawaii 7996.

Putative Keystone Taxa and Their Differential Abundance in the Tomato Rhizosphere

The potential candidate OTUs for the network hub, module hub, and connector were identified based on the rhizosphere

microbial community data of upland soil MF-treated (**Figure 4A**) and forest soil MF-treated tomato plants (**Figure 4B**). The module connectivity (*Zi*) and among-module connectivity (*Pi*) values (Deng et al., 2012) were measured in the rhizosphere soil of upland soil MF-treated and forest soil MF-treated Hawaii 7996 plants. The network analysis revealed that the peripherals were the most abundant nodes in the network hub. Additionally, no network hub was detected in the rhizosphere of upland and forest soil MF-treated tomato plants (**Figures 4C, D**). In total, two module hubs, OTUs of *Bacteroidetes*, were identified in the rhizosphere network of upland soil MF-treated plants. Five module connectors, OTUs of *Proteobacteria*, *Verrucomicrobia*, *Firmicutes*, *Bacteroidetes*, and *Planctomycetes Bacteroidetes* were also identified in the rhizosphere network of upland soil MF-treated Hawaii 7996 (**Figure 4C** and **Supplementary Table S5**). On the other hand, four module hubs belonging to *Proteobacteria* and two connectors, OTUs of *Proteobacteria* were detected in the rhizosphere network of forest soil MF-treated Hawaii 7996 (**Figure 4D** and **Supplementary Table S5**). The putative keystone taxa of the rhizosphere of upland soil MF-treated and forest soil MF-treated plants include OTUs from *Verrucomicrobia, Firmicutes, Bacteroidetes*, and *Planctomycetes* which were identified based on the *Pi* and *Zi* scores. However, they did not overlap in the network analysis between communities of two different treatment groups.

The RA of microbes in the module hubs ranged from 0.496 to 1.042% for upland soil MF-treated plants and from 0.451 to 1.158% for forest soil MF-treated plants (**Supplementary Figure S7**). The connectors also exhibited low RA (0.131 and 1.641%) (**Supplementary Figure S7**). The RA of putative keystone taxa in each treatment group revealed the differential abundance of keystone taxa in the rhizosphere of upland soil MF-treated and forest soil MF-treated plants (**Figure 5**). The number of network topological properties was compared among the rhizospheres of tomato treated with four different soil MFs. The properties included total nodes, total edges, the average degree, network diameter, network density, average clustering coefficient, and average path length (**Supplementary Table S6**). The microbiota network of the rhizosphere of upland soil MF-treated plants exhibited a higher number of network topological properties than the rhizosphere of forest soil MF-treated plants (**Supplementary Table S6**).

DISCUSSION

This study aimed to test our hypothesis that resistance of the well-known tomato cultivar Hawaii 7996 against BW is altered by rhizosphere microbiota. Rhizosphere microbiota is highly complicated and dependent on the surrounding soils. The structure of the rhizosphere microbiota is influenced by various biotic and abiotic factors. The critical factors determining the structure of rhizosphere microbiota are the soil type and soil physicochemical properties (Schutter et al., 2001; Bulluck et al., 2002; Marschner et al., 2004; İnceoğlu et al., 2012). Because the physiochemical properties of soil strongly influence the rhizosphere microbiome structure (Staley and Konopka, 1985; Amann et al., 1995; Hugenholtz et al., 1998; Lauber et al., 2008), we established the ASPMI method to evaluate the BW resistance of tomatoes treated with various soil MFs under sterile soil conditions. A previous study isolated microbes from field soils by excluding variations in soil physicochemical properties and treated isolated microbes with *Boechera stricta* seedlings to determine their effect on flowering phenology and time (Wagner et al., 2014). Our study adopted and modified the method developed by Wagner et al. (2014). The modifications mostly included two-step centrifugation of the soil suspension instead of filtration of soil suspension to establish the ASPMI protocol (**Supplementary Figure S1**). Our ASPMI protocol successfully excluded the abiotic factors in various soils (**Supplementary Table S2**). It is likely that application of soil MFs to aseptically germinated tomato seedlings aided in microbial colonization of the rhizosphere of tomato to sculpt a unique rhizosphere microbial community in the sterilized soil. Therefore, the microbial community may exhibit a priority effect in the tomato rhizosphere (Busby et al., 2017; Wei et al., 2019) as the soil MF was applied to germ-free tomato seedlings.

The rhizosphere microbiota plays an important role in protecting plants from pathogen invasion (Kwak et al., 2018) by preventing pathogen colonization or by facilitating the colonization of commensal bacteria. In this study, the upland soil MF-treated Hawaii 7996 cultivars exhibited higher BW disease resistance than the plants treated with other soil MFs. Interestingly, the soil MF treatment did not protect the BW-susceptible cultivar Moneymaker (**Figures 1A, B**). However, the MFs of upland soil and forest soil did not exhibit any antimicrobial effect against *R. solanacearum* (**Supplementary Figure S5**). This suggested that the observed BW resistance in Hawaii 7996 was the function of plant–microbiota interaction and was not due to the direct antagonistic effect. In fact, population dynamics of *R. solanacearum* SL341 showed that population of SL341 was not different at 5 dpi both in the roots and stems of Hawaii 7996 treated with either upland soil MF or forest soil MF (**Figure 2**). This suggested that pathogen invasion was not affected by microbiota transplant in tomato roots, and there might be no antagonistic effect by upland soil MF to bacterial pathogen.

Interestingly, in the same BW-resistant tomato cultivar, bacterial population of SL341 *in planta* was dramatically increased over time by forest soil MF treatment but not by upland soil MF treatment (**Figure 2**). It is likely that microbiota transplant somehow influenced the BW resistance of tomato Hawaii 7996 to have altered disease progress, *i.e.* tomato plants with upland soil-derived microbiota hindered the multiplication of bacterial pathogen *in planta*. Several reports have illustrated that *R. solanacearum* in the resistant tomato cultivar is limited to colonize inside of tomato plants and not able to multiply in the stem, although the resistant cultivar contains significant number of bacteria in the roots and shoots (Grimault et al., 1994; Saile et al., 1997). The colonization of *R. solanacearum* race 3 in tomato stems restricted by QTL was also reported by Carmeille et al. (2006). Our result suggests that the priming of the defense

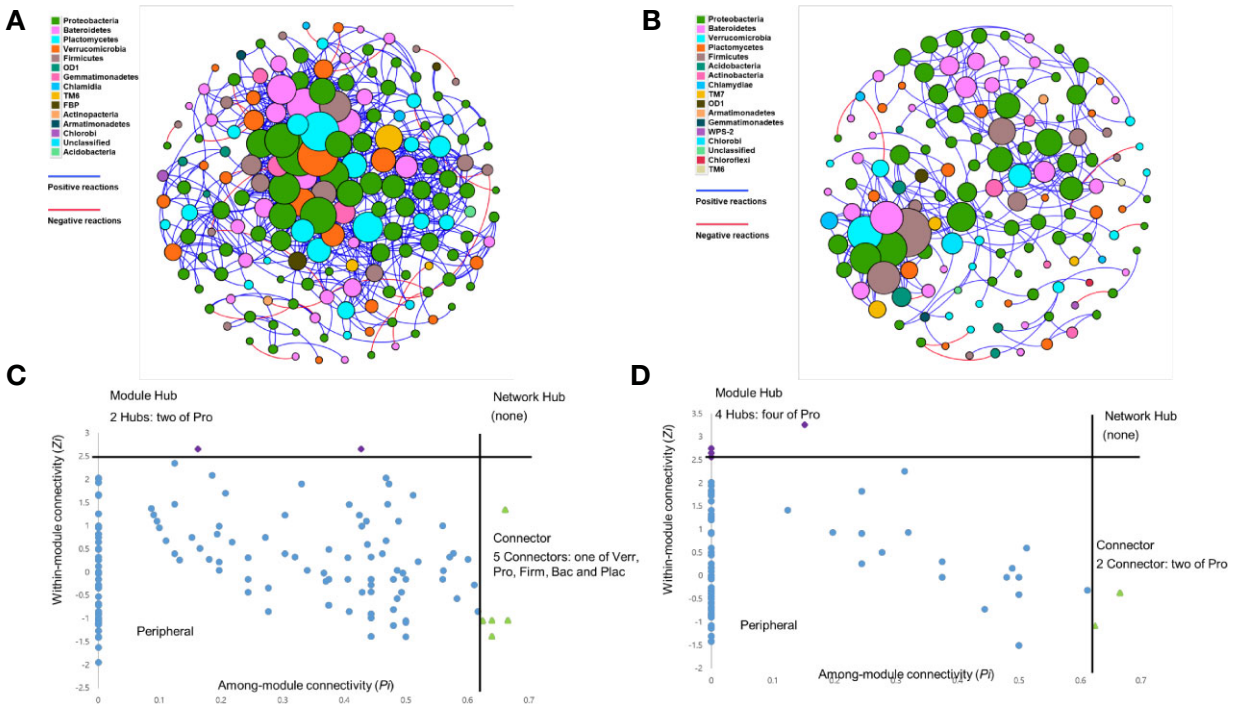

FIGURE 4 | Co-occurrence network of rhizosphere of tomato treated with upland soil microbial fraction (MF) **(A)** and forest soil MF **(B)**. Each node represents different operational taxonomic units (OTUs), while the edges indicate correlation between the nodes. Edges (lines) between nodes are colored blue for positive correlations between taxa; negative correlations are colored red. Node size corresponds to the number of edges. Node color represents each Phylum. Analysis of nodes to identify the putative keystone species in the rhizosphere networks **(C, D)**. Each symbol represents an OTU from rhizosphere network of upland soil MF-treated plants **(C)** and forest soil MF-treated plants **(D)** adopted for detailed module analysis. Network hub contains $Zi > 2.5$ and $Pi > 0.62$. Module hubs have $Zi > 2.5$, while the connectors retain $Pi > 0.62$. The taxonomy information of the module hubs (purple) and module connectors (green) is named on the plot using the following abbreviations; Bac, *Bacteroidetes*; Firm, *Firmicutes*; Plac, *Planctomycetes*; Pro, *Proteobacteria*; Verr, *Verrucomicrobia*.

response or alteration r of disease resistance trait is mediated by the rhizosphere microbiome in the Hawaii 7996 cultivar. The upland soil MF treatment conferred higher resistance to BW only in the BW-resistant cultivar, which further indicated that the alteration of BW disease resistance is mediated by the rhizosphere microbiome. One can argue if the differential entophytic community derived from different soil MFs transplant may be responsible for the altered BW-resistance. This needs to be investigated further. In contrast, the forest soil MF-treated Hawaii 7996 cultivars exhibited enhanced susceptibility to BW. It would be interesting to evaluate whether certain groups of microbial taxa or the microbial community enhance BW disease susceptibility in BW-resistant cultivars.

In this study, the ASPMI method was used to harvest microbes to obtain the soil MF from a variety of natural bulk soils and to analyze the biological effect of the isolated soil MF. In fact, the BW resistance of Hawaii 7996 conferred by upland soil MF transplant was completely abolished by heat-killed MF treatment. This result suggested that the plant phenotype (*i.e.*, BW resistance) could be regulated by the biological effect of treated soil MF. The tomato rhizosphere microbiota shaped by treating the sterilized nursery soil with various soil MFs may not fully represent the function of the original field soil microbiota because the soil properties under ASPMI were completely different from those of the original soil. However, the soil MF enabled the reproducible investigation of plant host response, *i.e.*, tomato BW resistance, to its microbiota compared to the field soil microbiota. This is because the field soil microbial composition is affected by fluctuating environmental factors that cannot be efficiently controlled. In this study, two soil MFs, upland soil MF and forest soil MF, displayed differential effects on the BW resistance of Hawaii 7996 under ASPMI. The bacterial community diversity and composition were markedly different between the fertilized, intensely managed grassland, and forest soils (Peiffer et al., 2013). Similarly, the rhizosphere bacterial community structure was significantly different between the upland soil MF-treated and forest soil MF-treated tomato plants (**Figure 3A**). These results indicate that the differential composition of bacterial taxa observed in the rhizosphere of the plants treated with various soil MFs may potentially influence the BW resistance. This differential effect on the plant phenotype may be due to the priority effect of initially colonized microbiota in the tomato rhizosphere (Wei et al., 2019).

In this study, most of the network nodes and connectors in the rhizosphere of upland soil MF-treated and forest soil MF-treated plants exhibited a relatively low abundance of putative keystone taxa (**Supplementary Figure S7**). This indicated that the rare taxa may be key to developing or maintaining the

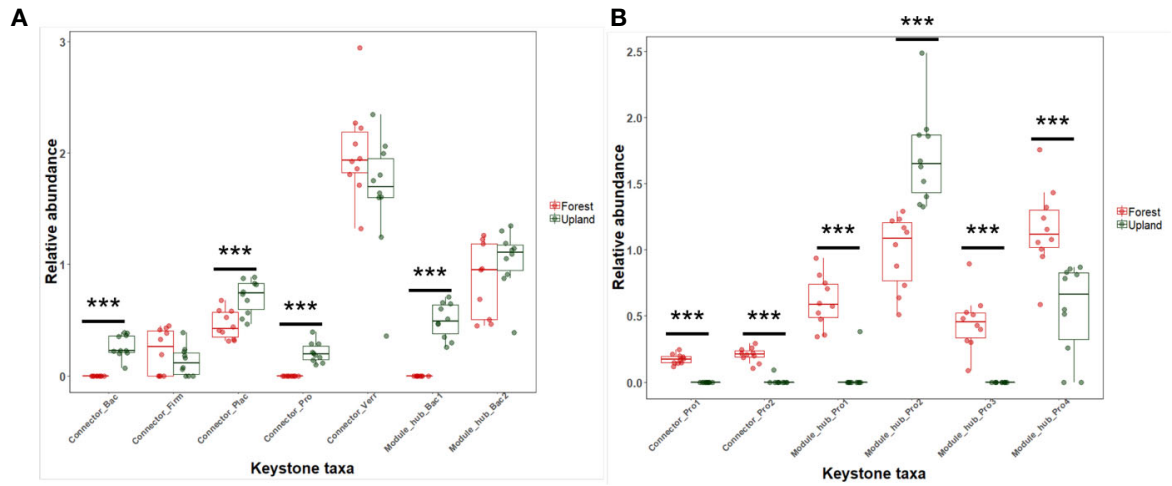

FIGURE 5 | Relative abundance (%) of the keystone taxa in co-occurrence network of rhizosphere in the upland soil MF-treated plants **(A)** and forest soil MF-treated plants **(B)**. Statistical significances between the treatment groups were evaluated by Wilcoxon signed ranks test using the R software (version 3.2.2). ***$p < 0.001$.

structure of the rhizosphere network. In fact, treatment of diluted upland soil MF lost the original upland soil MF activity of BW resistance (**Figure 1F**), suggesting that key players of upland soil MF to alter BW resistance in Hawaii 7996 could be members with low abundance. Similar conclusions were reported in other studies on microbial community analysis with macro- and micro-ecological networks in various ecosystems (Power et al., 1996; Lyons and Schwartz, 2001; Pester et al., 2010; Lupatini et al., 2014; Deng et al., 2016). The rhizosphere microbiome of upland soil MF-treated plants exhibited a higher complexity in the network in this study. An earlier study reported that the microbial community in the *Fusarium oxysporum* (*Fox*)-resistant cultivar was more complex than that in the *Fox*-susceptible cultivar (Mendes et al., 2017). High bacterial diversity was reported to confer enhanced resistance against pathogen invasion (Latz et al., 2012; Mallon et al., 2015). The disease resistance driven by the relationship between microbial diversity and pathogen invasion could be described by the fundamental interaction network architecture (Wei et al., 2015). The rhizosphere network of upland soil MF-treated plants contained more nodes and edges when compared to the rhizosphere network of forest soil MF-treated plants. The results of this study suggested that the highly connected and modular rhizosphere microbial community may be involved in conferring enhanced BW disease resistance to plants.

Genes and mechanisms underlying the quantitative resistance to BW in tomato Hawaii 7996 cultivar have yet to be identified and characterized. However, two major QTLs, *Bwr-12* and *Bwr-6*, have been identified in Hawaii 7996. *Bwr-12* confers phylotype I-specific BW resistance, and *Bwr-6* confers broad-spectrum BW resistance (Grimault and Prior, 1993). The *R. solanacearum* strain SL341 used in this study is phylotype I, and it is not clear how this strain could affect BW resistance in Hawaii 7996. Nonetheless, specific microbiota transplant, *i.e.*, upland soil MF, conferred stable resistance in Hawaii 7996 against the strain SL341. This result revealed the alteration of quantitative resistance in Hawaii 7996 by soil microbiota transplant, which will sculpt unique root microbiota. It would be interesting to investigate how the rhizosphere microbiota influences BW resistance in Hawaii 7996 once the genes are cloned. In conclusion, our ASPMI method can successfully be used to evaluate the effects of microbiota transplantation on the BW resistance of tomatoes, and this study is the first to show that quantitative traits of plant, such as disease resistance, can be altered by soil microbiota transplantation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra/?term=prjna559787.

AUTHOR CONTRIBUTIONS

S-WL conceived, organized, and supervised the project. KC, JC, RK, PL, and S-WL interpreted the result and prepared the manuscript. KC, JC, PL, NR, RK, HL, and HK performed the experiments. KC, HW, HK, PL, and S-WL edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01186/full#supplementary-material

REFERENCES

Amann, R. I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169.

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berg, G., and Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68, 1–13. doi: 10.1111/j.1574-6941.2009.00654.x

Bulgarelli, D., Rott, M., Schlaeppi, K., Themaat, E. V. L. V., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Themaat, E. V. L. V., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Bulluck, L., Brosius, M., Evanylo, G., and Ristaino, J. (2002). Organic and synthetic fertility amendments influence soil microbial, physical and chemical properties on organic and conventional farms. *Appl. Soil Ecol.* 19, 147–160. doi: 10.1371/journal.pone.0180442

Busby, P. E., Soman, C., Wagner, M. R., Friesen, M. L., Kremer, J., Bennett, A., et al. (2017). Research priorities for harnessing plant microbiomes in sustainable agriculture. *PLoS Biol.* 15, e2001793. doi: 10.1371/journal.pbio.2001793

Carmeille, A., Caranta, C., Dintinger, J., Prior, P., Luisetti, J., and Besse, P. (2006). Identification of QTLs for *Ralstonia solanacearum* race 3-phylotype II resistance in tomato. *Theor. Appl. Genet.* 113, 110–121. doi: 10.1007/s00122-006-0277-3

Chialva, M., Salvioli,, di Fossalunga, A., Daghino, S., Ghignone, S., Bagnaresi, P., et al. (2018). Native soils with their microbiotas elicit a state of alert in tomato plants. *New Phytol.* 220, 1296–1308. doi: 10.1111/nph.15014

Deng, Y., Jiang, Y.-H., Yang, Y., He, Z., Luo, F., and Zhou, J. (2012). Molecular ecological network analyses. *BMC Bioinform.* 13, 1471–2105. doi: 10.1186/1471-2105-13-113

Deng, Y., Zhang, P., Qin, Y., Tu, Q., Yang, Y., He, Z., et al. (2016). Network succession reveals the importance of competition in response to emulsified vegetable oil amendment for uranium bioremediation. *Environ. Microbiol.* 18, 205–218. doi: 10.1111/1462-2920.12981

Dennis, P. G., Miller, A. J., and Hirsch, P. R. (2010). Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiol. Ecol.* 72, 313–327. doi: 10.1111/j.1574-6941.2010.00860.x

Du, H., Chen, B., Zhang, X., Zhang, F., Miller, S. A., Rajashekara, G., et al. (2016). Evaluation of *Ralstonia solanacearum* Infection Dynamics in Resistant and Susceptible Pepper Lines Using Bioluminescence Imaging. *Plant Dis.* 101, 272–278. doi: 10.1094/PDIS-05-16-0714-RE

Engelbrecht, M. (1994). Modification of a semi-selective medium for the isolation and quantification of *Pseudomonas solanacearum*. ACIAR. *Bacterial Wilt Newslett.* 10, 3–5.

Grimault, V., and Prior, P. (1993). Tomato Bacterial wilt resistance associated with tolerance of vascular tissues to *Pseudomonas solanacearum*. *Plant Pathol.* 42, 589–594. doi: 10.1111/j.1365-3059.1993.tb01539.x

Grimault, V., Gélie, B., Lemattre, M., Prior, P., and Schmit, J. (1994). Comparative histology of resistant and susceptible tomato cultivars infected by *Pseudomonas solanacearum*. *Physiol. Mol. Plant P.* 44, 105–123. doi: 10.1016/S0885-5765(05)80105-5

Hanson, P. M., Licardo, O., Wang, J.-F., and Chen, J.-T. (1998). Diallel analysis of bacterial wilt resistance in tomato derived from different sources. *Plant Dis.* 82, 74–78. doi: 10.1094/PDIS.1998.82.1.74

Hassani, M. A., Durán, P., and Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome* 6, 58–74. doi: 10.1186/s40168-018-0445-0

Hayward, A. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* 29, 65–87. doi: 10.1146/annurev.py.29.090191.000433

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., and Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5, 1571–1579. doi: 10.1038/ismej.2011.41

Hikichi, Y., Mori, Y., Ishikawa, S., Hayashi, K., Ohnishi, K., Kiba, A., et al. (2017). Regulation involved in colonization of intercelluar spaces of host plants in *Ralstonia solanacearum*. *Front. Plant Sci.* 8, 967. doi: 10.3389/fpls.2017.00967

Hugenholtz, P., Goebel, B. M., and Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 4765–4774. doi: 10.1128/JB.180.18.4765-4774.1998

İnceoğlu, Ö., Salles, J. F., and van Elsas, J. D. (2012). Soil and cultivar type shape the bacterial community in the potato rhizosphere. *Microb. Ecol.* 63, 460–470. doi: 10.1007/s00248-011-9930-8

Kwak, M.-J., Kong, H. G., Choi, K., Kwon, S.-K., Song, J. Y., Lee, J., et al. (2018). Rhizosphere microbiome structure alters to enable wilt resistance in tomato. *Nat. Biotechnol.* 36, 1100–1109. doi: 10.1038/nbt.4232

Kyselková, M., Kopecký, J., Frapolli, M., Défago, G., Ságová-Marečková, M., Grundmann, G. L., et al. (2009). Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. *ISME J.* 3, 1127–1138. doi: 10.1038/ismej.2009.61

Latz, E., Eisenhauer, N., Rall, B. C., Allan, E., Roscher, C., Scheu, S., et al. (2012). Plant diversity improves protection against soil-borne pathogens by fostering antagonistic bacterial communities. *J. Ecol.* 100, 597–604. doi: 10.1111/j.1365-2745.2011.01940.x

Lau, J. A., and Lennon, J. T. (2012). Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proc. Natl. Acad. Sci. U. S. A.* 109, 14058–14062. doi: 10.1073/pnas.1202319109

Lauber, C. L., Strickland, M. S., Bradford, M. A., and Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol. Biochem.* 40, 2407–2415. doi: 10.1016/j.soilbio.2008.05.021

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., McDonald, M., et al. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* 349, 860–864. doi: 10.1126/science.aaa8764

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Lupatini, M., Suleiman, A. K., Jacques, R. J., Antoniolli, Z. I., de Siqueira Ferreira, A., Kuramae, E. E., et al. (2014). Network topology reveals high connectance levels and few key microbial genera within soils. *Front. Ecol. Environ.* 2:10. doi: 10.3389/fenvs.2014.00010

Lyons, K. G., and Schwartz, M. W. (2001). Rare species loss alters ecosystem function–invasion resistance. *Ecol. Lett.* 4, 358–365. doi: 10.1046/j.1461-0248.2001.00235.x

Mallon, C. A., van Elsas, J. D., and Salles, J. F. (2015). Microbial invasions: the process, patterns, and mechanisms. *Trends Microbiol.* 23, 719–729. doi: 10.1016/j.tim.2015.07.013

Marschner, H., Römheld, V., Horst, W., and Martin, P. (1986). Root-induced changes in the rhizosphere: Importance for the mineral nutrition of plants. *J. Plant Nutr. Soil Sci.* 149, 441–456. doi: 10.1080/01904168709363645

Marschner, P., Crowley, D., and Yang, C. H. (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* 261, 199–208. doi: 10.1023/b:plso.0000035569.80747.c5

Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Mendes, R., Garbeva, P., and Raaijmakers, J. M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.* 37, 634–663. doi: 10.1111/1574-6976.12028

Mendes, L. W., Raaijmakers, J. M., de Hollander, M., Mendes, R., and Tsai, S. M. (2017). Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. *ISME J.* 12, 212–224. doi: 10.1038/ismej.2017.158

Meng, Q., Yin, J., Rosenzweig, N., Douches, D., and Hao, J. J. (2012). Culture-based assessment of microbial communities in soil suppressive to potato common scab. *Plant Dis.* 96, 712–717. doi: 10.1094/PDIS-05-11-0441

Millet, Y. A., Danna, C. H., Clay, N. K., Songnuan, W., Simon, M. D., Werck-Reichhart, D., et al. (2010). Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. *Plant Cell* 22, 973–990. doi: 10.1105/tpc.109.069658

Mizrahi-Man, O., Davenport, E. R., and Gilad, Y. (2013). Taxonomic classification of bacterial 16S rRNA genes using short sequencing reads: evaluation of effective study designs. *PLoS One* 8, e53608. doi: 10.1371/journal.pone.0053608

Panke-Buisse, K., Poole, A. C., Goodrich, J. K., Ley, R. E., and Kao-Kniffin, J. (2015). Selection on soil microbiomes reveals reproducible impacts on plant function. *ISME J.* 9, 980–989. doi: 10.1038/ismej.2014.196

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U. S. A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Pester, M., Bittner, N., Deevong, P., Wagner, M., and Loy, A. (2010). A 'rare biosphere'microorganism contributes to sulfate reduction in a peatland. *ISME J.l* 4, 1591–1602. doi: 10.1038/ismej.2010.75

Power, M. E., Tilman, D., Estes, J. A., Menge, B. A., Bond, W. J., Mills, L. S., et al. (1996). Challenges in the quest for keystones. *Bioscience* 46, 609–620. doi: 10.2307/1312990

Robbins, C., Thiergart, T., Hacquard, S., Garrido-Oter, R., Gans, W., Peiter, E., et al. (2018). Root-associated bacterial and fungal community profiles of *Arabidopsis thaliana are* robust across contrasting soil P levels. *Phytobiomes* 2, 24–34. doi: 10.1094/PBIOMES-09-17-0042-R

Safni, I., Cleenwerck, I., De Vos, P., Fegan, M., Sly, L., and Kappler, U. (2014). Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *Int. J. Sys. Evol. Micr.* 64, 3087–3103. doi: 10.1099/ijs.0.066712-0

Saile, E., McGarvey, J. A., Schell, M. A., and Denny, T. P. (1997). Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. *Phytopathology* 87, 1264–1271. doi: 10.1094/PHYTO.1997.87.12.1264

Salgon, S., Jourda, C., Sauvage, C., Daunay, M.-C., Reynaud, B., Wicker, E., et al. (2017). Eggplant Resistance to the *Ralstonia solanacearum* Species Complex Involves Both Broad-Spectrum and Strain-Specific Quantitative Trait Loci. *Front. Plant Sci.* 8, 828. doi: 10.3389/fpls.2017.00828

Schutter, M., Sandeno, J., and Dick, R. (2001). Seasonal, soil type, and alternative management influences on microbial communities of vegetable cropping systems. *Biol. Fert. Soils* 34, 397–410. doi: 10.1007/s00374-001-0423-7

Scott, J. W., Wang, J. F., and Hanson, P. M. (2005). Breeding tomatoes for resistance to bacterial wilt, a global view. *Acta Hortic.* 695, 161–172. doi: 10.17660/ActaHortic.2005.695.18

Staley, J. T., and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Ann. Rev. Microbiol.* 39, 321–346. doi: 10.1146/annurev.mi.39.100185.001541

Thoquet, P., Olivier, J., Sperisen, C., Rogowsky, P., Laterrot, H., and Grimsley, N. (1996). Quantitative trait loci determining resistance to bacterial wilt in tomato cultivar Hawaii7996. *Mol. Plant Microbe Interact.* 9, 826–836. doi: 10.1094/MPMI-9-0826

Vasse, J., Frey, P., and Trigalet, A. (1995). Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. *Mol. Plant Microbe Interact.* 8, 241–251. doi: 10.1094/MPMI-8-0241

Vorholt, J. A., Vogel, C., Carlström, C. I., and Mueller, D. B. (2017). Establishing causality: opportunities of synthetic communities for plant microbiome research. *Cell Host Microbe* 22, 142–155. doi: 10.1016/j.chom.2017.07.004

Wagner, M. R., Lundberg, D. S., Coleman-Derr, D., Tringe, S. G., Dangl, J. L., and Mitchell-Olds, T. (2014). Natural soil microbes alter flowering phenology and the intensity of selection on flowering time in a wild Arabidopsis relative. *Ecol. Lett.* 17, 717–726. doi: 10.1111/ele.12276

Wang, J.-F., Hanson, P., and Barnes, J. (1998). "Worldwide evaluation of an international set of resistance sources to bacterial wilt in tomato," in *Bacterial wilt disease* (Berlin, Heidelberg: Springer), 269–270.

Wang, J.-F., Olivier, J., Thoquet, P., Mangin, B., Sauviac, L., and Grimsley, N. H. (2000). Resistance of tomato line Hawaii7996 to *Ralstonia solanacearum* Pss4 in Taiwan is controlled mainly by a major strain-specific locus. *Mol. Plant Microbe Interact.* 13, 6–13. doi: 10.1094/MPMI.2000.13.1.6

Wang, J.-F., Ho, F.-I., Truong, H. T. H., Huang, S.-M., Balatero, C. H., Dittapongpitch, V., et al. (2013). Identification of major QTLs associated with stable resistance of tomato cultivar 'Hawaii 7996' to *Ralstonia solanacearum*. *Euphytica* 190, 241–252. doi: 10.1007/s10681-012-0830-x

Wei, Z., Yang, T., Friman, V.-P., Xu, Y., Shen, Q., and Jousset, A. (2015). Trophic network architecture of root-associated bacterial communities determines pathogen invasion and plant health. *Nat. Commun.* 6, 8413. doi: 10.1038/ncomms9413

Wei, Z., Gu, Y., Friman, V. P., Kowalchuk, G. A., Xu, Y., Shen, Q., et al. (2019). Initial soil microbiome composition and functioning predetermine future plant health. *Sci. Adv.* 5, eaaw0759. doi: 10.1126/sciadv.aaw0759

Weller, D. M., Howie, W. J., and Cook, R. J. (1988). Relationship between in vitro inhibition of *Gaeumannomyces graminis* var. *tritici* and suppression of take-all of wheat by fluorescent pseudomonads. *Phytopathology* 78, 1094–1100. doi: 10.1094/phyto-78-1094

Zhou, J., Deng, Y., Luo, F., He, Z., Tu, Q., and Zhi, X. (2010). Functional molecular ecological networks. *MBio* 1, e00169–e00110. doi: 10.1128/mBio.00169-10

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Endophyte-Promoted Phosphorus Solubilization in *Populus*

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Phosphorus is one of the essential nutrients for plant growth, but it may be relatively unavailable to plants because of its chemistry. In soil, the majority of phosphorus is present in the form of a phosphate, usually as metal complexes making it bound to minerals or organic matter. Therefore, inorganic phosphate solubilization is an important process of plant growth promotion by plant associated bacteria and fungi. Non-nodulating plant species have been shown to thrive in low-nutrient environments, in some instances by relying on plant associated microorganisms called endophytes. These microorganisms live within the plant and help supply nutrients for the plant. Despite their potential enormous environmental importance, there are a limited number of studies looking at the direct molecular impact of phosphate solubilizing endophytic bacteria on the host plant. In this work, we studied the impact of two endophyte strains of wild poplar (*Populus trichocarpa*) that solubilize phosphate. Using a combination of x-ray imaging, spectroscopy methods, and proteomics, we report direct evidence of endophyte-promoted phosphorus uptake in poplar. We found that the solubilized phosphate may react and become insoluble once inside plant tissue, suggesting that endophytes may aid in the re-release of phosphate. Using synchrotron x-ray fluorescence spectromicroscopy, we visualized the nutrient phosphorus inside poplar roots inoculated by the selected endophytes and found the phosphorus in both forms of organic and inorganic phosphates inside the root. Tomography-based root imaging revealed a markedly different root biomass and root architecture for poplar samples inoculated with the phosphate solubilizing bacteria strains. Proteomics characterization on poplar roots coupled with protein network analysis revealed novel proteins and metabolic pathways with possible involvement in endophyte enriched phosphorus uptake. These findings suggest an important role of endophytes for phosphorus acquisition and provide a deeper understanding of the critical symbiotic associations between poplar and the endophytic bacteria.

Keywords: *Populus*, poplar, endophytes, phosphorus, solubilization, synchrotron x-ray fluorescence, x-ray absorption near edge structure, x-ray computed tomography

INTRODUCTION

Crop productivity is constrained by the bioavailability of water-soluble nutrients, especially phosphorus (P) in the form of phosphate. The efficiency of P acquisition, in which fine roots play a critical role, is important in addressing global food and bioenergy security issues that arise from increasing world population and climate change. In nutrient-limiting environments, plants are known to form associations with microorganisms capable of increasing the bioavailability of nutrients (Chhabra and Dowling, 2017). Phosphorus complexes with calcium, iron, and aluminum in soils, causing the bio-availability of this essential macronutrient to be low. Phosphorus must be in the form of orthophosphate anions for plants to uptake (Chhabra et al., 2013). Mycorrhizal fungal symbiotic associations with plants have long been known to increase transport of P (Clark and Zeto, 2000). However, it is becoming clear that bacterial associations can also increase nutrient acquisition (Richardson, 2001; Compant et al., 2010; Gaiero et al., 2013; Vandenkoornhuyse et al., 2015; Chhabra and Dowling, 2017). A wide variety of non-nodulating plant species are able to thrive in low-nutrient settings through symbiosis with internal microorganisms called endophytes (Santi et al., 2013). There has been a body of research on endophytes suggesting their important role in the growth of plants under nutrient-limiting conditions (Hardoim et al., 2008; Ryan et al., 2008; Gaiero et al., 2013).

Microbial mechanisms for solubilization of P include production of organic acid anions such as gluconic acid (Crespo et al., 2011; Oteino et al., 2015), malic acid (Jog et al., 2014), citric acid (Chen et al., 2014; Hane et al., 2014), salicylic acid, and benzeneacetic acid (Chen et al., 2014), as well as oxalic acid (Schneider et al., 2010; Mendes et al., 2014). Pyrroloquinoline quinone (PQQ) is involved in solubilization of both organic and inorganic phosphates as it is a redox cofactor of glucose dehydrogenase (GDH) for oxidation of glucose to gluconic acid (Misra et al., 2012). While screens for potentially symbiotic traits of plant-associated bacteria often include testing for solubilization of tricalcium phosphate [$Ca_3(PO_4)_2$], it is valuable to also test for the rarer ability to solubilize Fe-phosphate and Al-phosphate (Bashan et al., 2013).

Poplar (*Populus*) trees are early successional tree species that are known to be able to grow in nutrient-limited environments (Doty et al., 2016). Poplar, a member of the Salicaceae family, is considered important as a wood product, for bioenergy and for environmental services (Bradshaw and Stettler, 1995; Isebrands and Richardson, 2014). While it has been demonstrated that P-solubilizing bacteria can provide P that impacts plant growth (Chhabra et al., 2013; Oteino et al., 2015), such studies have not been done on ecologically important poplar trees. Recently, the role of endophytic bacteria in nitrogen (N) fixation was reported in wild poplar (Doty et al., 2016). Although many of the endophyte strains are shown to solubilize $Ca_3(PO_4)_2$ (Kandel et al., 2017), how plants interact with phosphate solubilizing endophytic bacteria remains understudied.

In this study, we tested the hypothesis that, apart from mycorrhizal networks, closely associated endophytic bacteria also contribute significant P to the host plants. We report evidence of endophytic activity to promote P uptake by comparing samples inoculated with two P-solubilizing endophytes and those uninoculated (controls). By comparing root masses from physical root measurements as well as root imaging by x-ray microtomography, we show indications of increased nutrient uptake in the plants inoculated with the endophytes. P inside the roots was visualized using synchrotron x-ray fluorescence (SR-XRF) microscopy. Using P K-edge x-ray absorption near edge structure (XANES), we determined P speciation inside the roots. Finally, our proteomics analysis points to differential protein enrichment between inoculated and uninoculated poplar roots as well as proteins that may have been involved in P solubilization and utilization. These findings suggest that endophytes are important for P acquisition and give us a deeper understanding of the biological relevance of the symbiosis between the plants and the endophytic bacteria.

MATERIALS AND METHODS

Microbial Strains

Diazotrophic endophyte strains from poplar (Doty et al., 2009; Khan et al., 2015; Kandel et al., 2017) had previously been screened for $Ca_3(PO_4)_2$ solubilization on National Botanical Research Institute's phosphate (NBRIP) growth medium plates as described before (Nautiyal, 1999; Khan et al., 2015). Strains with the highest phosphate solubilization index were further screened for the ability to grow on medium with aluminum phosphate or iron phosphate as the sole P sources. Based on these screens, strains WP5 (*Rahnella* sp.; Khan et al., 2015) and WP42 (*Burkholderia* sp.; Kandel et al., 2017) were selected for further study. All bacteria, and controls, for this experiment were incubated at 30°C with shaking at 150 rpm.

Quantification of Bacterial Growth and Phosphate Solubilization

Modified phosphate-free NBRIP liquid medium was prepared, with glucose 20 g/L and magnesium chloride 10 g/L as described for maximum phosphate solubilization (Nautiyal, 1999), and termed enhanced NBRIP (ENBRIP) broth. ENBRIP contained per liter: 20 g glucose, 5 g $Ca_3(PO_4)_2$, 10 g magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$), 0.25 g magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), 0.2 g potassium chloride (KCl), and 0.1 g ammonium sulfate [$(NH_4)_2SO_4$]. Fifty milligram of either $Ca_3(PO_4)_2$, iron phosphate, or aluminum phosphate was added to 125-ml Erlenmeyer flasks containing 10 ml of ENBRIP broth prior to autoclaving at 121°C for 25 min. The pH of the medium was adjusted to 7.0 prior to autoclaving. Starting cultures of strains WP5 and WP42 were grown overnight in 25 ml ENBRIP containing 3 g/L potassium phosphate as well as in 25 ml MG/L broth (Cangelosi et al., 1991) to ensure sufficient cells for the assays. The MG/L broth contained per liter: 5 g tryptone, 1.5 g yeast extract, 5 g sodium chloride (NaCl), 10 g mannitol, 2.32 g sodium glutamate, 0.5 g monobasic potassium phosphate (KH_2PO_4), and 0.2 g $MgSO_4{\cdot}7H_2O$. Cells were washed three times in ENBRIP without phosphate by centrifugation at 6,000 rpm for 20 min at 15°C. The cells

were then grown for several hours in ENBRIP to allow depletion of residual internal phosphate. The optical density at 600 nm of the washed cells were then measured by spectroscopy (Fisher) and the amount of culture needed to give an optical density of 0.1 in 10 ml was calculated. The flasks of ENBRIP with added phosphates, and a set without added phosphate, were inoculated in triplicate with each strain. Another set of 12 uninoculated control flasks was prepared, without the addition of cells. All flasks were incubated for 3 days before the cultures were transferred into 50-ml conicle tubes and allowed to settle for 90 min. Solubilized phosphate was quantified using the method of Murphy and Riley (1962) modified as follows: 5 µl of the settled cultures was added to 795 µl water and reacted with 200 µl of freshly prepared reagent B. Absorbance was read at 880 nm. Optical density of the settled cultures was read at 600 nm as an estimate of cell density.

Localization of the Endophyte Strains on Poplar Roots

Fluorescent tags were introduced into strains WP5 and WP42 through electroporation using standard protocols (Cangelosi et al., 1991). The green fluorescent protein (GFP) plasmid, pBHR-GFP (Stevens et al., 2005), conferring kanamycin resistance, and the purple fluorescent protein, pMMB67EH/Kate2, conferring gentamicin resistance, were obtained from the Greenberg and Miller labs (UW Microbiology Department). Tagged strains were verified by fluorescent microscopy using a Zeiss Imager M2 equipped with an AxioCam MRM and recorded with Zeiss Zen software (Karl Zeiss, LLC, Thornwood, NY, United States). Rooted, internally-sterile *Populus trichocarpa* clone Nisqually-1 poplar plants were co-cultivated in sterile 40 ml vials for 2 days with the tagged strains that had been brought to an OD_{600} of 0.5 in half-strength Murashige and Skoog (MS) medium without sucrose (Murashige and Skoog, 1962). Plants were then washed three times in sterile water and placed into fresh 0.5X MS. Colonization of root surfaces was visualized by fluorescent microscopy.

Plant Growth and Conditioning for P Solubilization Studies

Internally sterile *P. trichocarpa* clone Nisqually-1 plants were propagated on McCown's Woody Plant Medium (Phytotechnology Labs). Twelve-day old apical cuttings with fresh roots were transferred to Magenta vessels (Caisson Labs) containing 10 ml of ¼ strength MS broth without sucrose (pH 5.8). Endophyte strains, WP5 and WP42, were grown in N-Limited Combined Carbon Medium (Rennie, 1981) for 2 days. Colonies were inoculated into 10 ml NL-CCM broth in 125-ml flasks and grown on a shaker at 30°C for 2 days. Cells were pelleted by centrifugation and resuspended in N-free medium (Doty et al., 2009). Inoculum was prepared with a mixture of the two strains at equal densities to a final OD600 of 0.1 in ¼ X Hoagland's solution (Hoagland and Arnon, 1938). Half of the plants were co-cultivated in the inoculum while the control plants received the sterile broth. As soil medium, dried sand was thoroughly mixed with $Ca_3(PO_4)_2$ (Sigma-Aldrich) in a 200:1 ratio by weight before use (Oteino et al., 2015). The non-water soluble $Ca_3(PO_4)_2$ was chosen to confirm the capability of the endophytes to help the plant mobilize that compound. The ¼ X Hoagland's Solution (Hoagland and Arnon, 1938) was added to the soil, and the poplar plants were transferred into it. The samples that contained the endophyte strain were called P-mix samples, and those that did not were called Control samples. Plants were grown for 4 weeks in Magenta GA-7 vessels in growth chambers under 16 h/8 h light-dark regime (with light intensity of 250 µmol m^{-2} s^{-1}), temperature of 24°C day/18°C night, and relative humidity of 60%. A subset of plants grown under same condition were used for x-ray computed tomography (XCT) and µXRF/µXANES analyses (see below).

Soil Analysis

The same sandy soil mix was used for planting all P-mix and Control samples. In order to evaluate whether there was a significant difference between the total P concentrations of the P-mix and Control samples after the plants were harvested, Inductively Coupled Mass Spectrometry (ICP-MS) measurements were carried out on one soil sample from the P-mix group and one from the Control group. For acid extraction to bring all of the P into solution for the analysis, 20 g of sediment was weighed into 50-ml centrifuge tubes. Fifteen microliter of 2% nitric acid (trace metal grade) was added to the centrifuge tubes and vortexed for 24 h. The samples were then put in the 4°C fridge to let the solids settle. The supernatant was pulled off the next morning and all samples were left at 4°C until analysis.

Root Collection Procedure

Ten plants (five Control and five P-mix) were collected for root biomass, morphology analysis, and further proteomics characterization. The clippers and forceps were wiped down with ethanol between collecting each sample. Plants were gently removed from the planting boxes by tilting the boxes and rocking back and forth to expose roots. While holding onto the plant, the roots were gently freed from sand and gently rinsed with deionized water to remove the remaining sand. The plants were placed on large Kim-wipes to remove excess moisture and the longest root length was measured. The plant leaf mass was subsequently weighed and recorded. A picture of the plant was taken, then the roots were cut off, weighed, put in a labeled 50-ml Falcon tube and flash-frozen in liquid nitrogen. The roots were then placed in a −80°C freezer for proteomics analysis.

Proteomics Characterization

Protein Extraction and Digestion

Roots were flash frozen in liquid nitrogen and ground into a fine frozen powder using a Freezer/Mill (SPEX, Metuchen, NJ, United States) using a program that ran 2 cycles, with a 1 min precool, and a 2 min run time at 10 cycles/min. Two milliliters of ice-cold methanol was added to about 0.5–1 ml volume of frozen plant powder, and the sample was vortexed. Then, 1 ml of nanopure water and 1.8 ml of chloroform were added and the sample was shaken vigorously for about 15 s into an emulsion.

Each sample was centrifuged for 10 min at 4°C at 7,197 × *g*. The middle protein pellet was washed with 3 ml ice cold methanol and centrifuged at 10,000 × *g* for 10 min at 4°C three times to remove remaining metabolites from protein sample. Excess methanol was removed by drying the pellets gently under a flow of nitrogen for ~2 min. A protein solubilization solution containing 7 M urea, 2 M thiourea, 4% CHAPS, and 5 mM TCEP was added to completely cover each pellet, plus 500 µl more. Samples were then incubated at 4°C overnight. Debris/protein pellets from each sample were physically mixed into solution with a pipette tip and the slurry sonicated briefly in a sonoreactor. The protein slurries were then incubated at 60°C for 30 min, with samples vortexed and sonicated in a sonoreactor again for about 30 s. Each sample was then centrifuged for 10 min at 5,000 × *g* at 4°C. A Coomassie Plus protein assay (Pierce, Rockford, IL, United States) using a bovine serum albumin standard (BSA) was next performed on the individual supernatants to estimate protein concentration. Afterwards, the denatured samples were diluted tenfold with 50 mM ammonium bicarbonate, pH 8.0. $CaCl_2$ was added to a concentration of 2 mM and trypsin (Affymetrix, Santa Clara, CA, United States) was added at a trypsin:sample ratio of 1:50 (w/w). Samples were digested overnight at 37°C and alkylated with chloroacetamide at a concentration of 5 mM in the dark at 37°C for 30 min. The peptides from each treatment were desalted with C-18 SPE columns (SUPELCO Discovery) using a 0.1% TFA in nanopure water to wash the peptides and 80:20 acetonitrile:water with 0.1% TFA solvent was used to elute the peptides. Peptides were then quantified using a BCA assay (Pierce, Rockford, IL, United States) with a BSA standard.

iTRAQ Peptide Labeling

Peptides were labeled with 10-plex tandem mass tag (TMT) reagents (ThermoScientific, San Jose, CA, United States) according to manufacturer's instructions. After samples were labeled, they were dried down in a centrifugal vacuum concentrator.

Offline Fractionation of Peptides and Preparation of Proteome Samples

Labeled peptides were separated using an off-line high pH (pH 10) reversed-phase (RP) separation with a Waters XBridge C18 column (250 mm × 4.6 mm column containing 5 µm particles and a 4.6 mm × 20 mm guard column) using an Agilent 1200 HPLC System. The sample loaded onto the C18 column was washed for 15 min with Solvent A (10 mM ammonium formate, adjusted to pH 10 with ammonium hydroxide). The LC gradient started with a linear increase of Solvent B (10 mM ammonium formate, pH 10, 90:10 acetonitrile:water) to: 5% Solvent B over 10 min, 45% Solvent B over 65 min, and then a linear increase to 100% Solvent B over 15 min. Solvent B was held at 100% for 10 min, and then was changed to 100% Solvent A, this being held for 20 min to recondition the column. The flow rate was 0.5 ml/min. A total of 96 fractions were collected into a 96-well plate. The high pH RP fractions were then combined into 24 fractions using the concatenation strategy previously reported (Wang et al., 2011) excluding CHAPS containing wells (F2–F11). Peptide fractions were dried down and re-suspended in nanopure water at a concentration of 0.075 µg/µl for mass spectrometry analysis using an Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Scientific) system as described below.

Mass Spectrometry

All peptide samples were analyzed using an automated constant flow nano LC system (Agilent) coupled to Q Exactive Orbitrap (Thermo Fisher Scientific). Electrospray emitters were custom made using 150 µm o.d. × 20 µm o.d. × 20 µm i.d. chemically etched fused silica. An on-line 4-cm × 360 µm o.d. × 150 µm i.d. fused-silica capillary analytical column (3 µm Jupiter C18) was used. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid acetonitrile (B) operated at 300 nl/min with a gradient profile as follows (min: %B); 0:5, 2:8, 20:12, 75:35, 97:60, and 100:85.

The LTQ Orbitrap Velos mass spectrometer was operated in the data-dependent mode acquiring higher-energy collisional dissociation (HCD) scans (R = 7,500, 5 × 104 target ions) after each full MS scan (R = 30,000, 3 × 106 target ions) for the top 10 most abundant ions within the mass range of 300–1,800 m/z. An isolation window of 2.5 Th was used to isolate ions prior to HCD. All HCD scans used normalized collision energy of 45 and maximum injection time of 1,000 ms. The dynamic exclusion time was set to 60 s and charge state screening was enabled to reject unassigned and singly charged ions.

Peptide Identification and Quantification

For peptide identification, MS/MS spectra were searched against a decoy *P. trichocarpa* v3.1 protein database from Phytozome[1] using the algorithm SEQUEST (Eng et al., 1994). Search parameters included: no enzyme specificity for proteome data and trypsin enzyme specificity with a maximum of two missed cleaves, ±50 ppm precursor mass tolerance, ±0.05 Da product mass tolerance, and carbamidomethylation of cysteines and TMT labeling of lysines and peptide N-termini as fixed modifications. Allowed variable modifications were oxidation of methionine and proline. MSGF+ spectra probability values (Kim et al., 2008) were also calculated for peptides identified from SEQUEST searches. Measured mass accuracy and MSGF spectra probability were used to filter identified peptides to <0.4% false discovery rate (FDR) at spectrum level and <1% FDR at the peptide level using the decoy approach. TMT reporter ions were extracted using the MASIC software (Monroe et al., 2008) with a 10 ppm mass tolerance for each expected TMT reporter ion as determined from each MS/MS spectrum.

Protein Abundance Value and Significance Determination

Relative abundances of peptides were determined using TMT reporter ion intensity ratios from each MS/MS spectrum.

[1]https://genome.jgi.doe.gov/

Individual peptide intensity values were determined by dividing the base peak intensity by the relative ratio associated with each reporter ion. All peptide data were transformed to a log2 value, mean centered normalized, then each value was taken as an exponent of 2 to convert back to an unlogged value. Peptide abundance values were separated into two datasets, one of peptides unique to a single protein and peptides which may have been derived from two or more proteins. Peptides were rolled up to a protein value by summing the peptides that belong to each protein in each dataset. For the peptide data which could come from multiple proteins, these were concatenated into a group name to represent all proteins which could produce each unique peptide. Protein rollup calculations from the unique or shared/group peptide table were designated in the final protein rollup table. Kyoto Encyclopedia of Genes and Genomes (KEGG) protein function orthologs[2] (Kanehisa et al., 2017), gene ontologies[3] (Carbon et al., 2017), and other annotations were obtained from the *P. trichocarpa* v3.1 annotation file located at Phytozome[4] (Goodstein et al., 2012). A partial least-squares and Pearson's pairwise correlation plots were constructed using the proteomics software Inferno.[5] Log2 values were uploaded to MeV[6] (Wang et al., 2017), where a *t*-test was performed between Control and P-mix sample groups using a Welch approximation (assuming unequal group variances) and with a significance determined with a cut-off value of p less than 0.05 with a value of p based on a *t*-distribution.

STRING Network Analysis

A Search Tool for the Retrieval of Interacting Genes/Proteins (STRING): functional protein association network[7] (Szklarczyk et al., 2019) was performed on the proteins determined to be significantly changed from the *t*-test ($p < 0.05$) against co-expression, co-occurrence, databases, gene fusion, experiments, neighborhood, and textmining active interaction sources found in the STRING databases for *P. trichocarpa*. The minimum required interaction score was 0.400, and disconnected nodes in the network were hidden from the network image.

X-Ray Computed Tomography

To characterize root growth and architecture, inoculated and control samples were scanned using a microfocus XCT scanner (X-Tek/Metris XTH 320/225 kV, Nikon Metrology, Brighton, MI, United States). Scans were performed at 90 kV and 350 μA x-ray power. During scans, samples were rotated continuously with momentary stopping to collect each projection. A total of 2,000 projections were collected over 360° with an exposure time of 500 ms per projection. Images were collected at an isotropic voxel resolution of 40.0 μm, resulting in 32-bit gray-scale images. The raw images were reconstructed to create a three-dimensional (3D) dataset using the software CT Pro 3D (Nikon Metrology, Brighton, MI, United States). For higher resolution imaging, XCT data were collected at beamline 8.3.2 of the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory (LBNL). A double-multilayer monochromator was used to select 17 keV x-rays, and detection used a 0.5 mm LuAG scintillator and 2× lenses with a sCMOS PCO.Edge camera, giving a 3.3 μm pixel dimension, and a 8.4 mm horizontal field of view. The sample to scintillator distance was 15 mm. A 300 ms exposure time yielded 8,000 counts on the 16-bit camera (allowing a maximum of 65,535 counts). For each tomographic scan, 1,313 projections were acquired over a 180° rotation, with a total scan time of 15 min. The images were analyzed using Avizo (Thermo Fisher Scientific, Waltham, MA, United States) to segments the roots and generate root volume and surface area data.

Synchrotron X-Ray Fluorescence Microprobe

Petrographic thin sections (30-μm thick) of root samples were prepared by Spectrum Petrographics, Inc. Soda-lime petrographic glass slides of size 27 × 46 × 1.2 mm were mounted with a UV curing acrylic adhesive. High-purity fused quartz glass slides with superglue mounting adhesive were used for synchrotron sample preparations. The slides ranged in thickness from 0.030 to 0.040″. High-purity fused quartz cover glasses of about 200 μm thick were used to mount the samples with permanent adhesive. That sample/coverglass was then mounted in "piggyback" style with superglue (removable with acetone) onto a normal petrographic slide that had the necessary rigidity for further processing. Another set of samples were mounted on Si_3N_4 windows (5 × 5 mm size, 2 μm thick, Norcada Inc., Edmonton, Canada) after the following preparation. Roots from P-mix and Control samples were gently washed with MilliQ water. Root tips were then harvested and frozen at −80°C in 2.5% CMC using a 600-μl Eppendorf tube. Samples were then kept in the −80°C overnight tip orientation down. Samples were removed from the tube and mounted onto a chuck with 200-μl of Milliq water for fixing. Samples were cryosectioned on a Thermo NX-70 with the chuck set to −14°C and the blade set to −11°C. 60-μM sections were sliced both longitudinally and laterally to the tip of the root. Using tweezers kept at −20°C, sections were then placed onto the Si_3N_4 windows and thaw-mounted.

X-ray microprobe analyses were carried out at the ALS XFM beamline 10.3.2 at LBNL (Marcus et al., 2004). All data were recorded in fluorescence mode at room temperature, using a Si(111) monochromator, an Amptek FAST SDD fluorescence detector, and a He-filled chamber in the sample-detector path. Root and P reference powder samples were mounted on Magic Scotch 3 M tape, found to have the lowest detectable P content (no P, Si, or S detected). μXRF mapping was performed at an incident energy of 100 eV above the P K-edge (2,245 eV). This allowed for capturing the distribution of P as well as Ca and K (through harmonics), important elements in soils and roots. Coarse μXRF maps of the roots were first recorded with 35 μm pixel size, then specific regions of interest mapped at higher resolution (8 μm). XRF maps were displayed to the

[2]https://www.kegg.jp/
[3]http://geneontology.org/
[4]https://genome.jgi.doe.gov/
[5]https://omics.pnl.gov/software/infernordn
[6]http://mev.tm4.org/
[7]https://string-db.org

same intensity scale and analyzed for P distribution and concentration. Defocused and microfocused P K-edge XANES data were collected along with the μXRF images on the same samples to investigate P speciation. A variety of inorganic P reference compounds were used: $AlPO_4$, $FePO_4$, and multiple Ca-bound phosphates (see **Supplementary Figure S6**). Organically bound P was represented by phytate, DNA, and lecithin. Reference compounds were acquired by this project and from other studies (Barnes et al., 2019; O'Day et al., 2020). Bulk and microfocus spectra were deadtime corrected and deglitched using a custom LabVIEW software available at beamline 10.3.2. Spectra were then imported to the ATHENA software package (Demeter 0.9.20; Ravel and Newville, 2005) for calibration, baseline correction, normalization and linear combination fitting (LCF). Spectra were corrected for over-absorption prior to LCF using a custom LabVIEW software available at beamline 10.3.2. Calcium phosphate ($CaHPO_4$) with a white line at 2152.26 eV was used for calibration. Baseline correction and edge-step normalization parameters were varied for individual samples to reduce error associated with LCF (Werner and Prietzel, 2015). Fits were implemented with the component sum not forced to unity and a maximum of four reference compounds were allowed. A final fit was chosen based on the combination of reference compounds with lowest R-factor that also visually aligned with the unknown sample, and only fits within ±2.5% of 100% were accepted. Error associated with this technique is about 5–10% (Ajiboye et al., 2007; Werner and Prietzel, 2015), therefore, in instances where a reference fit with <5% of that compound, it was removed and the sample refit.

Additional high-resolution x-ray fluorescence data were collected at 2-ID-E beamline of the Advanced Photon Source (APS) at Argonne National Laboratory. X-ray energy was set to 10 KeV using 3.3 cm periodicity undulator and Si (111) crystal monochromator; beam was focused with Fresnel zone plate down to 400 × 400 μm spot on sample. Sample was placed in Helium and raster scanned using translation stages with 200 nm step in X and 300 nm step in Y and 10 msec/pixel at 45° angle to incident beam to minimize sample-to-detector distance and improve signal for low Z elements (P-Ca). Emitted x-ray spectra were recorded by four-element silicon-drift Vortex-ME detector (Hitachi), calibrated, and fitted by MAPS (Vogt, 2003) using thin-film standard AXO 1 X (AXO Dresden GmbH) to obtain 2D maps of elements.

The P concentrations inside the roots were determined from all high-resolution μXRF maps collected at beamline 2-ID-E at the APS. Using the average values, a final P concentration was generated to demonstrate any change in P content between P-mix and Control samples. The data were analyzed, fitted, and quantified using MAPS software (Vogt, 2003). The P concentrations were determined from two regions of interest (ROIs), from inside the root and substrate background from outside the root. In order to avoid soil traces within samples, ROIs were selected in areas with minimal interference from element, which are present in soil but have negligible uptake by plants, such as Si and Ti (van der Ent et al., 2018). In addition, other metals like Ca, K, and Fe, Mn, Cu, and Zn were measured from both the root and the background to correlate those with the P concentrations to see how it correlates with Ca, especially. The data points from each detector element for each region were normalized using downstream ion chamber and averaged. The substrate background elemental values were subtracted to get the true P concentration from inside the roots, as well as K, Ca, Fe, Cu, and Zn. Using the average values, a final P concentration was generated to demonstrate any change in P content between P-mix and Control samples.

RESULTS

Phosphate Solubilization by the Endophyte Strains

Poplar endophyte strains, WP5 and WP42, were previously tested using plate assays for the ability to solubilize $Ca_3(PO_4)_2$ (Khan et al., 2015; Kandel et al., 2017) and to grow on aluminum phosphate and iron phosphate (unpublished). To quantify bacterial growth and phosphate solubilization, liquid assays were performed. Both strains grew well only in the medium containing $Ca_3(PO_4)_2$, but appeared to have negligible growth beyond controls in media containing aluminum phosphate (**Figure 1C**). Results of the colorimetric method to quantify phosphate indicated that both strains solubilized $Ca_3(PO_4)_2$, with residual suspended solid phosphate seen in the control (**Figure 1A**). In addition, strains WP5 and WP42 both showed a limited ability to solubilize aluminum phosphate, with WP5 alone showing minor solubilization of iron phosphate above controls (**Figure 1B**). However, the cell concentration observed by optical density for both strains in iron phosphate was lower than the controls without added phosphate (**Figure 1C**).

Root Colonization by the Endophyte Strains

Since strains WP5 and WP42 were originally isolated as endophytes of poplar branches, we tested if they could also colonize root surfaces where their ability to solubilize phosphate from soils could be most advantageous to the host plant. Fluorescent microscopy images of poplar plants co-cultivated with WP5 tagged with purple fluorescent protein (PFP) and WP42 with GFP are shown in **Figure 2**, where colonization by the two strains is visualized simultaneously. Microbes, as a likely biofilm, are observed colonizing the root surface, covering the primary root as well as the root hairs. Microbial populations are, therefore, spatially available to manipulate external soil chemistry. When the junction of a primary root with a lateral root is investigated by focusing beyond the epidermis, endophytic fluorescence can be observed inside root tissue, showing availability for the manipulation of internal chemistry as well.

Soil Phosphorus Content

The starting total P concentration in the sand that was used as the root inhabiting medium was calculated to be approximately

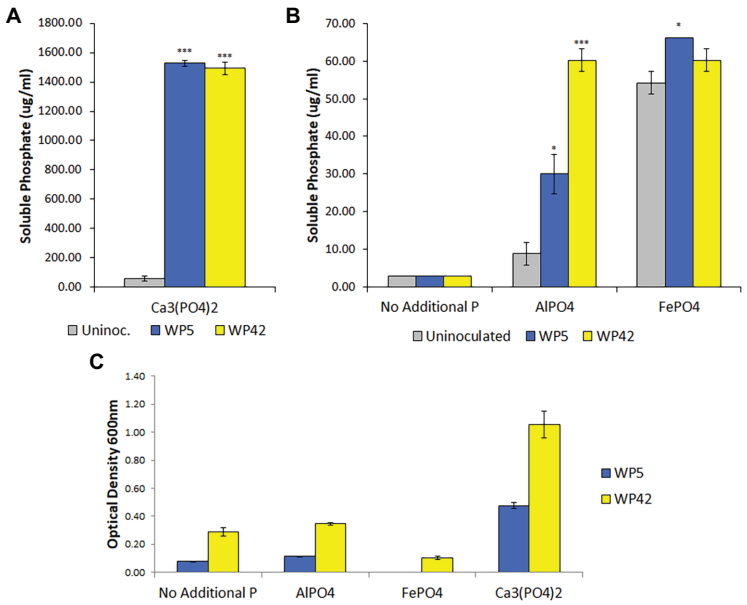

FIGURE 1 | (A,B) Solubilized phosphate in enhanced National Botanical Research Institute's phosphate (ENBRIP) media after 36 h incubation (n = 3, ±SE). Significantly different from uninoculated media (t-test, $^{*}p < 0.05$, $^{***}p < 0.001$). **(C)** Cell concentrations in ENBRIP media after 36 h incubation (n = 3, ±SE). Optical densities of uninoculated media were subtracted for each phosphate type to control for unsettled phosphate particulates.

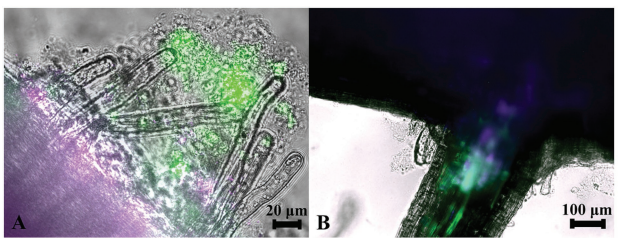

FIGURE 2 | Colonization of poplar roots by WP42 [tagged with green fluorescent protein (GFP)] and WP5 [purple fluorescent protein (PFP)]. **(A)** Microbes colonizing the root surface, as a likely biofilm, covering the primary root (seen bottom left of image) as well as the root hairs which eminate from it. Microbial populations are, therefore, spatially available to manipulate external soil chemistry. Scale bar 20 µm. **(B)** The junction of a primary root (seen top of image) with a lateral root. By focusing beyond the epidermis, endophytic fluorescence can be observed inside root tissue showing special availability for the manipulation of internal chemistry as well. Scale bar 100 µm.

3,500 µg/kg, based on the nutrient mix added and the weight and volume of the sand used in the pots. At the time of root harvesting, from ICP-MS, the total P concentration for the sand that contained a P-mix sample was reduced to 1,690 µg/kg, while for the Control sample it was reduced only to 2,680 µg/kg (see **Supplementary Figure S1**). Results from this complementary analysis were consistent with increased microbial P mineralization uptake of soluble P by endophyte inoculated plants.

Root Biomass and Morphology

We monitored poplar root phenotypic changes in P-mix inoculated and control plants. Roots and leaves from five biological replicates inoculated with the endophyte (P mix) and five Control samples were harvested for root length, mass, and leaf mass measurements prior to proteomics characterization. Representative Control and P-mix and the harvested plants are shown in **Supplementary Figure S2**. Root dry weight and root length showed similar values under both conditions and no significant changes were observed (see **Supplementary Table S1**).

Root Imaging by XCT

Since the comparison of root dry weight and root length values of the P-mix and Control samples was inconclusive, we turned to 3D imaging to look at the architecture and microstructure of the roots in more details. Root volume and surface area measurements were carried out by tomographic image-based analysis. Whole roots imaged by XCT revealed greater wet root volume in the P-mix samples and smaller wet root volume with greater fine root formation in the Control samples. High-resolution synchrotron micro-XCT images collected on small root sections confirmed the significant increase in root surface area for Control samples relative to the inoculated ones (**Figure 3**).

P Uptake Studied by SR-XRF

To further confirm and visualize P solubilization by the plant root, we conducted a combination of synchrotron x-ray micro-fluorescence (µXRF) imaging and P K-edge XANES experiments. Roots from poplar samples inoculated with the bacterial consortium and uninoculated samples were harvested for both longitudinal and cross sectioning and used for microprobe (µXRF and µXANES) analyses to investigate P distribution and speciation in the roots. Longitudinal sections showed a

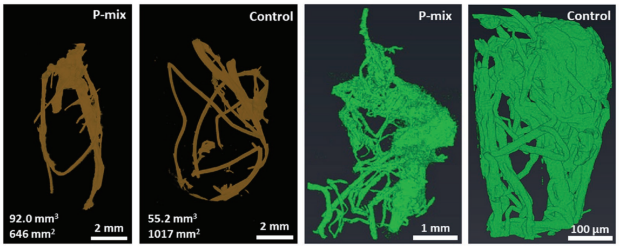

FIGURE 3 | CT analysis of a whole root (root of brown color) showing a P-mix sample with 92.0 mm^3 volume and 646 mm^2 surface area, and a control sample with 55.2 mm^3 volume and 1,017 mm^2 surface area. Analysis of synchrotron micro-CT images (green color) shows increased fine root formation in the Control; P-mix sample with a root volume 538.3 mm^3 exhibited a surface area of 1842.1 mm^2, while a Control sample with 27.4 mm^3 volume had a surface area of 2940.6 mm^2.

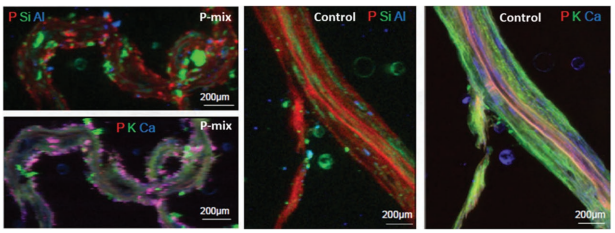

FIGURE 4 | Micro-x-ray fluorescence (µXRF) maps of longitudinal root sections show evidence of P uptake in plant that has the endophytic bacterial strains WP5 and WP42 ("P-mix"). P appears pink in bottom left and right most image due to P and Ca overlapping.

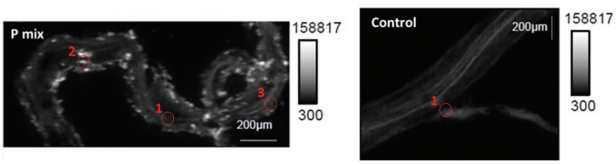

FIGURE 5 | Phosphorus x-ray fluorescence maps (grayscale) in poplar root longitudinal thin sections of the P-mix and Control samples. P in the roots is displayed on the same P intensity scale and same gamma value (white levels, expressed as counts in the maps, are the same). Red circles mark the spots where x-ray absorption near edge structure (XANES) spectra were collected (P-mix Long Spot 1, Spot 2, Spot 3, and Control Long in **Supplementary Material**).

marked difference in P distribution for the inoculated plant compared to controls. While the longitudinal sections appeared to show a uniform P distribution across the imaged root section for controls, the plants inoculated with P-mix exhibited distinct hot spots where P accumulated inside the root (**Figure 4**).

When the distribution of P in the roots is displayed on the same P intensity scale (**Figure 5**), we found a significant difference in the P distribution within the root from the endophyte-inoculated plant vs. the control. The P-mix sample also showed a higher P concentration in certain spots when compared to the Control sample. We presume that the Control sample has the homogeneously distributed P from the water-soluble ammonium phosphate in the Hoagland's solution (Hoagland and Arnon, 1938), while the P-mix samples have the additional, solubilized P from the insoluble phosphate, superimposed on the "background" P present in both groups. See **Supplementary Figures S4**, **S5** for additional µXRF images.

High-resolution x-ray fluorescence microprobe (µXRF) maps collected from inside the roots at the APS were used for a quantitative comparison of P concentrations between P-mix and Control samples. µXRF has been proven to be a useful approach for quantitative analysis of trace elements from environmental and biological samples (Twining et al., 2003; Fittschen and Falkenberg, 2011; Kirker et al., 2017). From quantitative elemental analysis perfomed on 17 different spots for P-mix and Control samples each, we found that the P concentrations inside the roots were consistently greater (by 20–30%) for all P-mix samples than for Control samples (see **Supplementary Figure S6** and **Supplementary Tables S2**–**S5**).

Chemical State of P From XANES

To learn about the chemical state of the P in those hot spots in **Figure 5**, XANES spectra were collected at the P K-edge both in defocused (overall P speciation of the roots) and focused modes (speciation of the hot spots) for root sections. We found species of inorganic and organic phosphates to be present in both the P-mix and Control samples (**Figure 6**). The inorganic P in both groups is mostly Ca-bound [$Ca_3(PO_4)_2$, $Ca(H_2PO_4)_2$, $CaHPO_4$, amorphous Ca phosphate, $Ca_5(PO_4)_3(OH,F,Cl)$, and $Ca_2P_2O_7$]; other inorganic phosphates identified are sodium pyrophosphate ($Na_4P_2O_7$) and aluminum phosphate ($AlPO_4$). The organic P could be identified as being chemically similar to Na- or Ca-phytate ($C_6H_{18}O_{24}P_6$) and DNA ($C_xH_xO_xN_xP$).

We found subtle differences between the P-mix and Control samples for P speciation collected in defocused mode (**Figure 6**; **Supplementary Figure S8** and **Supplementary Table S6**). Overall, P-mix defocused samples were dominated by organic species (DNA and phytate) which comprised 73.5% in Cross 1 and 68% in Cross 2. Control samples were inconsistent, as Control Cross Diffuse organic species represented 77% of total P and Control Cross Hot was only composed of 20.6%. Control Cross Hot was instead dominated by Ca-bound inorganic P (80.8%). Although composed of proportionally smaller amounts, inorganic P in the other defocused samples was also primarily associated with Ca. We examined P hot spots in the P-mix samples using microfocus mode at three locations (see **Supplementary Figure S8** and **Supplementary Table S6**). Our findings show two of the hot spots were dominated by inorganic Ca species with 51.6% (P-mix Long Spot 1) and 90.3% (P-mix Long Spot 2). Taken together, we hypothesize that the homogeneous P background observed in the maps of **Figure 5** can be attributed to the readily absorbed ammonium phosphate from the Hoagland's solution being used by the plant, while the P hot spots seen in the P-mix samples are related to the presence of $Ca_3(PO_4)_2$ that was taken in with the help of the endophytes. The increased P in the plant is most likely from the increased uptake of solubilized P due to the endophyte activity in solubilizing the $Ca_3(PO_4)_2$. Why do the microfocus

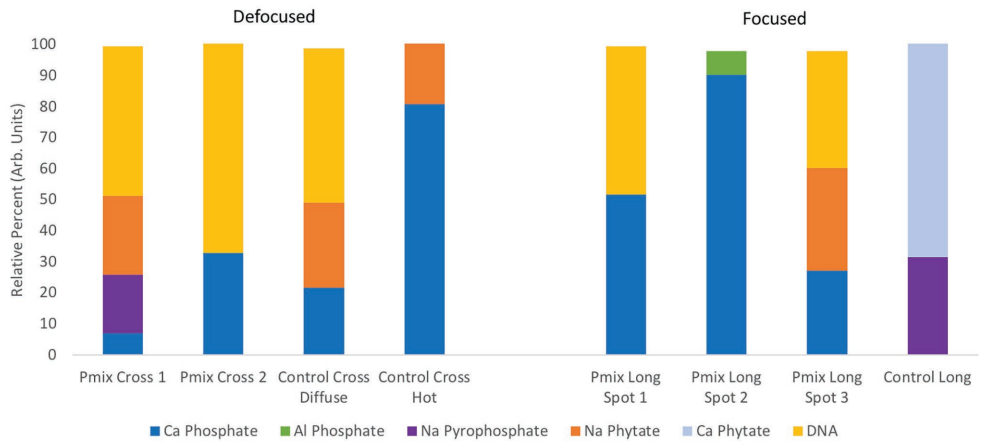

FIGURE 6 | Summary of defocused (bulk characterization) and focused (speciation of the hot spots identified in **Figure 5**) P K-edge XANES linear combination fit findings collected on longitudinal (i.e., long) and cross sections of P-mix and Control root samples.

spots look like Ca-bound P? As mentioned above, the once solubilized phosphate may have reacted with the Ca present in the plant becoming insoluble again. We found no evidence that endophytes made the phosphate insoluble. We expect a dynamic relationship of P going into and out of solution within the plant. We note that although the Control samples also had hot spots, they were overwhelmingly a characteristic of the P-mix group.

In summary, these μXRF/XANES results provide additional evidence that the endophytes possessed the capacity to solubilize phosphate. The chemical state of P found inside the root suggests that the solubilized P is a mix of inorganic and organic phosphates. The solubilization of the non-water-soluble phosphate $[Ca_3(PO_4)_2]$ was suggested by the presence of inorganic hot spots with chemistry similar to that of inorganic Ca-phosphates. Since the control plants could not solubilize the $Ca_3(PO_4)_2$ in the medium, they had less available calcium able to react with the phosphate within root tissue.

Proteomics Analysis

In order to further link our phenotypic and image-based P uptake observations to molecular signature of plant-endophyte interactions, and to gain insight into metabolic and system-wide protein changes in inoculated vs. uninoculated poplar roots, we conducted a global proteomics analysis. Phosphate limitation/starvation acts like a stress to plants, stimulating up-regulation of phosphate transporter genes, as well as nutrient sensing signaling networks both locally and systemically (Liu et al., 1998; Chiou and Lin, 2011). As transcriptional regulation only shows potential proteins involved in a process or perturbation, we opted, here, to explore the proteome in order to identify the actual protein machinery present and enriched between P-mix and Control roots. As significant protein enrichment can reveal differences in functional roles between samples, here, we show relative functional category distributions between the P-mix and Control roots. Significant protein enrichment was defined as those proteins increased in abundance in either the P-mix or Control samples and those that passed a Student's *t*-test with a $p < 0.05$ (**Figure 7**).

For proteins involved in metabolism (**Figure 7**, top panel), there were more proteins in total as well as more KEGG functional categories/metabolic pathways enriched in the P-mix inoculated roots than in the Control. This includes higher enrichment of proteins involved in primary carbohydrate metabolism like glycolysis, and starch/sucrose metabolism, which are dependent upon P as cosubstrate. Notable differences between the Control and P-mix group include the large enrichment of oxidative phosphorylation, photosynthesis – antenna proteins, and glutathione metabolism related proteins.

In the Genetic Information Processing analysis (**Figure 7**, middle panel) we identified the enrichment of several histones in the P-mix inoculated roots. We identified five histones (i.e., POPTR_0011s13490, POPTR_0013s01890, POPTR_0005s04260, POPTR_0018s01310, and Potri_013G028900) significantly increased in abundance in the P-mix inoculated roots and one histone modification protein, POPTR_0019s04940, which was significantly increased in abundance in the Control roots.

We were particularly interested in identifying transporters (related to P transport) enriched in either the P-mix or Control root samples. We observed in the Environmental Information Processing functional category (**Figure 7**, bottom panel) that the Control samples actually had a greater number of transporters enriched compared to the enrichment of a single transporter in the P-mix samples. The transporters we identified as enriched in the Control samples included an ATPase, an exportin, myosin V, and an aquaporin. However, the only transporter identified as being enriched in the P-mix inoculated samples was a KEGG defined ATPase POPTR_0010s23200, which has some homology to the anion transport protein in *Arabidopsis*. Other ATPases were identified as being significantly changed in the proteomics data but were not functionally annotated by KEGG so were not included in the funcational category comparisons in **Figure 7**. The ATPase POPTR_0009s12330 was found to be significantly increased in the P-mix trees.

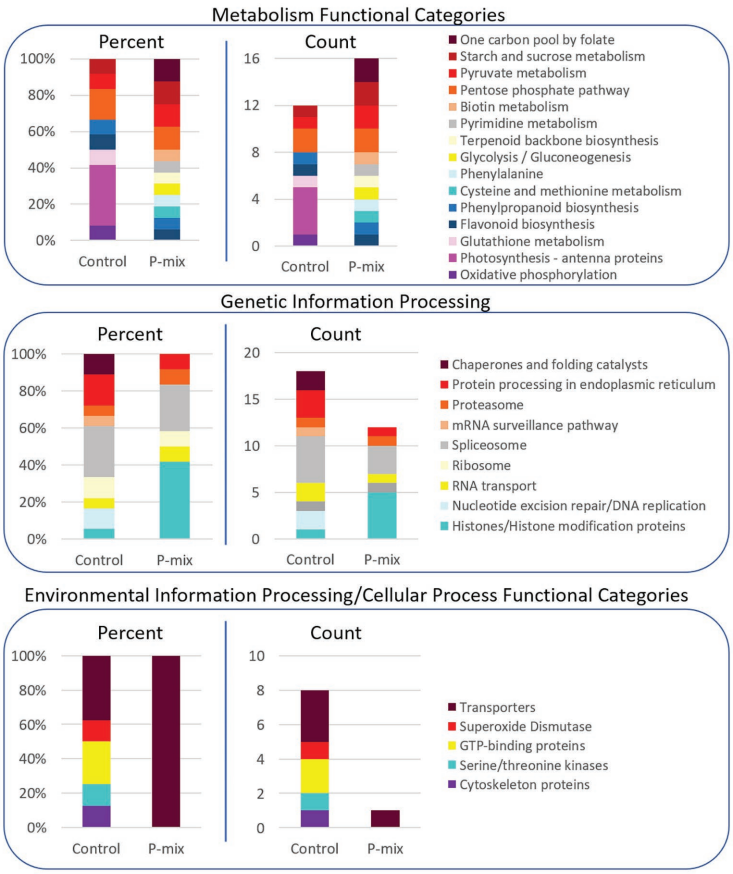

FIGURE 7 | KEGG functional category enrichment distributions displayed as an overall percent or absolute number of proteins identified in each category for those proteins determined to be significantly changed ($p < 0.05$) between Control and P-mix inoculated poplar roots using a *t*-test. A protein was designated to the Control or P-mix group depending on which group it displayed a higher abundance.

We only identified signaling related proteins to be enriched in the Control samples. However, we identified POPTR_0003s14620 which was significantly increased in abundance in the P-mix roots. This protein has high homology to an *Arabidopsis* tetratricopeptide repeat (TPR)-like superfamily protein. Three kinases, POPTR_0007s14380, POPTR_0001s14410, and POPTR_0013s14080, and one phosphatase, POPTR_0006s09720, were also found significantly enriched in the Control samples.

Protein Network Analysis

A STRING[8] (Szklarczyk et al., 2019) network analysis was performed on 100 significantly changed proteins found between the Control and P-mix root proteomics analyses. STRING network analysis works to identify known or predicted protein-protein associations between proteins utilizing prior analyses which include known interactions found in curated databases, experimentally determined databases, predicted interactions, including gene neighborhood, gene fusion, and gene co-occurrence analyses, and other predicted interactions based on text-mining, co-expression, and protein homology.

[8]https://string-db.org/

Figure 8 shows the interactions between 57 significantly changed poplar proteins found to have at least one protein-protein association in the STRING database. Each node in the figure represent a protein and each edge represents the association. The color of the edge represents the type of association known between the two proteins/nodes. Associations are meant to convey a variety of ways the proteins are related and do not necessarily mean they are physically binding/interacting *in vivo* with one another. Prominent clusters were further grouped using a black circle and the functional category or activity was noted.

DISCUSSION

Our solubilization experiments confirmed the earlier finding that poplar endophyte strains WP5 and WP42 had the ability to solubilize $Ca_3(PO_4)_2$ (Khan et al., 2015; Kandel et al., 2017). It is noted that most poplar microbiome studies lack the resolution to determine how common these particular bacterial species are in poplar (Ulrich et al., 2008; Gottel et al., 2011; Shakya et al., 2013; Beckers et al., 2017; Timm et al., 2018; Firrincieli et al., 2020); however, the genera of the two endophyte

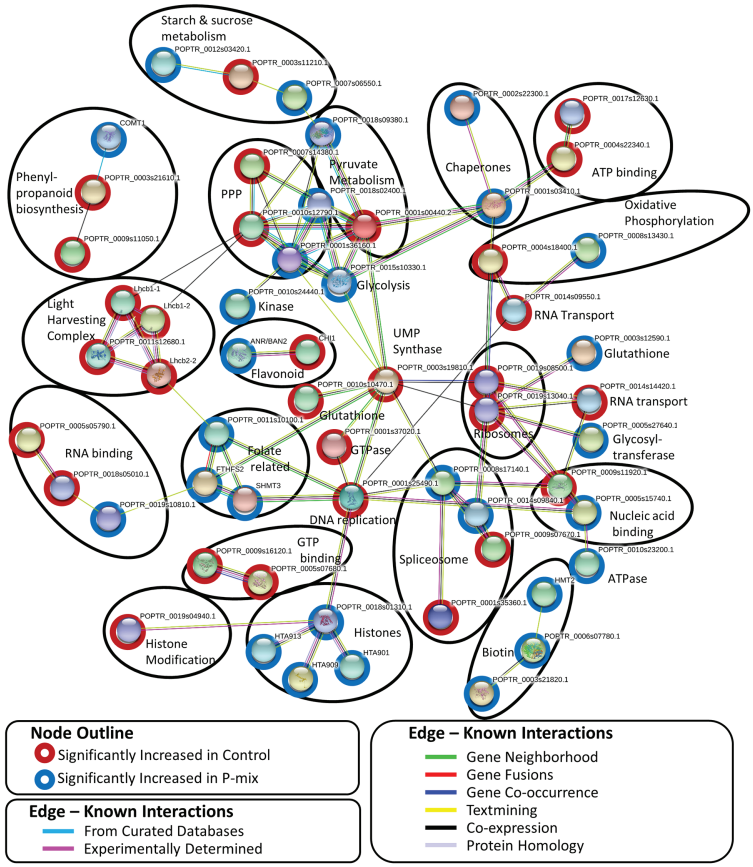

FIGURE 8 | STRING network analysis of proteins found to be significantly changed ($p < 0.05$, *t*-test) between phosphate limited Control and P-mix inoculated poplar roots. Nodes represent proteins and edges represent the protein-protein association. Black ovals designate functional groupings.

strains, *Rahnella* and *Burkholderia*, are fairly common culturable isolates from wild *P. trichocarpa* (Doty et al., 2009; Kandel et al., 2017). In a study of the poplar root microbiome, it was found that most of the core rhizosphere OTU's were within the order of Burkholderiales and Rhizobiales (Shakya et al., 2013). In another study, Burkholderiaceae comprised about 6% of the poplar rhizosphere (Beckers et al., 2017). Members of the *Rahnella* genus were one of the most abundant ASVs in *P. trichocarpa* in a poplar microbiome study that included the environment from which these strains were isolated (Firrincieli et al., 2020). Members of the family of Burkholderiaceae were up to about 20% of the microbial population in that study.

From tomography-based root imaging, we discovered that poplar samples inoculated with the phosphate solubilizing bacteria strains exhibited a root architecture with greater biomass (greater root volume), while the control samples exhibited increased fine root growth. This suggests an increased P uptake in the plants inoculated with the endophytes as plants that have more access to phosphorus typically produce fewer lateral roots (i.e., they produce less overall root surface area; Niu et al., 2013). High-resolution synchrotron micro-XCT images collected on small root sections confirmed the significant increase in root surface area due to fine root formation for control samples relative to the inoculated ones (**Figure 3**).

The macronutrient P was successfully visualized inside the root by synchrotron μXRF further indicating that the endophytes possessed the capacity to solubilize phosphate, thereby allowing the plant host to gain increased access to this nutrient. The quantitative μXRF based results that the P concentrations were greater in the P-mix samples are consistent with our micro-CT results that suggest markedly different root architecture and root volume for the samples inoculated with the endophytes. More available nutrient was expected to result in greater root mass growth.

X-ray absorption near edge structure studies into the chemical state of the P contained inside the root suggested that phosphorus within the plant is in the form of a mix of inorganic and organic phosphates. The solubilization of non-water-soluble phosphate was suggested by the presence of inorganic hot spots with chemistry similar to that of Ca-phosphates. We found that the inorganic P in both P-mix and Control groups was mostly Ca-bound, while the organic P could be identified as being chemically similar to phytate and DNA. By solubilizing the $Ca_3(PO_4)_2$ in the media, the endophyte strains make phosphate and calcium, both macronutrients required for growth, more

bioavailable to the plant. While the high reactivity of soluble phosphate with calcium is well-known, and the ability of many soil bacteria to solubilize inorganic P *in vitro* has been established, it was unknown why there was little correlation between solubilization ability and impact on plant growth (Collavino et al., 2010; Bashan et al., 2013). Our results point to a possible explanation for this incongruity. Once within the plant, phosphate seems to readily react with the calcium and other metals prevalent in the plant, becoming insoluble again. A benefit of endophytes over rhizobacteria may be that the phosphate could be stored in a non-reactive form inside the plant root and then re-released within the plant by endophytes as needed.

Considering the potential benefits of P solubilization enhanced by endophytes, the ability of WP5 and WP42 endophyte strains to fully solubilize Ca-phosphate could be beneficial to plants in alkaline soils, and the ability to at least partially solubilize Al-phosphate and Fe-phosphate could benefit plants in the more common acidic soils. While Ca-phosphate solubilization is commonly published as a symbiotic trait, $Ca_3(PO_4)_2$ is actually not difficult for many bacteria to dissolve (Bashan et al., 2013). The mechanism for solubilization of Ca-phosphate is primarily by organic acid production (Chhabra and Dowling, 2017), which is a fairly common trait. In screens of TCP-solubilizing bacteria for plant growth promotion, very few were positive. It is rarer to find Al-phosphate and Fe-phosphate solubilizers (Perez et al., 2007). Since solubility of Fe-phosphate decreases with lower pH, and Al-phosphate has the lowest solubility within pH 5.5 and 4.5, acidification through production of organic acids would not result in P solubilization in these cases (Bashan et al., 2013). The ability of WP5 and WP42 to solubilize, at least to some degree, all three common forms of phosphate is possibly reflective of the environment from which they were isolated. Both strains were isolated from wild poplar growing in primary substrates of cobble and sand with low organic matter. The source of the Snoqualmie River is high alpine snow melt; therefore, the environment is likely to be highly selective for the ability to make all sources of P bioavailable (Doty et al., 2009; Stettler, 2009). The growth data of both strains in ENBRIP do not fully align with this thinking; however, the mechanisms of solubilizing Al and Fe phosphate are less well understood. It may be that cells adhere to these phosphates during the solubilization process. If this is the case, then cells would have settled out with the excess phosphate and would not contribute to the optical density of the media. In support of this hypothesis, we often observed a high degree of cell clumping when dilutions of the phosphate cultures were plated but not from the inoculated no phosphate controls.

We explored the proteome in the plants in order to identify the actual protein machinery present and enriched between P-mix and Control roots. In this study, Control roots could be thought of as P-limited and potentially more stressed compared to the P-mix root where P was more available (by endophytes). An increased chlorosis observed in the Control trees compared to the P-mix trees may be indicative of this increased stess due to P being more limited. However, unfortunately, we did not have enough replicates to monitor these kinds of phenotyping responses.

The higher enrichment of proteins involved in primary carbohydrate metabolism (glycolysis and starch/sucrose metabolism, both dependent upon P as cosubstrate) indicates that the greater uptake of phosphorus brought about by P-solubilizing endophytes stimulated more metabolic pathways than when phosphate was less available and when metabolism presumably was slowed due to the relative limitations of this essential nutrient. The large enrichment of oxidative phosphorylation, photosynthesis – antenna proteins, and glutathione metabolism related proteins in the Control samples is connected to nutrient limitation and abiotic stress in that group. It is documented that sugars are redistributed to the roots when phosphate is less available (Ciereszko et al., 2005; Morcuende et al., 2007; Muller et al., 2007). Greater sugar accumulation would require increased oxidative phosphorylation capability to catabolize the sugars into ATP.

With regards to the histones that were found to be significantly increased in abundance in the P-mix inoculated roots and the one histone modification protein, which was significantly increased in abundance in the Control roots, phosphate availability is known to be involved in chromatin modification (Smith et al., 2010). In *Arabidopsis*, histone H2A.Z was found to regulate phosphate starvation response genes (Smith et al., 2010). Prior studies have shown that reactive oxygen species (ROS) concentrations are increased when P and other nutrients (i.e., nitrogen, potassium, and sulfur) are less available (Shin and Schachtman, 2004; Shin et al., 2005; Schachtman and Shin, 2007; Tyburski et al., 2009). ROS has also been associated with changes in root system architecture (RSA; Shin et al., 2005; Tyburski et al., 2009) and regulation of genes *via* oxidation reduction reactions. As a protection mechanism against ROS, plant cells utilize antioxidant and other oxidation ameliorating molecules, such as glutathione, super oxide dismutase (SOD), and light-harvesting antenna complex proteins (LHCBs), which are also induced in Control plants in this work. While the induction of antenna complex proteins in root tissues (which are devoid of photosynthesis capability) appears unusual, it is documented in the literature that antenna protein expression is coupled to abscisic acid signaling capable of being complexed with carotenoids and xanthophylls as well as chlorophyll, which are very efficient in ROS scavenging (Kobayashi et al., 2013) and have been found to be expressed ubiquitously in all different tissues, including roots of *Arabidopsis* (Smith et al., 2010; Xu et al., 2012). A common feature of endophytes is the ability to scavenge ROS, resulting in lowered stress responses in the host plant (Sessitsch et al., 2012). We previously reported that a consortium of endophyte strains, including strain WP5, reduced ROS levels in poplar (Khan et al., 2016).

Based on our exploration of which transporter proteins showed enrichment in the Control or the P-mix samples, it appears that greater phosphate availability does not necessarily equate into greater diversity of active transporters. Instead it appears as though the Control samples, where P was less available, required the enrichment of a greater number of transporters. One transporter that was identified as being enriched in the P-mix samples, and is related to the anion transport protein in *Arabidopsis*, may serve to aid transport of the phosphate provided by endophytes. The ATPase protein

that was found to be significantly increased in the P-mix trees is a four-way junction helicase. Helicases are involved in unwinding DNA during replication. This ATPase might, therefore, play a role in cell replication which could support the increased metabolic activity occurring in the roots of the P-mix trees.

The enrichment of signaling-related proteins in the Control samples indicated that more cell-signaling networks were activated and enriched in the roots when the trees had less P available than when the P-solubilizing endophytes were present. At the same time, the *Arabidopsis* TPR-like superfamily protein, which was significantly increased in abundance in the P-mix roots, is thought to be involved in the regulation of different cellular functions and plant hormone signaling like TPR proteins (Schapire et al., 2006). Specifically, these proteins have been found to be essential for abscisic acid, ethylene, cytokinin, gibberellin, and auxin which are all hormones involved in P sensing responses (Chiou and Lin, 2011).

In protein network analyses, centrality is a proxy for essentiality or importance. The protein/node uridine monophosphate (UMP) synthase (UMPS; POPTR_0003s19810) displayed the highest betweenness centrality. UMPS catalyzes the formation of uridine monophosphate which is a building block of RNA and pyrimidine synthesis. UMPS may also play a role as a negative regulator in increased availability of sugar in the roots. It is well-documented that when plants experience less phosphate in the roots, starch in shoot tissue is broken down and the released sugars are mobilized to the roots (Ciereszko et al., 2005; Morcuende et al., 2007; Muller et al., 2007). When UMPS expression was decreased in potato tubers, there were increased conversions of sucrose to starch and cell wall synthesis (Geigenberger et al., 2005). In our network analysis, UMPS displayed the highest betweenness centrality connecting major protein clusters related to genetic information processing (e.g., nucleic acid replication, chromatin remodeling, spliceosome, and ribosomal proteins) and carbohydrate metabolism. We hypothesize that disruption of this protein may cause interruption in sugar metabolism related to P availability.

In summary, the proteomics data revealed that more proteins involved in a diverse array of metabolic activities were enriched in the P-mix roots, whereas proteins related to abiotic stress, cell-signaling, and ROS amelioration were enriched in the Control samples which experienced less P availability.

Finally, we note that in order to visualize the effects of the inoculated strains on poplar, it was essential to begin with internally sterile plants. It is unknown how other members of the plant microbiome may affect the phosphorous solubilization performed by the two strains, WP5 and WP42. Microbial community dynamics can be complex, with both positive and negative interactions at play. However, these microbial interplays on which strains solubilize the phosphates would likely not affect the impact of bacterially solubilized phosphate on the host plant. Since bioavailable phosphate is an essential nutrient and solubilization is an exported activity, it is a "public good" subject to microbial "cheating" (recently reviewed in Smith and Schuster, 2019).

Further research is required to determine the mechanisms by which WP5 and WP42 solubilize the three phosphates. Also, in order to screen specific inorganic-organic associations related to P nutrient solubilization processes and to learn about the elemental distribution and speciation of the elements P and O on the nanoscale, future studies will employ scanning transmission x-ray microscopy in the soft x-ray energy region at 25–50 nm resolution combined with XANES.

CONCLUSION

Using laboratory and synchrotron XCT, synchrotron x-ray fluorescence spectromicroscopy and proteomics we found direct evidence of endophyte-promoted phosphorus uptake in poplar. Root imaging by XCT revealed greater root volume in the samples inoculated with the endophytes and smaller wet root volume with greater fine root formation in the control samples. This suggested an increased P uptake in the inoculated plants as plants that have more access to phosphorus typically produce fewer lateral roots. Using synchrotron x-ray fluorescence spectromicroscopy, we visualized the nutrient phosphorus inside poplar roots inoculated by the selected endophytes and found the phosphorus in both forms of organic and inorganic phosphates inside the root. Proteomics characterization on poplar roots coupled with protein network analysis revealed novel proteins and metabolic pathways with possible involvement in endophyte enriched phosphorus uptake, cell signaling, and metabolism. Our results have a significant implication: phosphate taken up by plants can form insoluble phosphate compounds within the plant; and therefore, rhizobacteria with the ability to solubilize P exclusively outside the plant tissue in soil may be at a disadvantage compared with endophytes. Being within the plant, endophytes may have the ability to continue re-releasing the phosphate for continued plant growth. In addition, since the endophytic bacteria in this study possess at least some ability to promote solubilization of all three common forms of phosphate, i.e., Ca-, Al, and Fe-phosphate, they may have an advantage as bioinoculants in both alkaline and acidic soils. In all, these findings suggest an important role of endophytes for phosphorus acquisition and provide a deeper understanding of the symbiotic associations between poplar and the endophytic bacteria.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD017325.

AUTHOR CONTRIBUTIONS

TV developed the study, carried out the x-ray-based (tomography and spectroscopy) experimental work, and prepared the first draft of the manuscript. KKH performed the proteomics characterization work with help from CDN, analyzed all proteomics data, and provided critical inputs for the study as well as to the writing of the manuscript. AHA directed the root collection process

performed by TEW and provided critical inputs for the study as well as during preparation of the manuscript. AWS performed all of the microbial experiments and subsequent data analysis, prepared **Figures 1** and **2** , and assisted in manuscript preparation. MEB performed critical analysis of the XANES data and provided critical inputs to its discussion in the manuscript. RKC prepared root thin sections for x-ray spectroscopy and prepared the soil for ICP-MS characterization. LRR and AKB helped with the tomography data processing and analysis. SCF helped to collect the micro-XRF and XANES data, created some of the micro-XRF maps (from the ALS), contributed to the XANES analysis as well as to the writing of the manuscript. OA helped to collect some of the micro-XRF maps (from the APS) and helped with related data analysis. DYP helped to collect the synchrotron XCT data. JRH performed the preliminary microbial experiments. SLD provided the plant samples with and without the microbial strains, assisted in the microbial experiments, provided critical inputs for the study as well as during preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.567918/full#supplementary-material

All proteomics data can be accessed through MassIVE (accession # MSV000084860) and ProteomeXchange (accession # PXD017325).

REFERENCES

Ajiboye, B., Akinremi, O. O., and Jurgensen, A. (2007). Experimental validation of quantitative XANES analysis for phosphorus speciation. *Soil Sci. Soc. Am. J.* 71, 1288–1291. doi: 10.2136/sssaj2007.0007

Barnes, M., O'Day, P., Young, R., Hart, S. C., and Berhe, A. A. (2019). Aeolian particulate matter as a source of bioavailable phosphorus to Sierra Nevada soil.

Bashan, Y., Kamnev, A. A., and De-Bashan, L. E. (2013). Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. *Biol. Fertil. Soils* 49, 465–479. doi: 10.1007/s00374-012-0737-7

Beckers, B., De Beeck, M. O., Weyens, N., Boerjan, W., and Vangronsveld, J. (2017). Structural variability and niche differentiation in the rhizosphere and endosphere bacterial microbiome of field-grown poplar trees. *Microbiome* 5:25. doi: 10.1186/s40168-017-0241-2

Bradshaw, H. D., and Stettler, R. F. (1995). Molecular-genetics of growth and development in *Populus*. IV. Mapping QTLs with large effects on growth, form, and phenology traits in a forest tree. *Genetics* 139, 963–973.

Cangelosi, G. A., Best, E. A., Martinetti, G., and Nester, E. W. (1991). Genetic-analysis of Agrobacterium. *Methods Enzymol.* 204, 384–397. doi: 10.1016/0076-6879(91)04020-o

Carbon, S., Dietze, H., Lewis, S. E., Mungall, C. J., Munoz-Torres, M. C., Basu, S., et al. (2017). Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Res.* 45, D331–D338. doi: 10.1093/nar/gkw1108

Chen, Y., Fan, J. B., Du, L., Xu, H., Zhang, Q. H., and He, Y. Q. (2014). The application of phosphate solubilizing endophyte *Pantoea dispersa* triggers the microbial community in red acidic soil. *Appl. Soil Ecol.* 84, 235–244. doi: 10.1016/j.apsoil.2014.05.014

Chhabra, S., Brazil, D., Morrissey, J., Burke, J. I., O'gara, F., and Dowling, D. N. (2013). Characterization of mineral phosphate solubilization traits from a barley rhizosphere soil functional metagenome. *Microbiology* 2, 717–724. doi: 10.1002/mbo3.110

Chhabra, S., and Dowling, D. N. (2017). "Endophyte-promoted nutrient acquisition: phosphorus and iron" in *Functional importance of the plant microbiome: Implications for agriculture, forestry and bioenergy*. ed. S. L. Doty (Cham, Switzerland: Springer), 21–42.

Chiou, T. J., and Lin, S. I. (2011). Signaling network in sensing phosphate availability in plants. *Annu. Rev. Plant Biol.* 62, 185–206. doi: 10.1146/annurev-arplant-042110-103849

Ciereszko, I., Johansson, H., and Kleczkowski, L. A. (2005). Interactive effects of phosphate deficiency, sucrose and light/dark conditions on gene expression of UDP-glucose pyrophosphory lase in *Arabidopsis*. *J. Plant Physiol.* 162, 343–353. doi: 10.1016/j.jplph.2004.08.003

Clark, R. B., and Zeto, S. K. (2000). Mineral acquisition by arbuscular mycorrhizal plants. *J. Plant Nutr.* 23, 867–902. doi: 10.1080/01904160009382068

Collavino, M. M., Sansberro, P. A., Mroginski, L. A., and Aguilar, O. M. (2010). Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biol. Fertil. Soils* 46, 727–738. doi: 10.1007/s00374-010-0480-x

Compant, S., Clement, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42, 669–678. doi: 10.1016/j.soilbio.2009.11.024

Crespo, J. M., Boiardi, J. L., and Luna, M. F. (2011). Mineral phosphate solubilization activity of gluconacetobacter diazotrophicus under P-limitation and plant root environment. *Agric. Sci.* 2, 16–22. doi: 10.4236/as.2011.21003

Doty, S. L., Oakley, B., Xin, G., Kang, J. W., Singleton, G., Khan, Z., et al. (2009). Diazotrophic endophytes of native black cottonwood and willow. *Symbiosis* 47, 23–33. doi: 10.1007/BF03179967

Doty, S. L., Sher, A. W., Fleck, N. D., Khorasani, M., Bumgarner, R. E., Khan, Z., et al. (2016). Variable nitrogen fixation in wild *Populus*. *PLoS One* 11:e0155979. doi: 10.1371/journal.pone.0155979

Eng, J. K., McCormack, A. L., and Yates, J. R. (1994). An approach to correlate tandem mass-spectral data of peptides with amino-acid-sequences in a protein database. *J. Am. Soc. Mass Spectrom.* 5, 976–989. doi: 10.1016/1044-0305(94)80016-2

Firrincieli, A., Khorasani, M., Frank, A. C., and Doty, S. L. (2020). Influences of climate on phyllosphere endophytic bacterial communities of wild poplar. *Front. Plant Sci.* 11:203. doi: 10.3389/fpls.2020.00203

Fittschen, U. E. A., and Falkenberg, G. (2011). Trends in environmental science using microscopic X-ray fluorescence. *Spectrochim. Acta Part B At. Spectrosc.* 66, 567–580. doi: 10.1016/j.sab.2011.06.006

Gaiero, J. R., McCall, C. A., Thompson, K. A., Day, N. J., Best, A. S., and Dunfield, K. E. (2013). Inside the root microbiome: bacterial root endophytes and plant growth promotion. *Am. J. Bot.* 100, 1738–1750. doi: 10.3732/ajb.1200572

Geigenberger, P., Regierer, B., Nunes-Nesi, A., Leisse, A., Urbanczyk-Wochniak, E., Springer, F., et al. (2005). Inhibition of de novo pyrimidine synthesis in growing potato tubers leads to a compensatory stimulation of the pyrimidine salvage pathway and a subsequent increase in biosynthetic performance. *Plant Cell* 17, 2077–2088. doi: 10.1105/tpc.105.033548

Goodstein, D. M., Shu, S. Q., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., et al. (2012). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* 40, D1178–D1186. doi: 10.1093/nar/gkr944

Gottel, N. R., Castro, H. F., Kerley, M., Yang, Z., Pelletier, D. A., Podar, M., et al. (2011). Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Appl. Environ. Microbiol.* 77, 5934–5944. doi: 10.1128/AEM.05255-11

Hane, J. K., Anderson, J. P., Williams, A. H., Sperschneider, J., and Singh, K. B. (2014). Genome sequencing and comparative genomics of the broad host-range pathogen rhizoctonia solani AG8. *PLoS Genet.* 10:e1004281. doi: 10.1371/journal.pgen.1004281

Hardoim, P. R., van Overbeek, L. S., and van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.* 16, 463–471. doi: 10.1016/j.tim.2008.07.008

Hoagland, D. R., and Arnon, D. I. (1938). *The water-culture method for growing plants without soil*. Berkeley, California: University of California, College of Agriculture, Agricultutal Experiment Station.

Isebrands, J. G., and Richardson, J. (2014). *Poplars and willows*. Rome, Italy: The Food and Agriculture Organization of the United Nations.

Jog, R., Pandya, M., Nareshkumar, G., and Rajkumar, S. (2014). Mechanism of phosphate solubilization and antifungal activity of *Streptomyces* spp. isolated from wheat roots and rhizosphere and their application in improving plant growth. *Microbiology* 160, 778–788. doi: 10.1099/mic.0.074146-0

Kandel, S. L., Firrincieli, A., Joubert, P. M., Okubara, P. A., Leston, N. D., McGeorge, K. M., et al. (2017). An in vitro study of bio-control and plant growth promotion potential of salicaceae endophytes. *Front. Microbiol.* 8:386. doi: 10.3389/fmicb.2017.00386

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* 45, D353–D361. doi: 10.1093/nar/gkw1092

Khan, Z., Kandel, S. L., Ramos, D. N., Ettl, G. J., Kim, S. H., and Doty, S. L. (2015). Increased biomass of nursery-grown Douglas-fir seedlings upon inoculation with diazotrophic endophytic consortia. *Forests* 6, 3582–3593. doi: 10.3390/f6103582

Khan, Z., Rho, H., Firrincieli, A., Hung, S. H., Luna, M. V., Masciarelli, O., et al. (2016). Growth enhancement and drought tolerance of hybrid poplar upon inoculation with endophyte consortia. *Curr. Plant Biol.* 6, 38–47. doi: 10.1016/j.cpb.2016.08.001

Kim, S., Gupta, N., and Pevzner, P. A. (2008). Spectral probabilities and generating functions of tandem mass spectra: a strike against decoy databases. *J. Proteome Res.* 7, 3354–3363. doi: 10.1021/pr8001244

Kirker, G., Zelinka, S., Gleber, S. C., Vine, D., Finney, L., Chen, S., et al. (2017). Synchrotron-based X-ray fluorescence microscopy enables multiscale spatial visualization of ions involved in fungal lignocellulose deconstruction. *Sci. Rep.* 7:41798. doi: 10.1038/srep41798

Kobayashi, K., Sasaki, D., Noguchi, K., Fujinuma, D., Komatsu, H., Kobayashi, M., et al. (2013). Photosynthesis of root chloroplasts developed in *Arabidopsis* lines overexpressing GOLDEN2-LIKE transcription factors. *Plant Cell Physiol.* 54, 1365–1377. doi: 10.1093/pcp/pct086

Liu, C. M., Muchhal, U. S., Uthappa, M., Kononowicz, A. K., and Raghothama, K. G. (1998). Tomato phosphate transporter genes are differentially regulated in plant tissues by phosphorus. *Plant Physiol.* 116, 91–99. doi: 10.1104/pp.116.1.91

Marcus, M. A., Macdowell, A. A., Celestre, R., Manceau, A., Miller, T., Padmore, H. A., et al. (2004). Beamline 10.3.2 at ALS: a hard X-ray microprobe for environmental and materials sciences. J. *Synchrotron Radiat.* 11, 239–247. doi: 10.1107/S0909049504005837

Mendes, G. D., de Freitas, A. L. M., Pereira, O. L., da Silva, I. R., Vassilev, N. B., and Costa, M. D. (2014). Mechanisms of phosphate solubilization by fungal isolates when exposed to different P sources. *Ann. Microbiol.* 64, 239–249. doi: 10.1007/s13213-013-0656-3

Misra, H. S., Rajpurohit, Y. S., and Khairnar, N. P. (2012). Pyrroloquinoline-quinone and its versatile roles in biological processes. *J. Biosci.* 37, 313–325. doi: 10.1007/s12038-012-9195-5

Monroe, M. E., Shaw, J. L., Daly, D. S., Adkins, J. N., and Smith, R. D. (2008). MASIC: a software program for fast quantitation and flexible visualization of chromatographic profiles from detected LC-MS(/MS) features. *Comput. Biol. Chem.* 32, 215–217. doi: 10.1016/j.compbiolchem.2008.02.006

Morcuende, R., Bari, R., Gibon, Y., Zheng, W., Pant, B. D., Bläsing, O., et al. (2007). Genome-wide reprogramming of metabolism and regulatory networks of *Arabidopsis* in response to phosphorus. *Plant Cell Environ.* 30, 85–112. doi: 10.1111/j.1365-3040.2006.01608.x

Muller, R., Morant, M., Jarmer, H., Nilsson, L., and Nielsen, T. H. (2007). Genome-wide analysis of the *Arabidopsis* leaf transcriptome reveals interaction of phosphate and sugar metabolism. *Plant Physiol.* 143, 156–171. doi: 10.1104/pp.106.090167

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Murphy, J., and Riley, J. P. (1962). A modified single solution method for determination of phosphate in natural waters. *Anal. Chim. Acta* 26, 678–681.

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* 170, 265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Niu, Y. F., Chai, R. S., Jin, G. L., Wang, H., Tang, C. X., and Zhang, Y. S. (2013). Responses of root architecture development to low phosphorus availability: a review. *Ann. Bot.* 112, 391–408. doi: 10.1093/aob/mcs285

O'Day, P., Nwosu, U. G., Barnes, M., Hart, S. C., Berhe, A. A., Christensen, J. N., et al. (2020). Phosphorus speciation in atmospherically deposited particulate matter and implications for terrestrial ecosystem productivity. *Environ. Sci. Technol.* 54, 4984–4994. doi: 10.1021/acs.est.9b06150

Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., et al. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. *Front. Microbiol.* 6:745. doi: 10.3389/fmicb.2015.00745

Perez, E., Sulbaran, M., Ball, M. M., and Yarzabal, L. A. (2007). Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. *Soil Biol. Biochem.* 39, 2905–2914. doi: 10.1016/j.soilbio.2007.06.017

Ravel, B., and Newville, M. (2005). ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT. *J. Synchrotron Radiat.* 12, 537–541. doi: 10.1107/S0909049505012719

Rennie, R. J. (1981). A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. *Can. J. Microbiol.* 27, 8–14. doi: 10.1139/m81-002

Richardson, A. E. (2001). Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Funct. Plant Biol.* 28, 897–906. doi: 10.1071/PP01093

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiol. Lett.* 278, 1–9. doi: 10.1111/j.1574-6968.2007.00918.x

Santi, C., Bogusz, D., and Franche, C. (2013). Biological nitrogen fixation in non-legume plants. *Ann. Bot.* 111, 743–767. doi: 10.1093/aob/mct048

Schachtman, D. P., and Shin, R. (2007). Nutrient sensing and signaling: NPKS. *Annu. Rev. Plant Biol.* 58, 47–69. doi: 10.1146/annurev.arplant.58.032806.103750

Schapire, A. L., Valpuesta, V., and Botella, M. A. (2006). TPR proteins in plant hormone signaling. *Plant Signal. Behav.* 1, 229–230. doi: 10.4161/psb.1.5.3491

Schneider, K. D., Van Straaten, P., De Orduna, R. M., Glasauer, S., Trevors, J., Fallow, D., et al. (2010). Comparing phosphorus mobilization strategies using *Aspergillus niger* for the mineral dissolution of three phosphate rocks. *J. Appl. Microbiol.* 108, 366–374. doi: 10.1111/j.1365-2672.2009.04489.x

Sessitsch, A., Hardoim, P., Döring, J., Weilharter, A., Krause, A., Woyke, T., et al. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Mol. Plant Microbe Interact.* 25, 28–36. doi: 10.1094/MPMI-08-11-0204

Shakya, M., Gottel, N., Castro, H., Yang, Z. K., Gunter, L., Labbé, J., et al. (2013). A multifactor analysis of fungal and bacterial community structure in the root microbiome of mature *Populus deltoides* trees. *PLoS One* 8:e76382. doi: 10.1371/journal.pone.0076382

Shin, R., Berg, R. H., and Schachtman, D. P. (2005). Reactive oxygen species and root hairs in Arabidopsis root response to nitrogen, phosphorus and potassium deficiency. *Plant Cell Physiol.* 46, 1350–1357. doi: 10.1093/pcp/pci145

Shin, R., and Schachtman, D. P. (2004). Hydrogen peroxide mediates plant root cell response to nutrient deprivation. *Proc. Natl. Acad. Sci. U. S. A.* 101, 8827–8832. doi: 10.1073/pnas.0401707101

Smith, A. P., Jain, A., Deal, R. B., Nagarajan, V. K., Poling, M. D., Raghothama, K. G., et al. (2010). Histone H2A.Z regulates the expression of several classes of phosphate starvation response genes but not as a transcriptional activator. *Plant Physiol.* 152, 217–225. doi: 10.1104/pp.109.145532

Smith, P., and Schuster, M. (2019). Public goods and cheating in microbes. *Curr. Biol.* 29, R442–R447. doi: 10.1016/j.cub.2019.03.001

Stettler, R. F. (2009). *Cottonwood and the river of time: On trees, evolution, and society*. Seattle, WA, United States: University of Washington Press.

Stevens, J. M., Ulrich, R. L., Taylor, L. A., Wood, M. W., Deshazer, D., Stevens, M. P., et al. (2005). Actin-binding proteins from *Burkholderia mallei* and *Burkholderia thailandensis* can functionally compensate for the actin-based motility defect of a *Burkholderia pseudomallei* bimA mutant. *J. Bacteriol.* 187, 7857–7862. doi: 10.1128/JB.187.22.7857-7862.2005

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 47, D607–D613. doi: 10.1093/nar/gky1131

Timm, C. M., Carter, K. R., Carrell, A. A., Jun, S. R., Jawdym, S. S., Vélez, J. M., et al. (2018). Abiotic stresses shift belowground populus-associated bacteria toward a core stress microbiome. *mSystems* 3, e00070–e00017. doi: 10.1128/mSystems.00070-17

Twining, B. S., Baines, S. B., Fisher, N. S., Maser, J., Vogt, S., Jacobsen, C., et al. (2003). Quantifying trace elements in individual aquatic protist cells with a synchrotron X-ray fluorescence microprobe. *Anal. Chem.* 75, 3806–3816. doi: 10.1021/ac034227z

Tyburski, J., Dunajska, K., and Tretyn, A. (2009). Reactive oxygen species localization in roots of *Arabidopsis thaliana* seedlings grown under phosphate deficiency. *Plant Growth Regul.* 59, 27–36. doi: 10.1007/s10725-009-9385-9

Ulrich, K., Ulrich, A., and Ewald, D. (2008). Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiol. Ecol.* 63, 169–180. doi: 10.1111/j.1574-6941.2007.00419.x

van der Ent, A., Przybylowicz, W. J., de Jonge, M. D., Harris, H. H., Ryan, C. G., Tylko, G., et al. (2018). X-ray elemental mapping techniques for elucidating the ecophysiology of hyperaccumulator plants. *New Phytol.* 218, 432–452. doi: 10.1111/nph.14810

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Vogt, S. (2003). MAPS: a set of software tools for analysis and visualization of 3D X-ray fluorescence data sets. *J. Phys. IV* 104, 635–638. doi: 10.1051/jp4:20030160

Wang, Y. Y. E., Kutnetsov, L., Partensky, A., Farid, J., and Quackenbush, J. (2017). WebMeV: a cloud platform for analyzing and visualizing cancer genomic data. *Cancer Res.* 77, E11–E14. doi: 10.1158/0008-5472.CAN-17-0802

Wang, Y. X., Yang, F., Gritsenko, M. A., Wang, Y., Clauss, T., Liu, T., et al. (2011). Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. *Proteomics* 11, 2019–2026. doi: 10.1002/pmic.201000722

Werner, F., and Prietzel, J. (2015). Standard protocol and quality assessment of soil phosphorus speciation by P K-edge XANES spectroscopy. *Environ. Sci. Technol.* 49, 10521–10528. doi: 10.1021/acs.est.5b03096

Xu, Y. H., Liu, R., Yan, L., Liu, Z. Q., Jiang, S. C., Shen, Y. Y., et al. (2012). Light-harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in *Arabidopsis*. *J. Exp. Bot.* 63, 1095–1106. doi: 10.1093/jxb/err315

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Effect of Inorganic N Top Dressing and *Trichoderma harzianum* Seed-Inoculation on Crop Yield and the Shaping of Root Microbial Communities of Wheat Plants Cultivated Under High Basal N Fertilization

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Wheat crop production needs nitrogen (N) for ensuring yield and quality. High doses of inorganic N fertilizer are applied to soil before sowing (basal dressing), with additional doses supplied along the cultivation (top dressing). Here, a long-term wheat field trial (12 plots), including four conditions (control, N top dressing, *Trichoderma harzianum* T34 seed-inoculation, and top dressing plus T34) in triplicate, was performed to assess, under high basal N fertilization, the influence of these treatments on crop yield and root microbial community shaping. Crop yield was not affected by top dressing and *T. harzianum* T34, but top dressing significantly increased grain protein and gluten contents. Twenty-seven-week old wheat plants were collected at 12 days after top dressing application and sampled as bulk soil, rhizosphere and root endosphere compartments in order to analyze their bacterial and fungal assemblies by 16S rDNA and ITS2 high-throughput sequencing, respectively. Significant differences for bacterial and fungal richness and diversity were detected among the three compartments with a microbial decline from bulk soil to root endosphere. The most abundant wheat root phyla were Proteobacteria and Actinobacteria for bacteria, and Ascomycota and Basidiomycota for fungi. An enrichment of genera commonly associated with soils subjected to chemical N fertilization was observed: *Kaistobacter*, *Mortierella,* and *Solicoccozyma* in bulk soil, *Olpidium* in rhizosphere, and *Janthinobacterium* and *Pedobacter* in root endosphere. Taxa whose abundance significantly differed among conditions within each compartment were identified. Results show that: (i) single or strain T34-combined application of N top dressing affected to a greater extent the bulk soil bacterial levels than the use of T34 alone; (ii) when N top dressing and T34 were applied in combination, the N fertilizer played a more decisive role in the bacterial microbiome

than T34; (iii) many genera of plant beneficial bacteria, negatively affected by N top dressing, were increased by the application of T34 alone; (iv) bulk soil and rhizosphere fungal microbiomes were affected by any of the three treatments assayed; and (v) all treatments reduced *Claroideoglomus* in bulk soil but the single application of T34 raised the rhizosphere levels of this mycorrhizal fungus.

Keywords: bacterial composition, fungal composition, chemical fertilization, bulk soil, rhizosphere, root endosphere

INTRODUCTION

Wheat is one of the most important crops worldwide, with figures like a harvested area of 214.3 million ha and a global production of 734 million tons in 2018 (FAOSTAT, 2020). Given the fact that wheat grain provides about one-fifth of both calories and proteins to human diet, there is, therefore, a need for increasing the production of this crop in order to feed the world's-growing population (International Wheat Genome Sequencing Consortium [IWGSC], 2018). Conventional extensive agriculture has an absolute requirement of nitrogen (N) for ensuring the yield and high quality of wheat crops (Zörb et al., 2018). However, it is well known that this and other widespread cereal crops use only 30–40% of the applied N fertilizers, while the rest remains unused causing severe environmental pollution (Rockström et al., 2009; Curci et al., 2017). Although the EU is suggesting a reduction in N fertilization to quantities of 170 kg/ha/year, in countries like Spain, where 2.4 million ha are devoted to this crop, this figure can still reach as high as 500 kg/ha/year. It is a common practice in Spain to apply 240 kg/ha as basal nitrogen fertilizer and to add a higher quantity as top dressings along the wheat crop.

Several studies have reported a wide range of beneficial effects of the microbiome members on plants, including disease suppression, priming of the plant immune system leading to the induction of systemic resistance, increased nutrient acquisition, increased tolerance to abiotic stresses or adaptation to environmental variations (Hassani et al., 2018). It is now evident that the root system provides many more traits than just anchorage and uptake of nutrients and water, and therefore all the interconnected factors that influence the complex ecosystem of the rhizosphere, considering it as an integrated whole, including numerous and multiple kinds of microorganisms that interact in various ways need to be taken into account (Mendes et al., 2011). Besides the well-known mycorrhizal fungi, N-fixing bacteria, and growth-promoting bacteria, plant microbiomes include a high diversity of microorganisms that become apparent when comparing microbial species and strains even at the level of the genotypes from a same species (Vandenkoornhuyse et al., 2002; Bulgarelli et al., 2012; Peiffer et al., 2013; Rossmann et al., 2020).

Advances in next-generation sequencing (NGS) technologies have marked the beginning of a new era in gathering information on the genetic repertoires of microbial communities (Fricker et al., 2019). The Proteobacteria, mostly alpha and beta classes, usually dominate in root-associated samples. Other major bacterial groups that are often present in the roots include Actinobacteria, Acidobacteria, Cyanobacteria, Firmicutes, FCB (Fibrobacteres-Chlorobi-Bacteroidetes), particularly Bacteroidetes and Gemmatimonadetes, and PVC (Planctomycetes-Verrucomicrobia-Chlamydiae), especially Planctomycetes and Verrucomicrobia (Philippot et al., 2013; Turner et al., 2013). A large number of research reports have explored the fungal communities associated with plant roots, revealing a staggering diversity of fungi, mainly belonging to the two major phyla Ascomycetes and Basidiomycetes (Porras-Alfaro and Bayman, 2011; Rossmann et al., 2020; Wang et al., 2020). Fungal communities are not randomly assembled but instead appear to be specifically filtered by their plant host which recruits a particular microbial consortium to adapt to the environmental conditions at a microscale (Lê Van et al., 2017). At least three distinct microbiomes thriving at the root-soil interface have been identified (Bulgarelli et al., 2012; Hirsch and Mauchline, 2012), depending on whether they belong to bulk soil, rhizosphere or endosphere. In almost all cases, an apparent decrease in the diversity of species was recorded from the rhizosphere to the endosphere, indicating that exists a strong habitat filtering mechanism and that it may shape the composition of each microbiome compartment (Vandenkoornhuyse et al., 2015). It has been described that the bacterial rhizosphere changes much more than the bulk soil community in wheat cropping systems (Donn et al., 2015). Moreover, soil nutrient availability constitutes a driving factor in shaping the wheat endophytic bacterial microbiome (Robinson et al., 2015), although the use of N fertilization negatively affects bacterial assemblages in the wheat rhizosphere (Kavamura et al., 2018).

Most *Trichoderma* spp. have been linked to biocontrol against plant pathogenic fungi, oomycetes, and even nematodes (Medeiros et al., 2017; Debbi et al., 2018). Moreover, rhizosphere competent strains have proved to be beneficial for plants (Hermosa et al., 2012). *Trichoderma* species are frequently found as common inhabitants of the soil and the rhizosphere, and even though many of them may become facultative endophytes, the number of truly endophytic *Trichoderma* spp. is scarce (Bae et al., 2009; Carrero-Carrón et al., 2018). In addition to rhizosphere colonization, nutrient uptake facilitation and plant growth promotion (Hermosa et al., 2012; Samolski et al., 2012), the application of *Trichoderma* strains may also affect the soil bacterial and fungal communities in a pH- and N supply dependent manner, respectively (Zhang et al., 2018). It has been reported that biofertilizers based on *Trichoderma* strains when used alone or in combination with organic fertilizers (compost) provoke changes in the rhizosphere microbial community of crop plants (Zhang et al., 2013; Pang et al., 2017; Ros et al., 2017; Qiao et al., 2019). Specifically, *Trichoderma* spp. have been directly related to the increased levels of Acidobacteria detected

in different agricultural soils such as those from maize and black pepper (Saravanakumar et al., 2017; Umadevi et al., 2017; Singh et al., 2018). The combined application of *Trichoderma*, and other beneficial microorganisms such as *Bacillus*, to crop soils fosters the recruitment of other plant beneficial bacteria and fungi in the rhizosphere (Wang et al., 2019).

The previous studies showed positive effects of *T. harzianum* T34 on tomato plant growth under greenhouse conditions (Rubio et al., 2017) and also the ability of this strain to increase wheat systemic defense after culturing under *in vitro* conditions (Rubio et al., 2019). However, little is known about the effects caused by the application of *Trichoderma* or N-based fertilizers on the microbiota of wheat plants under field conditions, and whether the microbial communities are randomly assembled or specifically filtered by the host plant to create a particular microbial assemblage to meet the new requirements of the environment. The conventional agronomic practices for wheat crop in the Spanish region of Castile and Leon include the application of high doses of N fertilizer to the soil before the sowing (basal) and along the cultivation (top dressing). We lack of a complete understanding of how bacterial and fungal communities are structured in crop plants, how fertilization practices can alter microbial communities, how such practices might affect microbe performance, and how they are in turn linked to their potential microbial preys. Here, the aim of our work has been to assess the diversity and structure of both bacterial and fungal communities in the root system of wheat crop plants subjected to three different treatments (top dressing, *T. harzianum* T34, and strain T34 plus top dressing) in order to explore, under high basal N dosage (control), the influence of inorganic N top dressing and *Trichoderma* application in the microbiome distribution at the bulk soil, rhizosphere and endosphere compartments.

MATERIALS AND METHODS

Field Wheat Experiment and Sample Collection

A field trial was performed in Ventosa de la Cuesta (Valladolid, Spain), a region with continental Mediterranean climate and an average annual temperature and precipitation of 12.5°C and 415 mm, respectively. The experimental field was preceded by fallow for 1 year and this last by a barley crop. The trial was carried out over 1 year from 2018 to 2019 and included 12 experimental plots containing four conditions (C1, C2, C3, and C4) with three replicates in a randomized complete block design (**Supplementary Figure S1**). Each experimental plot had 12.75 m^2 (8.5 × 1.5 m) with a plantation framework of 425 seeds/m^2, corresponding approximately to 240 kg seeds/ha, and using wheat of the Berdun R variety.

The four conditions were designed as follows: C1 (control: soil amended with basal chemical fertilizer), C2 (soil amended with basal chemical fertilizer and two top dressing applications), C3 (soil amended with basal chemical fertilizer and strain *T. harzianum* T34), and C4 (soil amended with basal chemical fertilizer, and both strain T34 and two top dressing applications). Following conventional agronomic practices in this region, 2 days before sowing 240 kg/ha of NPK 8-15-15 and 30 kg/ha of KCl were applied as basal chemical fertilization. The first top dressing application was performed 12 weeks after sowing with the 60% of N requirement (157 kg/ha of calcium nitrate, CAN) in conditions C2 and C4, and the second CAN dosage was supplied similarly 27 weeks after sowing with the 40% of total N requirement (105 kg/ha) in these two conditions. *Trichoderma harzianum* CECT 2413 (Spanish Type Culture Collection, Valencia, Spain), also referred to as strain T34, was grown on potato dextrose agar medium (PDA, Difco Laboratories, Detroit, MI, United States) and spores were harvested as previously described (Rubio et al., 2017). Strain T34 was seed-coating applied at a concentration of 2×10^6 conidia/seed in the C3 and C4 conditions. The procedure was carried on through the addition of 15 mL of a T34 suspension (6.7×10^8 conidia/mL) and 10 mL of a commercial Arabic gum solution (Pelikan, Barcelona, Spain) to plastic bags containing 250 g of wheat seeds and subsequent manual mixing. The seed-inoculated bags were kept open for 20 h in a laminar flow cabinet for drying. Furthermore, 15 mL of sterile water and 10 mL of Arabic gum solution were added to each bag with seeds for C1 and C2 conditions.

Sampling process was carried out at 27 weeks after sowing (12 days after applying the second top dressing where indicated). Soil and wheat samples were collected from five spots selected within each plot (assayed condition) and were considered as a single sample. Three biological replicates per condition were considered. The five sample spots were uniformly selected across plots (**Supplementary Figure S1**). From each spot a total of 20 plants were harvested by digging a hole (up to about 30 cm deep and 15 cm wide) around the pool of plants with a trench shovel in order to collect the whole root system of the plants (bulk soil and rhizosphere) as well as the areal part. The 20 plants from each of the five spots were carefully placed in one plastic bag and labeled with the condition, replicate, and sampling plot. In addition, soil samples were collected from each hole for chemical analysis. Approximately, 100 g of fine earth was collected from each hole with a hand shovel, once the pool of plants was taking out, and placed in a 50 mL sterile tube. The soil samples from the five spots per plot were combined in a single sample and three replicates per condition were considered. Soil and plant samples were taken to the laboratory for processing. The crop was harvested on June 28th, 2019 and the grains were used to calculate crop yield (kg/ha), macro- and microelements content, and quality parameters such as protein and gluten contents.

Root Sample Preparation

In order to carry out the microbiota analysis, the whole root system of the plants (set of 100 plants per plot) was processed to isolate three different samples: (i) bulk soil, considered as the soil at a distance of 2–6 cm of the root surface; (ii) rhizosphere, considered as the loosely adhering soil from the root system; and (iii) root endosphere, considered as the inside of surface-disinfected roots. Samples were prepared according to the methodology previously described (D'Amico et al., 2018; Yamamoto et al., 2018) with slight modifications. Briefly, for each plant set, 10 g of bulk soil was uniformly hand-collected, taking

care not to disturb any root, and placed in a 50 mL sterile tube, frozen in liquid N, and stored at −80°C. Once the soil attached to the roots was removed, the root systems of the set of plants were laid on a flat bench as a unit and transversally cut with scissors. From these roots, 3.5 g were collected, cut into segments and washed twice with 20 mL PBS-S buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3mMNaH_2PO_4, pH 7.0, 0.02% Silwet L-77), by shaking at 180 rpm in a 50 mL tube for 20 min. Washed roots were transferred to a 50 mL sterile tube and the remained liquid was filtered throught nylon. The obtained liquid, about 38 mL, was centrifuged at 3200 rpm and 4°C for 15 min. The generated pellet, called as the rhizosphere, was frozen in liquid N, and stored at −80°C. The roots were subsequently washed by shaking as described above once in 35 mL of 2% commercial sodium hypochlorite and three times in 35 mL of PBS-S buffer. Then, the roots were transferred to a 50 mL sterile tube with 35 mL PBS-S buffer, sonicated for 20 min with a water bath sonicator at 40 kHz (Model 5510, Branson Ultrasonics Corporation, Danbury, CT, United States), and washed again in 35 mL of PBS buffer using the same procedure described above. Roots were dried on 50 mm diameter Whatman filter paper, transferred to a 50 mL sterile tube, and then frozen in liquid nitrogen for storage at −80°C. The three sample types obtained were used for DNA extraction.

Chemical Properties of Soil and Grains

All measurements were quantified by the IRNASA's analytical service (CSIC, Salamanca, Spain), apart from protein and gluten contents in wheat grain. For the soil, the 12 sample sets of 0.5 kg were sieved and an aliquot of 100 g used for determination of pH and content in $CaCO_3$, carbon (C), organic matter, N and phosphorus (P). For the 12 sets of pooled wheat grain, the samples were powdered and 500 mg used for quantification of C, N, macro- and microelements.

The pH of the soil was determined in a soil/water suspension (1:2.5, w/v ratio) with a glass electrode. N and organic C contents, expressed as percentage (g per 100 g sampled material), were determined by dry combustion (Dumas, 1831) in a CN628 automatic carbon-nitrogen analyzer (LECO Instruments S.L., Madrid, Spain) following the manufacturer's instructions. Organic C data were used to calculate the organic matter percentage. The content of inorganic carbon was determined as $CaCO_3$ with a Bernard calcimeter. The available P in soil samples was estimated by extraction with sodium bicarbonate (Olsen et al., 1954).

The content of macro- [sulphur (S), P, magnesium (Mg), potassium (K) and calcium (Ca)] and microelements [iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu)] was determined by mineralization in a mixture of nitric acid and hydrogen peroxide (4:1 v/v) using an Ethos Up High Performance Microwave Digestion System (Milestone, Sorisole, Italy). Samples were subjected to the microwave heating with a temperature ramp ranging from room temperature to 200°C for 40 min, followed by maintaining at 200°C for 15 min. After cooling, solutions were quantitatively transferred into 25 mL volumetric flasks and brought up to volume with ultrapure water. The content of such elements was analyzed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry, iCAP 6300 DUO, Thermo Electron Corporation, Rugby, United Kingdom), as previously described (Jiménez et al., 2019).

Protein and wet gluten contents were determined in 0.5 kg of pooled wheat grains per plot by near-infrared spectroscopy (NIR) technology (Chen et al., 2017), using a portable Zeltex ZX50 NIR analyzer (Zeltex Inc., Hagerstown, MD, United States). Values were expressed as percentage.

DNA Extraction, PCR Amplifications and Illumina Sequencing

DNA was extracted from all the 36 sample sets, 12 from each soil, rhizosphere and root endosphere compartments. Root endosphere samples were previously lyophilized and ground to a fine powder with a sterilized mortar and a pestle. Total DNA of bulk soil and rhizosphere samples was extracted using the FastDNA Spin Kit for Soil (MP Biomedical LLC, Irvine, CA, United States) and that of root endosphere samples using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany), following manufacturer's instructions. Each sample had three replicates in our experiment, and the triplicate DNA samples were pooled. Approximately 30 ng of DNA for each sample was sent to the Genomics Unit (Parque Científico de Madrid, Madrid, Spain) for amplification, library preparation and sequencing.

The 16S rRNA and ITS region were used to determine bacterial and fungal communities, respectively, in all the 36 sample sets from soil, rhizosphere and root endosphere. DNA concentration was determined in the samples using Quant-IT PicoGreen reagent (ThermoFisher Scientific, Waltham, MA, United States). Purified DNAs (3 ng) and the universal primers 341f (5′-CCTACGGGNGGCWGCAG-3′) and 785r (5′-GACTACHVGGGTATCTAATCC-3′) were employed to amplify the V3-V4 region of the bacterial 16S rRNA gene, and the primers ITS86F (5′-GTGAATCATCGAATCTTTGAA-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS2 region of the fungal ITS. The PCR mix was prepared as previously described (Zhang et al., 2018). The PCR thermal cycling program consisted of initial denaturation at 98°C for 30 s, followed by 20 (for 16S) or 21 (for ITS) cycles of denaturation at 95°C for 10 s, annealing at 55°C for 20 s and extension at 72°C for 20 s, and a final extension step at 72°C for 2 min. Each sample was amplified in triplicate and subsequently the PCR products were pooled. PCR products (approximately 450 and 300 pb in size for bacterial and fungal samples, respectively) included extension tails which allowed sample barcoding and the addition of specific Illumina sequences in a second low cycle number PCR. The obtained amplicons were validated and quantified by a Bioanalyzer, and an equimolecular pool of 16S and ITS PCR products was purified using AMPure beads and titrated by quantitative PCR using the "Kapa-SYBR FAST qPCR kit for Light Cycler 480" and a reference standard for quantification. The pool of amplicons was denatured before seeding on a flowcell of an Illumina Miseq platform at a density of 10 pM, and the cluster were formed and sequenced using a "MiSeq Reagent Nano Kit v2" and a 2 × 250 pair-end sequencing run. Illumina sequencing resulted in a total of 3,621,101 reads for 16S and 4,019,719 reads for ITS that

passed Illumina quality control (**Supplementary Tables S1, S2**). The obtained bacterial 16S and fungal ITS sequences data are available at the Sequence Read Archive (SRA), operated by the National Center for Biotechnology Information (NCBI), under the accession number PRJNA639567.

Bioinformatics Processing and Taxonomy Assignment

Sequence quality was evaluated for raw forward and reverse Illumina ITS and 16S reads with FastQC (Andrews, 2010). Preprocessing and quality control filtering, operational taxonomic unit (OTU) clustering, taxonomy assignment and construction of the abundance tables were performed using USEARCH v11.1 (Edgar, 2010). Sequences which could not be assembled, singletons, chimeras, and sequences with a low quality score were discarded.

For both bacterial and fungal communities, OTUs were clustered with at least 97% similarity threshold using UPARSE-OTU algorithm (Edgar, 2013) and were taxonomically assigned using the GreenGenes v13.5 (DeSantis et al., 2006) and UNITE USEARCH/UTAX release for fungi version 18.11.2018 (Kõljalg et al., 2013), a database specifically modified for USEARCH pipeline, respectively. Only taxonomic annotations with a 97% confidence estimate as provided by the SINTAX algorithm (Edgar, 2016) command were accepted. Taxonomy assignment provided the available annotation of each OTU to the different taxonomy levels (kingdom, phylum, class, order, family, genus, and species). The low abundance OTUs were eliminated from the OTU table if they did not have a total of at least 10 counts across all the dataset, moreover, OTUs assigned to mitochondria (o__Rickettsiales/f__mitochondria) and chloroplasts (p__Cyanobacteria/c__Chloroplast) were removed before downstream analysis. Taxonomic prediction was explored. A phylogenetic tree was generated using *cluster_tree* command from USEARCH v11.1.

Statistical Analyses

Metagenomics Data

All metagenomic data analyses were conducted in RStudio v3.6.2 (R Core Team, 2019). Rarefaction curves were constructed for each sample individually per compartment (bulk soil, rhizosphere and root endosphere) and condition type (C1–C4) using *rarefy_even_depth* command from *phyloseq* package (McMurdie and Holmes, 2013). Redundancy analysis (RDA) was performed based on *vegan* package (Oksanen et al., 2015) to evaluate the taxonomic structure of bacterial and fungal communities and to correlate them with compartment and condition type using Hellinger distance. A hierarchical clustering was performed to examine whether there were clusters between samples and relate them to the environmental conditions using the euclidean distance and the complete linkage method.

Sample richness and evenness were estimated using total number of observed OTUs and the alpha-diversity indices [Chao1 and abundance-based coverage estimator (ACE), Shannon, Simpson, Pielou and Phylogenetic Diversity (PD)]. The PD index was calculated using the *picante* package (Kembel et al., 2010), the rest of indices were calculated using the *phyloseq* package. Kruskal–Wallis sum-rank test was used to compare difference in medians of alpha-diversity indices across the three compartments and the four conditions types. Wilcoxon rank-sum test was further employed to test for pairwise significant differences. Bacterial and fungal beta-diversity was estimated according to the Bray–Curtis and un/weighted UniFrac distances from the abundance matrix across samples. A Permutational Multivariate Analysis of Variance (PERMANOVA) test was performed to determine whether bacterial and fungal communities were significantly influenced by compartment and condition types, with 999 permutations, and a multivariate pairwise test for pairwise comparisons using the *adonis* command from *vegan* package and *pairwise.adonis* from *PairwiseAdonis* (Martinez-Arbizu, 2017), respectively. Principal coordinates analysis (PCoA) based on these beta-diversity distances were used to visualize the dissimilarities among the compartments and condition types.

The relative abundance of taxa at the phylum, family, genus and species levels was calculated and depicted by stacked barplots. The differential abundance testing was conducted using ALDEx2 (Fernandes et al., 2013) in order to explore whether the abundance for bacteria and fungi data varied at the genus level among compartments and among conditions within a given compartment. ALDEx2 uses the centered log-ratio (clr) transform which ensures that the data is scale invariant and compositional consistent. Before carrying out this analysis, a filtering was performed, excluding those OTUs with a relative abundance of less than 0.001%. While ALDEx2 provides both parametric and non-parametric statistical tests, only non-parametric test results are reported in this study, Kruskal–Wallis test followed by Wilcoxon test were used for pairwise comparisons. Significance was measured based on the Benjamini–Hochberg corrected *P*-value for both tests (significance threshold, $P < 0.05$). In pairwise comparisons, ALDEx2 also provides an effect size and a 95% confidence interval (95% CI).

Finally, the linear discriminant analysis (LDA) effect size (LEfSe) method from Huttenhower Lab (Segata et al., 2011), which is based on the Kruskal–Wallis sum-rank test for comparison classes, was also used to identify genera significantly different among compartments and, within compartments, among conditions. An LDA threshold score > 4.0 for compartments and >2.0 for conditions, and a significance $P < 0.05$ threshold for conditions as well as a sample normalization to 1 M, which is usually applied for metagenomic data in which the relative abundances are taken into account, were used. Different LEfSe-generated taxonomic cladograms from phylum to genus were produced.

Agronomic Data

All data were collected from three biological replicates. The homogeneity of variances and normality tests were performed by Levene's and Shapiro–Wilk's tests. The data of soil parameter, agronomic traits, and micro- and macroelements content agreed with the parametric statistics assumptions were further analyzed. One-way and two-way ANOVA were performed followed by a post-hoc Tukey's test using the IBM SPSS Statistics for Windows,

version 25 (IBM Corp., Armonk, NY, United States) and setting confidence intervals of 95%.

RESULTS

To explore the soil parameters existing at the time of collecting the microbiome samples, soil samples were also collected, pH measured, and content in organic matter, $CaCO_3$, C, N, and P was analyzed. Non-variability among conditions was detected (**Table 1**). In addition, there was not significant effect of combining T34 and top dressing upon the outcome of these values.

In order to determinate associations between microbiome data and crop traits, parameters such as final crop production, specific weight, and protein and gluten contents were calculated in the grain samples that were harvested from this wheat trial (**Table 2**). No statistical differences were observed for yield values among the four conditions ($P > 0.05$), although compared to control (C1) conditions C2, C3, and C4 tended to increase final production. Gluten values recorded for conditions C2 and C4, both supplemented with CAN top dressing, were significantly higher than those of C1. Regarding grain protein content, significant higher percentages were only observed for CAN top dressing (C2) when compared to C1. A two-way ANOVA statistical analysis of the data from the four crop parameters indicated above showed that the top dressing application increased protein ($P = 0.024$) and gluten ($P = 0.017$) in grain when top dressing-applied and not applied conditions were compared. These parameters were not significantly modified by the T34 application relative to the T34-unapplied conditions, and non-significant changes for these four parameters were detected in the double T34 and top dressing interaction. In terms of micro- and macroelements content in harvested grain (**Table 3**), no significant differences among the tested conditions were detected for N, Ca and the four microelements analyzed. Compared to control (C1), the single application of T34 (C3) significantly increased the Mg and K contents but reduced that of C, while the top dressing application alone (C2) or in combination with T34 (C4) increased the S content. Interestingly, the combined application of T34 and top dressing (C4) decreased the content of P when compared to the single application of T34 (C3). Despite the effect caused by the T34 and top dressing applications on the content of some elements, only the Mg content was significantly affected by the combination of both factors ($P < 0.05$).

Bacterial Microbiome Assembly in Wheat Crop Plants Under Different Conditions

Exploratory Analysis of Bacterial Libraries

We obtained 3,621,101 raw reads for V3–V4 region from the Illumina Miseq of the 36 samples (99.6% of them with Phred score of 20). After filtering the 3,130,161 clean sequences, a total of 2,541,261 high-quality reads were obtained with an average of 70,608 ± 24,328 per sample (**Supplementary Table S1**). The sequence reads were clustered into 5,984 OTUs at 97% identity and, after removing low abundance OTUs, a total of 4,990 OTUs were used to analyze bacterial diversity and composition.

The RDA used to explore the differences across the 36 samples showed that RDA1 (compartment) and RDA2 (condition) explained 48.7% ($P < 0.001$) and 9.6% ($P < 0.001$) variability, respectively. Moreover, the composition of communities was significantly affected by the factor compartment ($P < 0.001$) but not by the factor tested condition ($P = 0.267$). In any case, further bacterial composition analyses allowed to study the effect of the condition factor within each compartment. Considering the degree of bacterial taxonomy prediction, 97% of the OTUs were assigned at phylum level and the percentage was gradually reduced at class (91.9), order (76.8) and family (44.2) level, until it reached 13.6 and 0.24% at genus and species level, respectively.

Diversity of Bacterial Communities

All metrics used to calculate richness and/or biodiversity, including observed OTUs, Shannon index and Faith's PD (**Figure 1A**), exhibited similar trends among the three compartments. Values of Shannon index for bulk soil and rhizosphere samples were >5.5 which was indicative of a moderate-high bacterial diversity, while values of Simpson index were close to 1 for these two compartment samples which indicated dominance of some taxons. Moreover, root endosphere samples pointed out to a moderate diversity (Shannon index: 3 to under 5). A significantly decrease in bacterial alpha-diversity, estimated by Shannon and Faith's PD index, from bulk soil to root endosphere was observed (Kruskal–Wallis test, $P < 0.001$), and there were not differences among conditions ($P > 0.05$). Regarding beta-diversity PCoA, similar PCoA plots were observed using weighted/unweighted UniFrac and Bray-Curtis distance models. All of them revealed significant separation among compartments ($P < 0.001$) but no segregation among tested conditions ($P > 0.9$). The index of weighted UniFrac distance (**Figures 2A,B**) was able to explain the 72.6% variability reached, such distance being only significantly affected by the factor compartment (PERMANOVA, $P < 0.001$; and for all pairwise comparisons post-hoc *Adonis* $P < 0.001$, P adjusted 0.003). Moreover, root endosphere samples displayed the highest dispersion degree.

Composition of Bacterial Communities

Considering the relative abundance of taxonomically assigned OTUs, members of the phyla Proteobacteria (74.3–16.5%) and Actinobacteria (63.1–12.7%) dominated in the 36 samples, followed by Bacteroidetes (18.3–4.8%), Acidobacteria (14.4–0.2%), Gemmatimonadetes (6.9–0.1%) and Chloroflexi (5.2–0.2%). The wide range of these percentages indicates that there is a high variability among the 36 samples. At phylum and family levels, bulk soil and rhizosphere samples showed closer composition patterns than those from root endosphere (**Supplementary Table S3**). Nevertheless, the bacterial composition differed in relative abundance among the three compartments analyzed. Actinobacteria and Proteobacteria were the most relatively abundant bacteria in root endosphere, while Bacteroidetes increased their presence in bulk soil. The relative abundance of Acidobacteria, Gemmatimonadetes, Chloroflexi, Verrucomicrobia, Planctomycetes and Cyanobacteria decreased

from bulk soil to root endosphere. A summary of the genera with assigned names and relative abundance higher than 1% is shown in **Figure 3A**.

When abundance differences among compartments were explored at the genus level by ALDEx2 analysis, 231 taxa showing differential abundance (corrected Kruskal–Wallis,

TABLE 1 | Soil parameters' analysis in samples from a field wheat trial under four different conditions collected at 27 weeks after sowing and 12 days after second top dressing application where corresponded.

Parameters[a]	Conditions[b]						
	C1	C2	C3	C4	P_{T34}[c]	P_{TD}	$P_{T34 \times TD}$
pH	7.81 ± 0.45[a]	7.71 ± 0.83[a]	8.04 ± 0.43[a]	7.07 ± 0.50[a]	ns	ns	ns
$CaCO_3$ (%)	1.05 ± 0.74[a]	1.06 ± 0.92[a]	1.48 ± 0.96[a]	0.27 ± 0.47[a]	ns	ns	ns
C (%)	0.54 ± 0.11[a]	0.56 ± 0.14[a]	0.53 ± 0.17[a]	0.59 ± 0.04[a]	ns	ns	ns
Organic matter (%)	0.93 ± 0.19[a]	0.97 ± 0.25[a]	0.91 ± 0.30[a]	1.02 ± 0.06[a]	ns	ns	ns
N (%)	0.07 ± 0.01[a]	0.06 ± 0.01[a]	0.07 ± 0.01[a]	0.07 ± 0.00[a]	ns	ns	ns
C/N ratio	8.27 ± 1.82[a]	9.00 ± 1.71[a]	7.93 ± 3.15[a]	8.17 ± 0.35[a]	ns	ns	ns
P (ppm)	21.72 ± 8.55[a]	19.06 ± 4.01[a]	25.09 ± 2.65[a]	19.07 ± 6.11[a]	ns	ns	ns

[a]*For each parameter, values are means of three plots by treatment (n = 3). Values in the same row with same letter are not significantly different according to Tukey's test (P > 0.05).* [b]*The four conditions, under high basal chemical N fertilization, were as follow: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, T. harzianum T34 (T34); and C4, T34 plus two applications of calcium nitrate as TD.* [c]*Significant effects were determined by a two-way analysis of variance (ANOVA) for T34, TD, and the interactions between both factors (T34 × TD) (Tukey's test, P < 0.05). ns, no statistical differences.*

TABLE 2 | Agronomic traits of wheat grain harvested from a field trial under four different conditions.

Traits[a]	Conditions[b]						
	C1	C2	C3	C4	P_{T34}[c]	P_{TD}	$P_{T34 \times TD}$
Yield (kg/ha)	6154.0 ± 188.09[a]	6613.0 ± 362.93[a]	7098.5 ± 931.26[a]	6950.3 ± 722.39[a]	ns	ns	ns
Wet gluten (%)	24.30 ± 0.28[b]	27.00 ± 0.82[a]	25.45 ± 0.78[ab]	26.57 ± 1.18[a]	ns	*	ns
Proteins (%)	10.67 ± 0.05[b]	11.28 ± 0.15[a]	10.90 ± 0.16[ab]	11.07 ± 0.29[ab]	ns	*	ns
Specific weight (kg/hl)	80.90 ± 0.57[a]	80.93 ± 0.31[a]	80.80 ± 0.00[a]	81.03 ± 0.32[a]	ns	ns	ns

[a]*For each parameter, values are means of three plots by condition (n = 3). Values in the same row with same letter are not significantly different according to Duncan's test (P > 0.05).* [b]*The four conditions, under high basal chemical N fertilization, were as follows: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, T. harzianum T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD.* [c]*Significant effects were determined by a two-way analysis of variance (ANOVA) for T34, TD, and the interactions between both factors (T34 × TD) (Duncan's test, P < 0.05). ns, no significant differences.*

TABLE 3 | Measurement of micro- and macroelements content on wheat grain harvested from the field trial at 27 weeks after sowing and 12 days after second top dressing application where corresponded.

Elements[a]		Conditions[b]						
		C1	C2	C3	C4	P_{T34}[c]	P_{TD}	$P_{T34 \times TD}$
Macro-elements	C	45.53 ± 0.14[b]	45.59 ± 0.08[b]	45.14 ± 0.12[a]	45.31 ± 0.22[ab]	*	ns	ns
	N	1.85 ± 0.03[a]	1.96 ± 0.02[a]	1.88 ± 0.08[a]	1.96 ± 0.07[a]	ns	*	ns
	Ca	0.44 ± 0.02[a]	0.44 ± 0.02[a]	0.43 ± 0.01[a]	0.44 ± 0.02[a]	ns	ns	ns
	K	3.22 ± 0.11[a]	3.20 ± 0.10[a]	3.51 ± 0.10[b]	3.33 ± 0.15[ab]	*	ns	ns
	Mg	1.20 ± 0.02[a]	1.17 ± 0.01[a]	1.25 ± 0.03[b]	1.16 ± 0.02[a]	ns	*	*
	P	2.51 ± 0.10[ab]	2.49 ± 0.05[ab]	2.75 ± 0.11[b]	2.48 ± 0.13[a]	ns	*	ns
	S	1.47 ± 0.05[a]	1.64 ± 0.01[b]	1.55 ± 0.08[ab]	1.62 ± 0.04[b]	ns	*	ns
Micro-elements	Cu	3.22 ± 0.30[a]	2.91 ± 0.25[a]	3.15 ± 0.13[a]	2.82 ± 0.25[a]	ns	*	ns
	Fe	42.85 ± 21.91[a]	61.79 ± 29–40[a]	45.80 ± 13.72[a]	50.25 ± 17.16[a]	ns	ns	ns
	Mn	22.63 ± 1.12[a]	22.32 ± 2–26[a]	23.43 ± 1.68[a]	22.18 ± 1.82[a]	ns	ns	ns
	Zn	11.30 ± 1.65[a]	11.89 ± 1.11[a]	12.53 ± 1.25[a]	12.32 ± 0.68[a]	ns	ns	ns

[a]*Macroelements: C and N = %; Ca, K, Mg, P, and S = g/kg of grain. Microelements = mg/kg of grain. Data are the mean of three plots for each condition (n = 3). Values in the same row with different letter are significantly different according to Tukey's test (P > 0.05).* [b]*The four conditions, under high basal chemical N fertilization, was as follow: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, T. harzianum T34 (T34); and C4, T34 plus two applications of calcium nitrate as TD.* [c]*Significant effects were determined by a two-way analysis of variance (ANOVA) for T34, TD, and the interactions between both factors (T34 x TD) (Tukey's test, P < 0.05), ns: no statistical differences.*

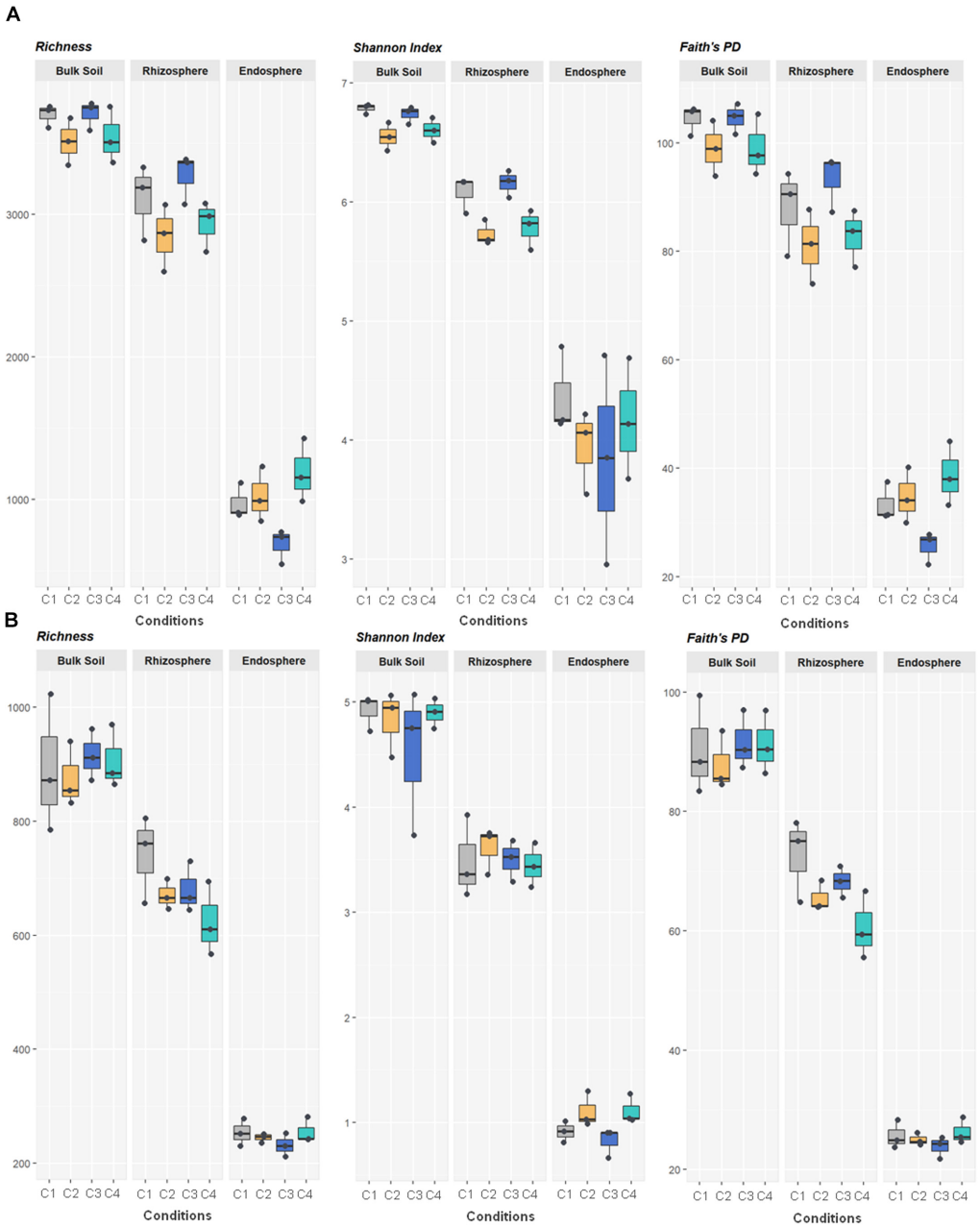

FIGURE 1 | Bacterial **(A)** and fungal **(B)** variety in 36 samples of wheat crop plants under four different conditions, using the total number of OTUs observed (richness) and the indices of Shannon and Faith's Phylogenetic Diversity. The four conditions, under high basal inorganic N fertilization, were as follows: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. Whiskers represent the minimum and maximum values. All other points are contained within the box, and the bar represents the median. For all metrics, bulk soil, rhizosphere and root endosphere samples were significantly separated (Kruskal–Wallis test, $P < 0.001$).

$P < 0.05$) were identified: 195 corresponded to endospore vs. bulk soil; 163 to rhizosphere vs. bulk soil; and 108 to endosphere vs. rhizosphere (**Supplementary Table S4**). Based on the effect size (95% CI), still a larger number of taxa whose abundance differed among compartments were observed. Bearing in mind these pairwise comparison results, it can be pointed out that the levels of genera such as *Pedobacter*, *Janthinobacterium*, *Agrobacterium*, *Flavobacter* or *Chitinophaga* were gradually increased from bulk soil to root endosphere, while *Kaistobacter* followed an opposite direction showing the highest levels in the bulk soil. Results also showed that genera such as *Devosia*, *Rhizobium,* and *Sphingomonas* were increased in rhizosphere and root endosphere. The 20 bacterial taxa with the highest values

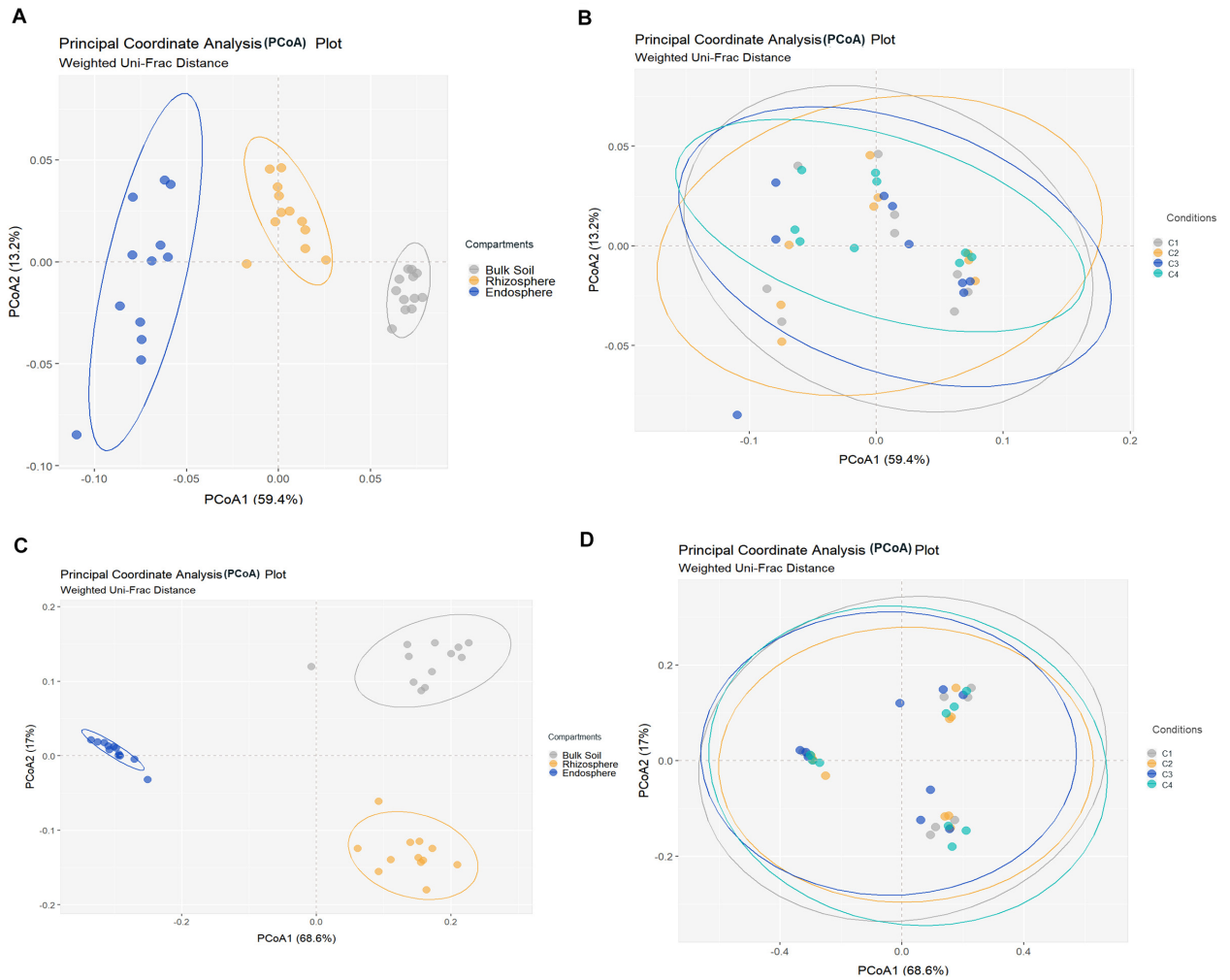

FIGURE 2 | Principal coordinates analysis (PCoA) of bacterial **(A,B)** and fungal **(C,D)** community structures in different samples of wheat crop plants, based on the weighted UniFrac distance model. Bacterial **(A)** and fungal **(C)** PCoAs show significant segregation among bulk soil, rhizosphere and root endosphere samples. Bacterial **(B)** and fungal **(D)** PCoAs show no separation among conditions [C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD] samples. Permutational multivariate PERMANOVA based on distance matrices (Adonis), $P = 0.001$.

of average relative abundance (mean proportions) are presented in **Figure 4A**.

Differential abundance among compartments was also analyzed at the genus level by the LEfSe method, and 41 bacterial taxa showing differences in abundance (LDA > 4, $P < 0.05$) were identified (**Supplementary Table S5**). In order to better understand the changes occurring from phylum to genus, a LEfSe taxonomic cladodram was generated (**Supplementary Figure S2A**). Different considerations could be taken into account for the factor compartment: (i) most changes due to conditions occurred in bulk soil; (ii) the phyla Acidobacteria, Gemmatimonadetes, Chloroflexi, Verrucomicrobia, and Plactomycetes in bulk soil, and Enterobactereriaceae in endosphere, were increased, and (iii) the order Rhizobiales increased in the rhizosphere. At the genus level, 11 taxa showed to be differentially more abundant (LDA > 4, $P < 0.05$) in one of the compartments (**Supplementary Figure S2B**). They were distributed in this way: (i) *Kaistobacter* and one member from each of the following taxa: family Sphingomonadaceae, order Solirubrobacterales and the Acidobacteria order iii1-15, in bulk soil; (ii) one member of order Rhizobiales and another from family Nocardioidaceae, in rhizosphere; and (iii) *Janthinobacterium* and the FCB *Pedobacter*, and three members belonging to the order Actinomycetales and the families Streptomycetaceae and Enterobacteriaceae, in root endosphere.

Although our ALDEx2 and LEfSe analyses performed at the genus level could not identify taxa differing in abundance among conditions through all three compartments, many taxa were identified when such differences were explored within each of the three compartments by ALDEx2 (**Supplementary Table S4**). After pairwise comparisons, between conditions C2, C3, or C4 and C1 (control), differences in abundance (effect

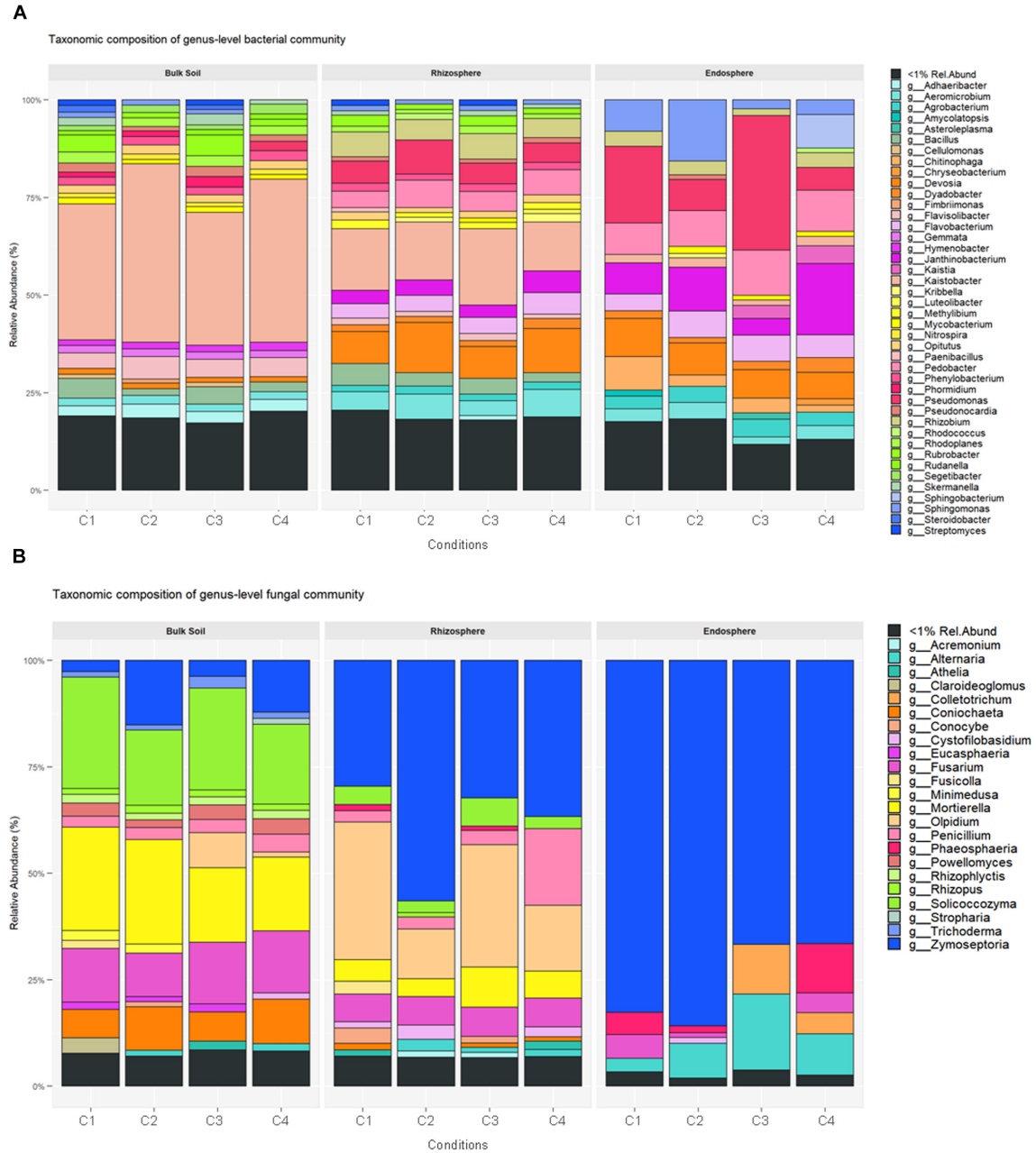

FIGURE 3 | Average of relative abundance of bacteria **(A)** and fungi **(B)** in bulk soil, rhizosphere and root endosphere samples of wheat crop plants under four different conditions [C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD]. Relative abundance at genus level was used for comparisons, and mean value of the three sample replicates for each condition is shown.

size, 95% CI) were detected in 81, 16 and 9 taxa for bulk soil, rhizosphere and root endosphere samples, respectively. As expected, most of these taxa corresponded to genera annotated as unclassified for the three compartments. Results show that most changes associated to conditions occurred in bulk soil. Taxa showing significant differences in abundance among conditions within bulk soil, rhizosphere and root endosphere are respectively included in **Figures 4B–D**. As they were many in bulk soil, only the 20 taxa with the highest values of average relative abundance (mean proportions) were depicted. According to the abundance differences detected in bulk soil: (i) application of the top dressing alone (C2) caused a decrease in the levels of *Streptomyces*, *Cellulomonas*, *Nonomuraea*, *Rubrobacter*, *Haliangium* and *Brevibacillus*, but increased those of *Aeromicrobium*, *Kaistobacter*, *Gemmatimonas*, *Luteolibacter*, *Flavisolibacter,* and *Opitutus*; (ii) no changes were associated to

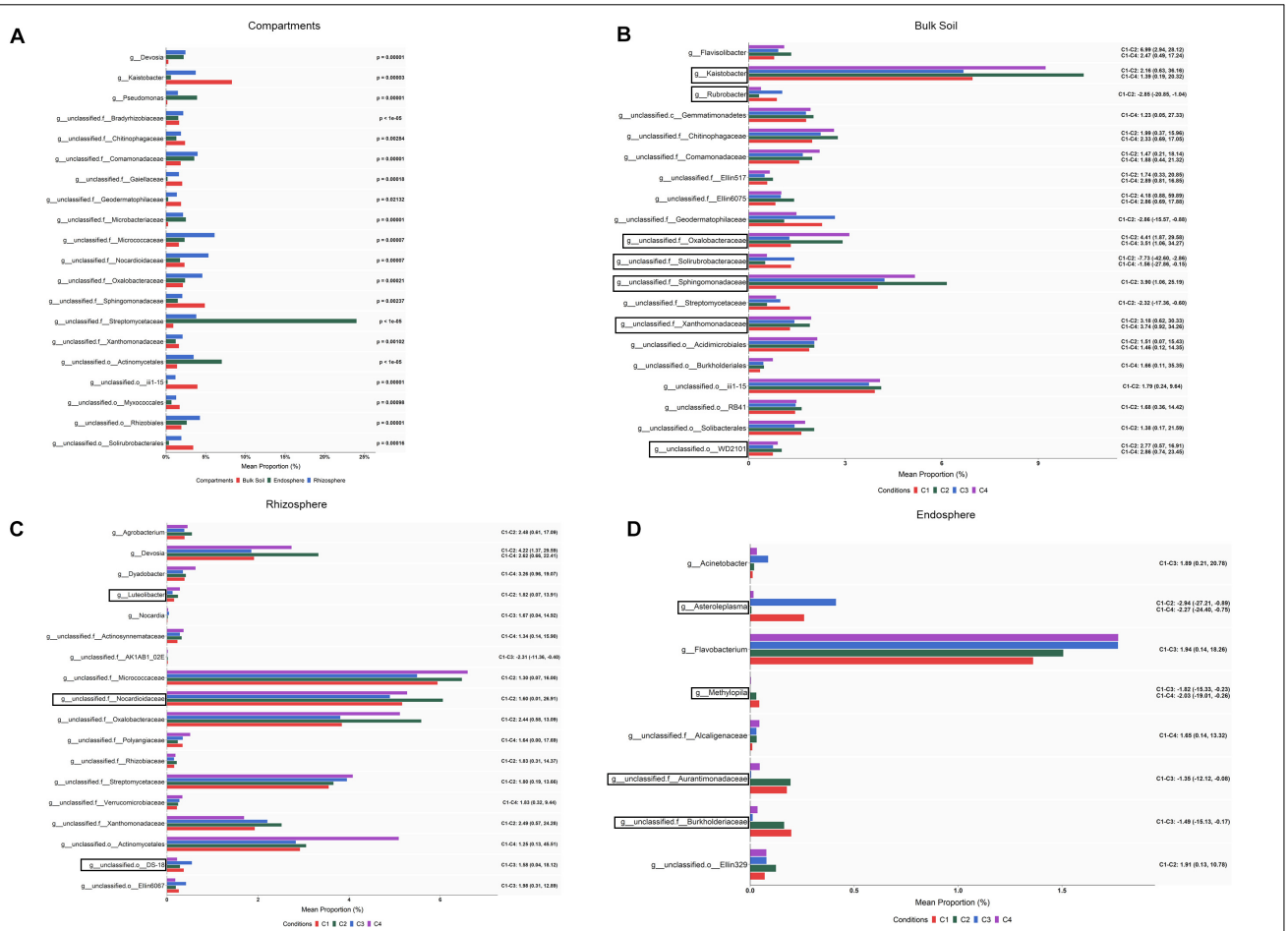

FIGURE 4 | Bacterial taxa showing differential abundance among compartments **(A)** and conditions within bulk soil **(B)**, rhizosphere **(C)** and root endosphere **(D)** of wheat crop plants. Abundance is shown as mean proportion and was calculated by ALDEx2 at the genus level. Significance for compartments is based on corrected Kruskal–Wallis test ($P < 0.05$) and for conditions in effect size (95% CI) ($n = 3$). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. For panels **(A,B)**, only the 20 most abundant taxa are shown. Black boxes refer to the taxa identified by both ALDEx2 and LEfSe analyses. For panels **(B–D)**, significant pairwise comparisons with median effect size values and in brackets their lower and upper limits are indicated. Black boxes refer to the taxa identified by both ALDEx2 and LEfSe analyses.

the single application of strain T34 (C3); and (iii) the combined application of top dressing and strain T34 reduced the levels of *Williamsia*, *Haliangium* and *Steroidobacter*, but increased those of *Kaistobacter*, *Gemmatimonas*, *Luteolibacter*, *Flavisolibacter*, *Janthinobacterium,* and *Lysobacter*. The differences in abundance shared by conditions C2 and C4, for at least five genera, indicate that they are due to top dressing. The rhizospheric levels of *Devosia*, *Luteolibacter,* and *Agrobacterium* in the condition C2, *Nocardia* in C3, and *Dyadobacter* in C4 were significantly increased when compared with those of C1. The fact that increased levels of *Devosia* were also detected in C4 and that a taxa of the order Actynomicetales was only differentially increased in that condition, is an example of the particular effects caused by the combined application of top dressing and strain T34 on the different rhizospheric bacterial taxa (**Figure 4C**). The lowest number of taxa showing changes in abundance associated with conditions was recorded in the root endosphere (**Figure 4D**). Compared to the control condition, the endosphere changes showed that the single application of strain T34 (C3) increases the levels of *Acinetobacter* and when applied in combination with top dressing (C4) reduces *Methylopila* and *Asteroleplasma*, although lower levels of the latter genus were also observed when top dressing is applied alone (C2) (**Figure 4D**).

When the category condition was explored by LEfSe for each compartment (LDA > 2, $P < 0.05$), a total of 87, 27 and 21 taxa showing differential abundance in bulk soil, rhizosphere and endosphere, respectively, were identified (**Figures 5**, **6**). Changes occurring from phylum to genus are shown in cladograms (**Figures 5A**, **6A,C**), and taxa identified at the genus level according to their differential abundance among conditions are separately displayed (**Figures 5B**, **6B,D**). For bulk soil, in addition to several unclassified genus taxa (**Figure 5B**), increased levels of the genera *Bacillus*, *Catellatospora* and *Virgisporangium* in condition C1, *Kaistobacter* in C2, and *Rubrobacter*, *Streptosporangium,* and *Haliangium* in C3, were detected. The analysis of rhizosphere taxa identified that

the abundance of 12 of them was affected by the factor condition (**Figure 6B**), with increases for *Paenibacillus* in C1, *Asteroleplasma* in C3 and *Luteolibacter* in C4. In the endosphere, *Methylopila* in C1 and *Asteroleplasma* in C3 were two of the eight taxa with increased levels detected under any of the conditions (**Figure 6D**).

Fungal Microbiome Assembly in Wheat Crop Plants Under Different Conditions

Exploratory Analysis of Fungal Libraries

We obtained 4,019,719 raw reads for the ITS2 region from the Illumina Miseq of the 36 samples (98.5% of them with Phred score of 20). After filtering the 3,397,598 clean sequences, a total of 3,386,201 high-quality reads were obtained with an average of 94,061 ± 15,261 per sample (**Supplementary Table S2**). The sequence reads were clustered into 3,497 OTUs at 97% identity and, after removing low abundance OTUs, a total of 2,056 OTUs were used to analyze fungal diversity and composition.

The RDA used to explore the differences across the 36 fungal libraries showed that compartment and condition variables explained 48.7% ($P < 0.001$) and 9.6% ($P < 0.001$) variability, respectively. As observed for bacterial samples, the separation of fungal ones was affected by the factor compartment ($P < 0.001$) but not by the factor condition ($P = 0.11$). Subsequent fungal composition analyses allowed to study the effect of the condition factor within each compartment. It can be pointed out that the predicted degree of the reached fungal taxonomy was low. At phylum level, only 26.4% of the OTUs were assigned, and the percentage decreased at class (19.7%), order (17.2%), family (11.4%), genus (6.91%), and species (1.8%) levels.

Diversity of Fungal Communities

Fungal richness and alpha-diversity obtained across the 36 samples (**Figure 1B**) were only significantly affected by the factor compartment (Kruskal–Wallis test, $P < 0.001$). The lowest richness (total observed OTUs, Chao1, ACE) corresponded to root endosphere samples. Regarding estimated alpha-diversity, three groups were separated by Shannon index values of ca. 1 (root endosphere), above 3 (rhizosphere), and close to 5 (bulk soil), being indicative of low, low-moderate and moderate diversity, respectively (**Figure 1B**). There was taxa dominance for bulk soil and rhizosphere samples, as supported by Simpson index values close to 1, and this did not occur in root endosphere samples. Similar results were obtained for the beta-diversity estimated by weighted/unweighted UniFrac and Bray-Curtis distance models, confirming that the variable compartment was a significant factor for the spatial separation of the 36 samples in the three groups. The most discriminative PCoA plot was observed for the distance model weighted UniFrac (**Figures 2C,D**) that explained the reached 85.6% variability, of which 68.6% was due to the component compartment (PERMANOVA, $P < 0.001$; and for all pairwise comparisons *post hoc Adonis* $P < 0.001$, *P* adjusted 0.003). Moreover, root endosphere samples showed the lowest dispersion degree within a given group (root endosphere vs. bulk soil, $P = 2.993\ 10^{-10}$; and root endosphere vs. rhizosphere, $P = 1.580\ 10^{-7}$).

Composition of Fungal Communities

The relative abundance calculated at the different taxonomy levels led to a picture of compositional structure extremely uneven for the three compartments concerned. A total of 46 phyla with a relative abundance > 1% showed differences among samples, where the seven most abundant predicted phyla were Ascomycota, Basidiomycota, Olpidiomycota, Chytridiomycota, Mortierellomycota, Glomeromycota and Mucoromycota (**Supplementary Table S6**). However, their relative abundance differed for the three compartments and ranged as follow: Ascomycota in bulk soil (39.04–20.5%), rhizosphere (35.33–18.1%), and root endosphere (7.48–1.43%); and Basidiomycota in rhizosphere (14.52–4.07%), bulk soil (9.12–3.2%), and root endosphere (0.94–0.02%) samples. Moreover, the phyla Mortierellomycota and Olpidiomycota were increased in bulk soil and rhizosphere samples, respectively. The fungal genera with assigned name and relative abundance higher than 1% are presented in **Figure 3B**.

An ALDEx2 analysis of the differences in abundance at the genus level among compartments identified changes (corrected Kruskal–Wallis, $P < 0.05$) in 64 fungal taxa: 51 corresponded to endosphere vs. bulk soil, 31 to endosphere vs. rhizosphere, and 46 to rhizosphere vs. bulk soil (**Supplementary Table S7**). Lower numbers of taxa whose abundance differed among compartments were detected considering the effect size. Results (effect size, 95% CI) showed that the levels of *Solicoccozyma*, *Mortierella*, *Eucasphaeria*, *Rhizopus*, *Powellomyces*, *Coniochaeta*, *Rhizophlyctis,* and *Trichoderma* decreased from bulk soil to rhizosphere, and many of these were not present in root endosphere. The 20 differential taxa showing the highest percentages of average relative abundance, calculated as mean proportions, are shown in **Figure 7A**. It can be also observed that the levels of genera such as *Olpidium*, *Penicillium,* and *Zymoseptoria* were increased in rhizosphere, and those of *Fusarium* in bulk soil.

The LEfSe-based differential abundance of fungi was also explored at the genus level among compartments and a total of 41 fungal taxa showing differences (LDA > 4, $P < 0.001$) was identified (**Supplementary Table S8** and **Supplementary Figure S3**). Changes from phylum to genus among compartments are represented in a taxonomic cladogram (**Supplementary Figure S3A**). The genera *Solicoccozyma* and *Mortierella* as well as one member from each of the following taxa: phylum Ascomycota, order Hypocreales and class Sordariomycetes, were more abundant in bulk soil, while *Olpidium* and a member of phylum Basidiomycota were more abundant in rhizosphere.

When differences in abundance were investigated by ALDEx2 at the genus level within each of the three compartments, several taxa were identified (**Supplementary Table S7**). After pairwise comparing each condition with the control (C1), 29, 13 and one taxa presented differential abundance (effect size, 95% CI) among some of the compared conditions in bulk soil, rhizosphere and root endosphere, respectively (**Figures 7B–D**). In the case of bulk soil taxa, only 20 with the highest abundance are shown in **Figure 7B**. According to the differences detected in bulk soil (effect size, 95% CI) for genera with assigned name (**Figure 7B**),

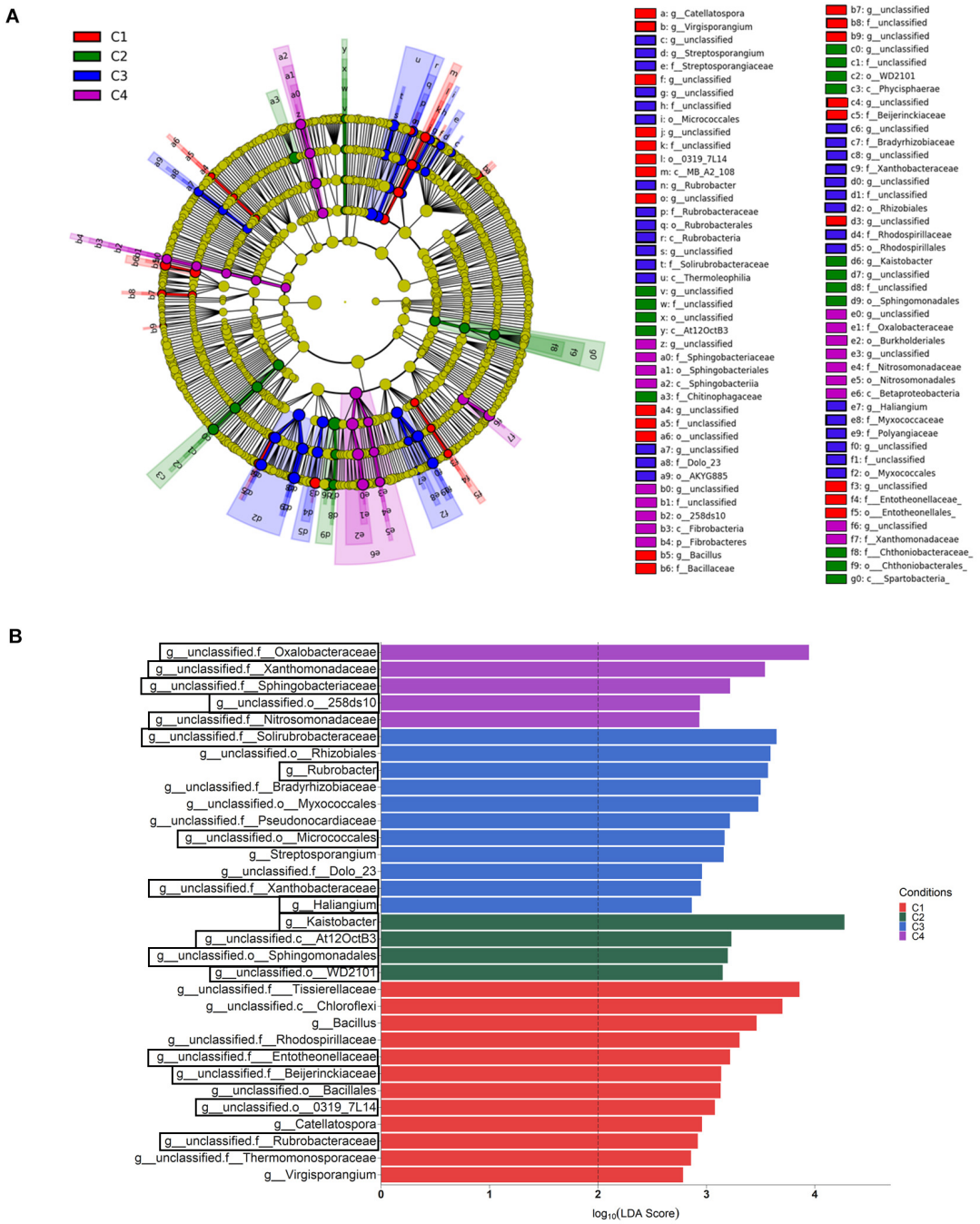

FIGURE 5 | Bulk soil bacterial taxa overrepresented in the four tested conditions on wheat crop plants after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and bacterial taxa with LDA > 2 ($P < 0.05$) **(B)** ($n = 3$). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

it can be deduced that: (i) the single application of top dressing (C2) caused a decrease of *Claroideoglomus* and *Geminibasidium* but also an increase of *Conocybe* and *Zymoseptoria* levels; (ii) the single application of strain T34 (C3) reduced the levels of *Claroideoglomus*, *Fusicolla* and *Holtermanniella*, and increased those of *Trichoderma*; and (iii) the combined application of top dressing and strain T34 (C4) also reduced the levels of *Claroideoglomus*, *Fusicolla*, *Geminibasidium*, *Holtermanniella*, *Minimedusa* and *Striatibotrys*, with *Alternaria* being increased. Lower and higher rhizospheric levels of *Trichoderma* and

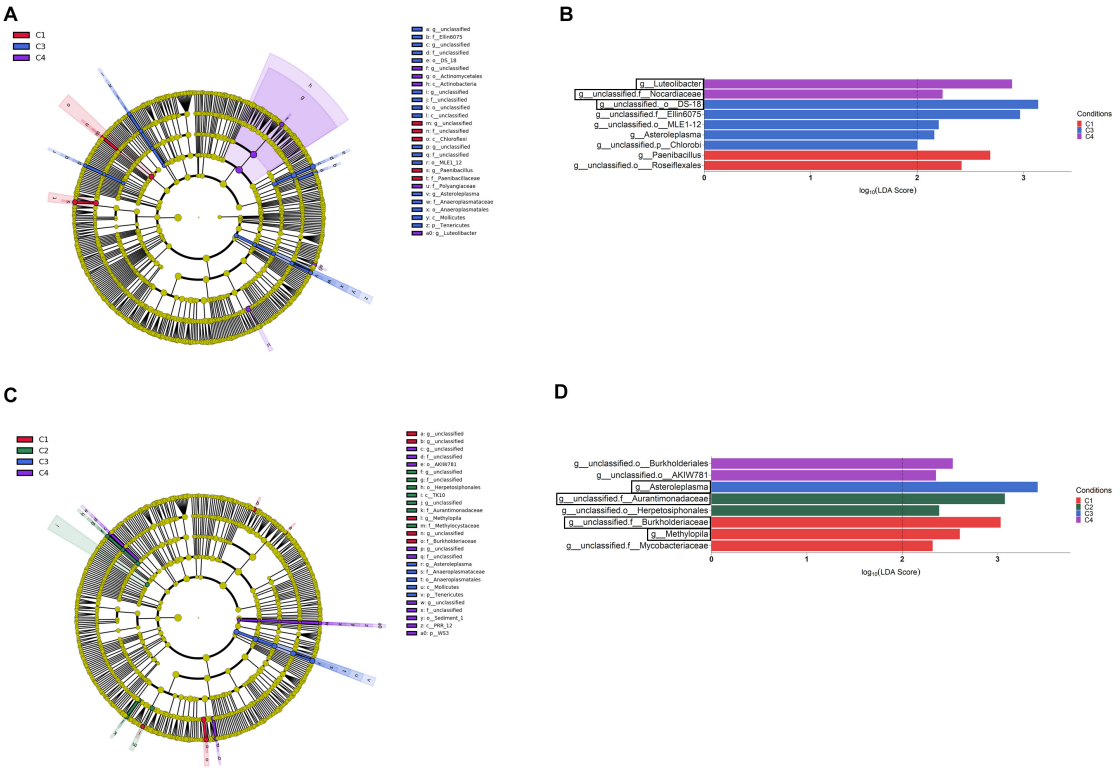

FIGURE 6 | Rhizosphere **(A,B)** and root endosphere **(C,D)** bacterial taxa overrepresented in the four tested conditions on wheat crop plants after LEfSe analysis at the genus level. Taxonomic cladograms showing differences from phylum to genus level in the rhizosphere **(A)** and the root endosphere **(C)**, and bacterial taxa with LDA > 2 ($P < 0.05$) in the rhizosphere **(B)** and the root endosphere **(D)** ($n = 3$). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. In cladograms, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In barplots, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Udeniomyces were respectively detected in C2 (**Figure 7C**). As *Trichoderma* was also decreased in the condition C4, such reduction can be associated with the application of top dressing. In the root endosphere (**Figure 7D**), only significant changes were observed for the yeast *Sporobolomyces,* which levels were raised by the single application of top dressing.

The LEfSe analysis at the genus level among conditions (LDA > 2, $P < 0.05$) let us to identify a total of four, five and one taxa showing differential abundance in bulk soil, rhizosphere and root endosphere, respectively (**Figures 8A–F**). Considering the differences observed in genera with assigned name, it can be established that: (i) the levels of *Claroideoglomus* in bulk soil, and *Itersonilia* and *Trichoderma* in rhizosphere were negatively affected by any of the three treatments assayed; (ii) the single application of strain T34 increased the rhizospheric levels of *Gymnoascus* and *Claroideoglomus*; and (iii) the single application of top dressing increased the levels of *Mortierella* in the root endosphere.

DISCUSSION

A wheat microbiome study was performed in a non-irrigated field trial, under the conventional agronomic practices for this crop in Spain, to explore the influence of inorganic N top dressing, *T. harzianum* T34 and their combination on root microbial community shaping and production traits. Yield results showed that nor did the application of top dressing or strain T34 influence the crop yield. A recent study has reported that low N fertilization increases sweet potato yield, whereas high N fertilization inhibits biological N fixation and produces unintended environmental consequences (Ding et al., 2020). We have seen that the effect of the T34 strain upon the growth of wheat plants is significantly determined by the concentration of chemical N fertilizer (Rubio et al., 2019). Thus, we should not rule out the fact that the basal chemical N dosage (240 kg/ha) could have been so high that led to not improvement in the crop yield for neither top dressing application nor strain T34 seed-inoculation. Even though the absence of a N basal fertilization condition might be considered as a flaw in the experimental design, it is worth noting that the application of high basal N fertilization is a very common practice in wheat intensive production in the region where the trial took place, so the possibility of following the conventional agronomic practices was considered the best and true to customs approach. Soil parameters analyses, including N (%), did not show differences among conditions (**Table 1**). Although we cannot exclude the possibility that part of the N applied as top dressing could be

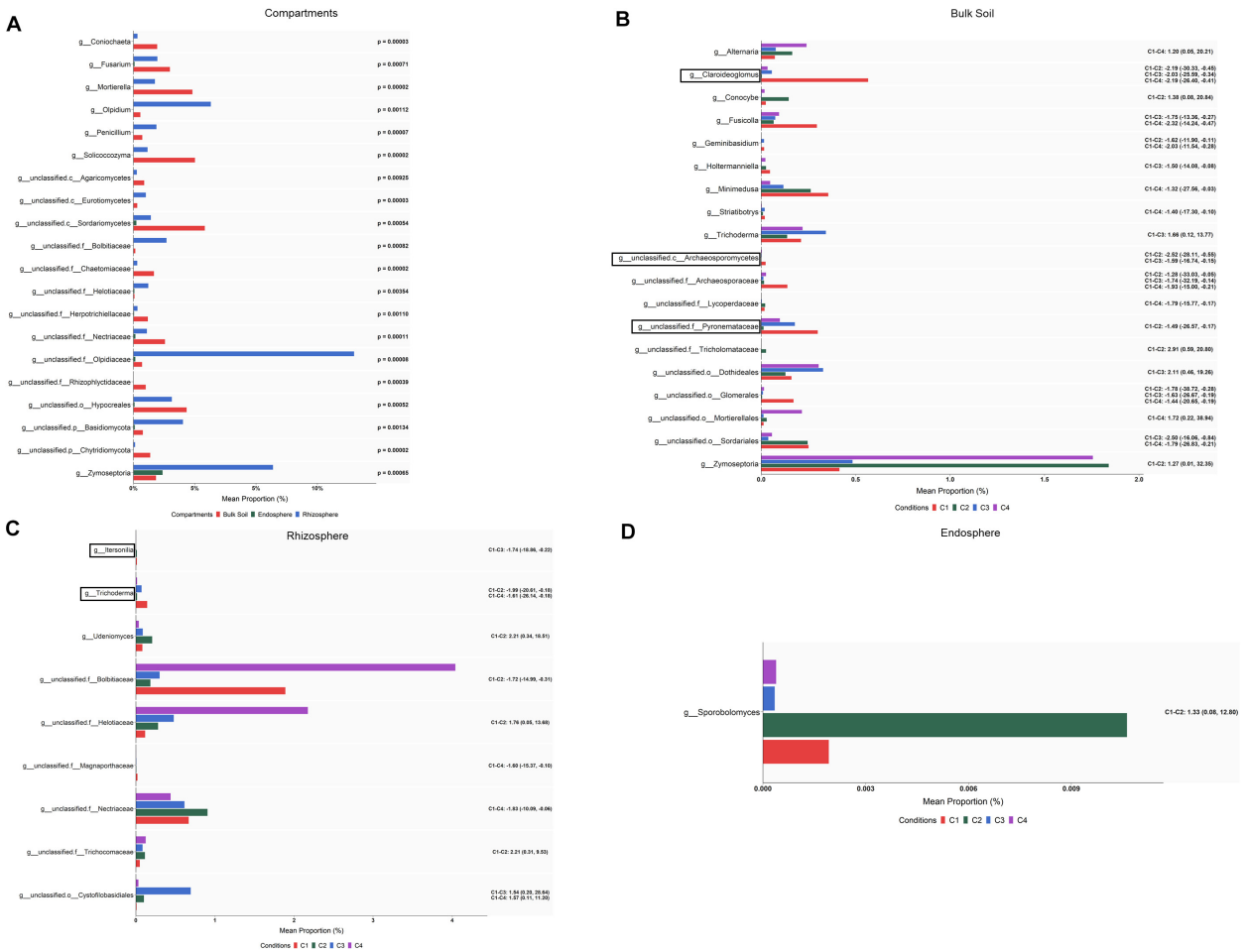

FIGURE 7 | Fungal taxa showing differential abundance among compartments **(A)** and conditions within bulk soil **(B)**, rhizosphere **(C),** and root endosphere **(D)** of wheat crop plants. Abundance is shown as mean proportion and was calculated by ALDEx2 at the genus level. Significance for compartments is based on corrected Kruskal–Wallis test ($P < 0.05$) and for conditions in effect size (95% CI) ($n = 3$). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. For panels **(A,B)**, only the 20 most abundant taxa are shown. For panels **(B–D)**, significant pairwise comparisons with median effect size values and in brackets their lower and upper limits are indicated. Black boxes refer to the taxa identified by both ALDEx2 and LEfSe analyses.

lost, other part of that N could be uptaken by plants. This statement is based on the positive effects of top dressing on grain gluten and protein contents detected for the conditions C2 and C4 (**Table 2**). In this sense, a two-way ANOVA showed that the N content of grains, as well as that of Mg, P, S, and Cu, were significantly increased by CAN top dressing application (**Table 3**), demonstrating in any case the practical value of this treatment. Particularly, the grain gluten content is a highly valuable quality parameter by the flour industry. However, several considerations linked to the use of chemical fertilization on wheat crop should be taken into account as chemical N fertilization costs are high and N is one of the major inputs for intensive production. It is well known that the unused N by plants ends up polluting the environment, and so the adjustment of chemical N fertilizer dosages or even the replacement with biofertilizers are needed goals. Either way, there is still a lack of knowledge upon the use of beneficial organisms such as *Trichoderma* on wheat crops and their effects on fertilization (Meena et al., 2016; Mahato et al., 2018).

Many microbiome studies in wheat cropping systems have been focused on bacterial communities (Donn et al., 2015; Robinson et al., 2015; Rascovan et al., 2016; Kavamura et al., 2018), but the most recent ones include both bacterial and fungal analyses (Friberg et al., 2019; Schlatter et al., 2019; Rossmann et al., 2020). Our results show an overall different microbial layout in the three compartments analyzed and, for bacterial and fungal communities, the differences involving richness, diversity and relative composition. All comparison analyses performed across the 36 bacterial and the 36 fungal samples showed that only the factor compartment explained their separation in three groups corresponding to bulk soil, rhizosphere and root endosphere ($P < 0.001$). In accordance with this premise, the effect of the condition factor on the composition of the microbial communities within each compartment was analyzed.

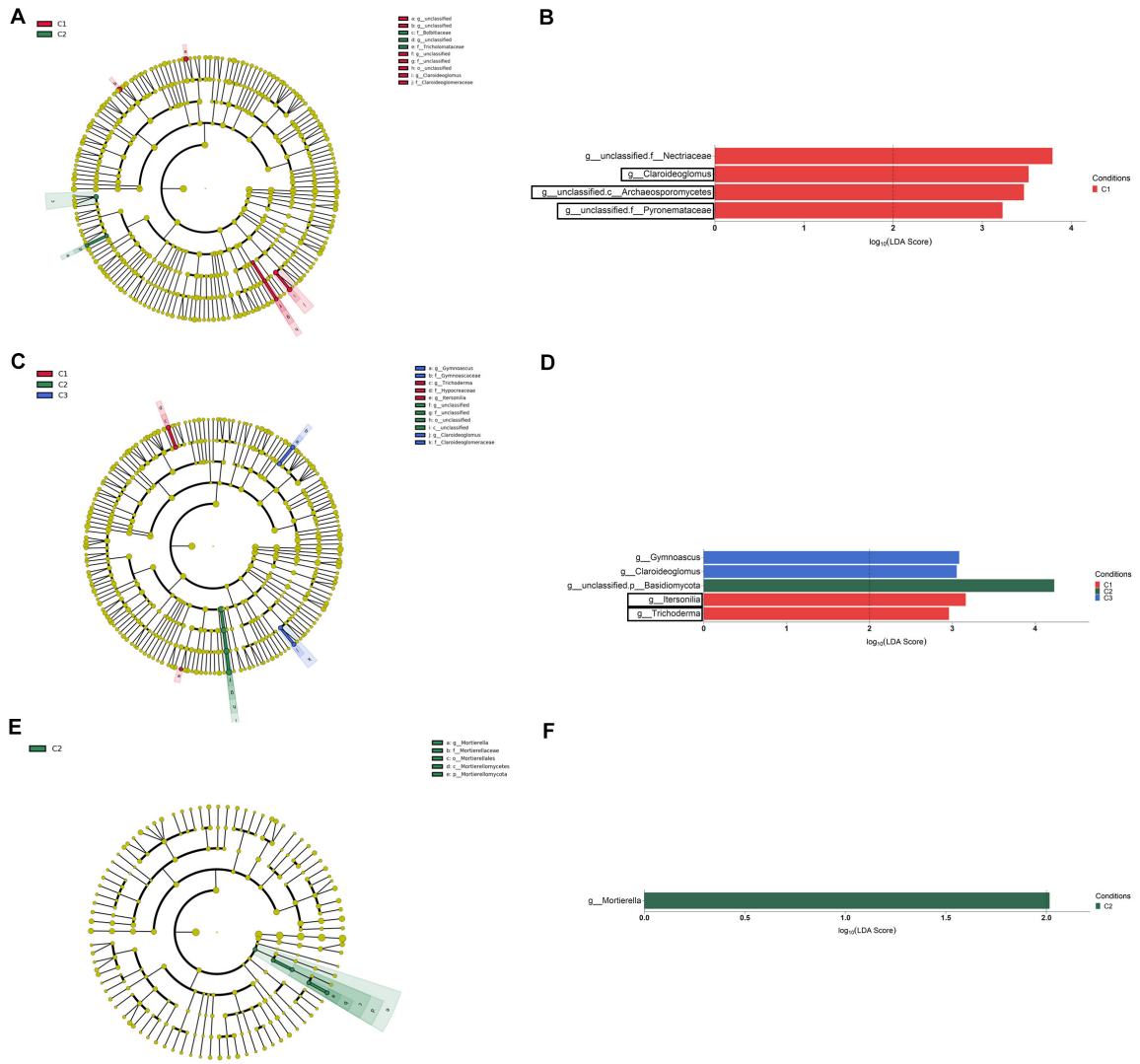

FIGURE 8 | Bulk soil **(A,B)**, rhizosphere **(C,D)**, and root endosphere **(E,F)** fungal taxa overrepresented in the four tested conditions on wheat crop plants after LEfSe analysis at the genus level. Taxonomic cladograms showing differences from phylum to genus level in bulk soil **(A)**, rhizosphere **(C)**, and root endosphere (**E**), and fungal taxa with LDA > 2 ($P < 0.05$) in bulk soil **(B)**, rhizosphere **(D)**, and the root endosphere **(F)** ($n = 3$). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. In cladograms, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In barplots, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Our results are in agreement with the description of the existence of at least three distinct microbiomes thriving at the root-soil interface (Bulgarelli et al., 2012; Hirsch and Mauchline, 2012). Based on Shannon index estimations, we have observed that the bacterial diversity within each compartment was always higher than that recorded for fungi. We have also observed a decrease of bacterial and fungal diversity from bulk soil to root endosphere of wheat plants, as previously described in microbiome studies from different plants and crop systems (Yamamoto et al., 2018; Ding et al., 2020). The microbial diversity differences detected in wheat plants were accompanied by different composition pictures in relative abundance at the different taxonomical levels investigated (phylum, family, and genus) in the three microhabitats. These observations are in agreement with previous reports that indicate the major role of plants in shaping the composition of each compartment (Vandenkoornhuyse et al., 2015; D'Amico et al., 2018; Rossmann et al., 2020) which should be considered separately.

Our study shows that the bacterial dominant taxa within each compartment were the phyla Proteobacteria and Actinobacteria and that other phyla present across samples differed in relative abundance among compartments. A dominance of Proteobacteria, Acidobacteria and Actinobacteria has been observed in rhizosphere of landraces and modern varieties of wheat (Rossmann et al., 2020). Our data indicate an enrichment in Proteobacteria, Bacterioidetes

and Actinobacteria in root endosphere samples. A similar behavior has been found in the root endosphere of grapevines (D'Amico et al., 2018). Results also show a decrease in Acidobacteria, Gemmatimonadetes, Chloroflexi, Verrucomicrobia, Planctomycetes and Cyanobacteria from bulk soil to root endosphere. This should come as no surprise, since the increased number of microbiome studies available (Kavamura et al., 2020; Rossmann et al., 2020) suggests that the particular conditions of every study impact on the microbial communities outcome.

After ALDEx2 and LEfSe analyses, our study discriminated bacterial genera with tropism toward wheat microecosystems. We have seen that *Kaistobacter* was significantly increased in bulk soil, whereas *Flavobacterium*, *Rhizobium* or *Devosia* were overrepresented in the rhizosphere, and *Sphingomonas* in rhizosphere and root endosphere. *Kaistobacter* has been described as one of the most abundant bacterial genera in soil globally (Delgado-Vaquerizo et al., 2018), including those from wheat crops (Schlatter et al., 2019; Zhou et al., 2020). It is not surprising the abundance of *Rhizobium* and *Devosia* close to the root system as they are rhizobacteria with a symbiotic lifestyle with plants (Zhou et al., 2020). In addition, it has been reported the use of antagonistic *Sphingomonas* for the biological control of wheat pathogens (Wachowska et al., 2013). In this sense, diseased wheat was not observed in our field trial. The inclusion of a fallow period of 1 year between barley and wheat crop seasons could have had a positive effect on the maintenance of the plant health status.

Although no significant differences in bacterial abundance were detected among the four conditions when the whole set of samples was compared, there were bacterial taxa showing differential abundance when pairwise comparisons of conditions within a given compartment were performed by ALDEx2, which also includes possibility of doing effect size and significance testing to identify features that are different between groups (Fernandes et al., 2013). Additionally, overrepresented bacterial genera in one of the tested conditions in each compartment were detected in a LEfSe analysis. Considering both approaches, the largest number of differentially abundant taxa was recorded in bulk soil (17 genera) while in the rhizosphere and root endosphere there were three and four, respectively. In general, many differential taxa were recorded as unclassified genus and this is clearly because the generic boundaries in many soil-borne microbes with importance in agriculture (i.e., plant pathogens, biocontrol agents) are still poorly defined. Most of the changes observed in bulk soil were due to the application of CAN top dressing alone or in combination with strain T34. In this sense, the use of CAN top dressing seems to reduce the levels of Actinobacteria but it also increases the levels of *Kaistobacter* together with FCB and PVC bacteria. Although the abundance levels of many taxa were similar for the conditions C2 and C4 (i.e., *Flavisolibacter*, *Kaistobacter*) (**Figure 4B**), some of them showed no significant differences for the C1–C4 pairwise comparison (i.e., *Rubrobacter*, g_unclassified.f_Sphingomonadaceae), as a result of the enormous variability shown by samples of the C4 condition. ALDEx2 results showed that the application of strain T34 (C3) did not lead to significant changes of bacterial genera in bulk soil compared to the control (C1). This is a confirmation of harmlessness of the use of *Trichoderma* against soil bacteria. Nevertheless, the application of strain T34 was associated by a LEfSe analysis to increased levels of 11 taxa including members of Rhizobiales and Actinobacteria, but also with a reduction of 12 taxa among which are other members of Actinobacteria and *Bacillus* (**Figure 5B**). In any case, such reductions are not exclusive to apply strain T34 since they can also be associated to the implementation of CAN. Similar to what was observed in the bulk soil, most of the changes detected by ALDEx2 in the rhizosphere are associated with the CAN top dressing and the absence of significance of some taxa in C4, with levels similar to those of C2, may be due to the high abundance variability of the C4 samples (**Figure 4C**, i.e., *Agrobacterium*). The progressive increase of rhizobia toward the wheat rhizosphere seems to be helped by the application of CAN top dressing. Contrary to what happens in bulk soil, strain T34 applied alone or combined with CAN top dressing seemed to affect the abundance levels of some taxa, although some of the increases, such as those of *Dyadobacter* and a member of Actinomycetales, would be consequence of the combination of both treatments.

It has been reported that inorganic N fertilization can negatively affect wheat rhizosphere bacterial communities (Kavamura et al., 2018), but we have detected that CAN top dressing application was associated with increased levels of *Kaistobacter*, *Gemmatimonas, Flavisolibacter* or *Aeromicrobium* in bulk soil, and *Agrobacterium*, *Devosia* and *Luteolibacter* in rhizosphere. However, similarly to Kavamura et al. (2018), we observed for the condition C2 a reduction in the levels of several genera of bacteria (*Brevibacillus*, *Cellulomonas*, *Rubrobacter*, *Streptomyces,* or *Haliangium*) in bulk soil. Many of these top dressing-impacted genera contain plant beneficial microorganisms used as biocontrol agents and biofertilizers (Bargaz et al., 2018; Begum et al., 2019). Interestingly, the increased levels of biocontrol agents in bulk soil, such as *Rubrobacter*, *Streptosporangium,* and *Haliangium*, were negatively affected by CAN top dressing, and they could be associated with the application of strain T34. As a positive feature of the use of strain T34, it should be noted that the C2-negatively affected strict anaerobic mollicutes *Asteroleplasma* was also favored in its colonization of rhizosphere and endosphere by the application of *Trichoderma*.

Our fungal approach indicates that Ascomycota and Basidiomycota were the most frequent phyla in the wheat microbiome. Although relative abundance data also indicate that Olpidiomycota was the phylum significantly increased in rhizosphere, Ascomycota was overrepresented in root endosphere, and Mortierellomycota and Chytridiomycota were overrepresented in bulk soil. Interestingly, the phylum Chytridiomycota was not present in root endosphere samples and the phylum Glomeromycota was only detected in one of the endosphere samples as it would be expected for AMF and zoospore-forming fungi. A recent study has reported that fungal communities of the wheat rhizosphere are dominated by Ascomycota, followed by Chytridiomycota and Basidiomycota (Rossmann et al., 2020), and it has been reported that saprophytic fungal genera are frequent in the rhizosphere of different crops,

including wheat, and that root pathogens are abundant in the wheat rhizosphere (Schlatter et al., 2019). We have observed from ALDEx2 analysis performed at the genus level that saprophytic fungi such as *Mortierella, Solicoccozyma* and *Trichoderma* are overrepresented in bulk soil, and the pathogenic *Zymoseptoria* in the rhizosphere. Likewise, *Fusarium* was also increased in bulk soil and even though some species of *Fusarium* are pathogenic to wheat, the absence of disease in our field assay would be supporting that an important amount of the detected fusaria could be not detrimental or even beneficial for the crop. In addition, the absence of disease could be a consequence of the observed presence of bacteria and fungi with potential activity of biocontrol, as occurs with *Kaistobacter*, *Streptomyces*, *Pseudomonas, Sphingomonas,* and *Trichoderma* (Jung et al., 2013; Liu et al., 2016; Mehrabi et al., 2016; Rubio et al., 2019). Moreover, as indicated above, such absence could be also related to the cultivation history of the experimental field, since the wheat crop was preceded by fallow land. Our data show a comprehensive picture of the significant impact of factor compartment in the relative abundance of fungal taxa observed in wheat, it being also in agreement with the idea of the plant modulating microbial communities assemblage (Bulgarelli et al., 2012; Hirsch and Mauchline, 2012). As it could be expected for a field trial performed under high basal N fertilization, *Mortierella* and *Solicoccozyma*, were amongst the genera with increased abundance in bulk soil detected by LEfSe, and they were previously associated to soil subjected to chemical N fertilization (Ding et al., 2020).

As in the bacterial study, there were fungal taxa showing differential abundance within a given compartment after ALDEx2 analysis and the largest number of taxa with differential abundance was found in bulk soil. Contrary to that observed in the bacterial analysis, several fungal taxa were identified as differentially abundant in the condition C3, this indicating that fungi are affected in a greater extent than bacteria by the application of strain T34. Our results are indicative of the enormous variability of soil fungal systems and their dependence on the treatment applied. In this sense, the changes in abundance observed for several taxa in bulk soil and rhizosphere seem to be a consequence of the applications of CAN top dressing (i.e., *Conocybe*, *Zymoseptoria*), strain T34 (*Trichoderma*) or their combination (*Alternaria,* unclassified.o_Mortierellales). Particularly, *Conocybe* and *Alternaria* have been proposed as bioindicators of intensive crop soils subjected to N fertilization (Schöps et al., 2018). Our results show that the strain T34 increased the levels of the *Trichoderma* genus in bulk soil while the single or T34-combined application of CAN top dressing reduced those levels in the rhizosphere. In this sense, several reports have shown that microbial communities of different crops are affected by the introduction of a *Trichoderma* strain (Umadevi et al., 2017; Schöps et al., 2018; Singh et al., 2018) but the opposite has been also described (Ganuza et al., 2019; Wang et al., 2019), illustrating that the increased availability of nutrients is not the sole mechanism which explains this fact. In other way, it is well known that the use of AMF inoculants increases the production of many crops, including wheat under drought stress conditions (Begum et al., 2019), and we have previously reported that strain T34 facilitates the access of AMF to non-host Brassicaceae *Arabidopsis* and rapeseed roots, with increased production (Poveda et al., 2019). Moreover, the AMF *Claroideoglomus* has been described as an abundant genus in the wheat rhizosphere under intensive chemical N fertilization (Sommermann et al., 2018). Our study shows that *Claroideoglomus* levels in bulk soil were negatively affected by the application of CAN top dressing, strain T34 or both. However, the single application of T34 favored the presence of *Claroideoglomus* in the rhizosphere. Thus, the trophic dependencies derived from N fertilization and *Trichoderma* application (Rubio et al., 2019) impact on the AMF abundance in wheat bulk soil and rhizosphere microbiome. Two beneficial fungi such as the yeast *Sporobolomyces* and *Mortierella* were increased in the endosphere by CAN top dressing application. This result was to be expected since *Mortierella* is considered a root-associated fungal metacommunity hub (Wani et al., 2017) proposed as soil fungal bioindicator after chemical N fertilization (Ding et al., 2020) and it has been reported that CAN supports the growth of *Sporobolomyces* when colonizing wheat plants (Frossard et al., 1983).

CONCLUSION

Although the factor top dressing increased wheat quality parameters, neither CAN applications nor seed-inoculated strain T34 impacted the crop yield in our field trial. The significant differences observed in bacterial and fungal richness, diversity and relative composition among bulk soil, rhizosphere and root endosphere show a specific trophic behavior in these three wheat microhabitats. Bulk soil overrepresented bacterial and fungal genera here recorded are microbes associated to soils with an abuse of chemical N fertilization history, and most changes in microbial abundance associated to conditions occurred in this compartment. The single or strain T34-combined application of CAN top dressing affected to a greater extent the bulk soil bacterial levels than the use of T34 alone. When combined, CAN top dressing played a more decisive role in the bacterial microbiome of wheat than strain T34. Particularly, the three tested treatments reduced the levels of the AMF *Claroideoglomus* in bulk soil, although strain T34 increased the rhizhosphere abundance of this mycorrhizal fungus as well as that of plant beneficial rhizobacteria. The fungal microbiome of wheat bulk soil and rhizosphere was notably affected and to varying degrees by any of the three treatments assayed. Interestingly, bacterial and fungal genera negatively affected by CAN top dressing were increased in their bulk soil and rhizosphere levels after the implementation of strain T34. The results obtained can provide the basis for future trials using lower doses of inorganic N fertilization aimed at favoring a specific microbiome able to allow acceptable agronomic traits with less environmental impact. Further studies focused on isolating potential biofertilizers from the wheat rhizosphere to be used as synthetic communities to favor microbiota recruitment in exhausted crop soils are considered important. The application of microbial synthetic communities particularly selected for wheat cropping could help

to modulate root microbiomes in order to sustain plant health and consequently productivity.

DATA AVAILABILITY STATEMENT

The raw sequencing data (16S and ITS rRNA gene fastq files) are publicly available in the NCBI Sequence Read Archive (SRA), Bioproject PRJNA639567 (bacterial libraries, run numbers SRR12018013 to SSR12018048; and fungal libraries, SSR12023978 to SSR12024013).

AUTHOR CONTRIBUTIONS

RH and EM conceived the research. MI, MR, VH-R, CN, and RH performed the experiments. MI, MR, MM-D, EM, and RH analyzed the data. MI, MM-D, and AM prepared the tables and figures. EM and RH contributed to reagents, materials, and analysis tools. EM and RH wrote the manuscript with all authors contributing to the discussion of the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.575861/full#supplementary-material

Supplementary Figure 1 | Schematic diagram of the wheat field trial. **(A)** Layout of the field trial showing the randomized distribution of the four conditions (C1–C4) and their three biological replicates. **(B)** Details of a plot with its 6 beds (white boxes) and the five spots where the soil (S1–S5) and plant samples (P1–P5) were collected.

Supplementary Figure 2 | Bacterial taxa overrepresented in wheat crop plant compartments (bulk soil, rhizosphere, endosphere) after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and bacterial taxa with LDA > 4 ($P < 0.05$) **(B)** ($n = 3$). In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Supplementary Figure 3 | Fungal taxa overrepresented in wheat crop plant compartments (bulk soil, rhizosphere, and root endosphere) after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and fungal taxa with LDA > 4 ($P < 0.05$) **(B)** ($n = 3$). In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Supplementary Table 1 | Information about statistics and quality of 16S sequences.

Supplementary Table 2 | Information about statistics and quality of ITS sequences.

Supplementary Table 3 | Bacterial OTU lists, assigned at phylum and family levels, showing differences in percentage of relative abundance among bulk soil, rhizosphere and root endosphere samples of wheat crop plants (ALDEx2 analysis, $P < 0.05$, $n = 3$).

Supplementary Table 4 | Bacterial abundance statistical analysis inferred from an ALDEx2 compositional analysis at the genus level. Relative abundance is calculated as mean proportion. Pairwise comparisons among compartments (bulk soil, rhizosphere, endosphere) and among conditions (C1, C2, C3, C4) are presented ($n = 3$).

Supplementary Table 5 | Bacterial taxa enriched in compartments (bulk soil, rhizosphere, endosphere) and conditions (C1, C2, C3, C4) within a given compartment, inferred from a LEfSe analysis at the genus level. LDA threshold score > 4.0 for compartments and >2.0 for conditions ($P < 0.05$) ($n = 3$).

Supplementary Table 6 | Fungal OTU lists, assigned at phylum and family levels, showing differences in percentage of relative abundance among bulk soil, rhizosphere and root endosphere samples of wheat crop plants (ALDEx2 analysis, $P < 0.05$, $n = 3$).

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Supplementary Table 8 | Fungal taxa enriched in compartments (bulk soil, rhizosphere, endosphere) and conditions (C1, C2, C3, C4) within a given compartment, inferred from a LEfSe analysis at the genus level. LDA threshold score > 4.0 for compartments and >2.0 for conditions ($P < 0.05$) ($n = 3$).

REFERENCES

Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available at: www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed May 20, 2020).

Bae, H., Sicher, R. C., Kim, M. S., Kim, S. H., Strem, M. D., Melnick, R. L., et al. (2009). The beneficial endophyte Trichoderma hamatum isolate DIS219b promotes growth and delays the onset of the drought response in Theobroma cacao. *J. Exp. Bot.* 60, 3279–3295. doi: 10.1093/jxb/erp165

Bargaz, A., Lyamlouli, K., Chtouki, M., Zeroual, Y., and Dhiba, D. (2018). Soil microbial resources for improving fertilizers efficiency in an integrated plant nutrient management system. *Front. Microbiol.* 9:1606. doi: 10.3389/fmicb.2018.01606

Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., et al. (2019). Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. *Front. Plant Sci.* 10:1068. doi: 10.3389/fpls.2019.01068

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11237

Carrero-Carrón, I., Rubio, M. B., Niño-Sánchez, J., Navas, J. A., Jiménez-Díaz, R. M., Monte, E., et al. (2018). Interactions between *Trichoderma harzianum* and defoliating *Verticillium dahliae* in resistant and susceptible wild olive clones. *Plant Pathol.* 67, 1758–1767. doi: 10.111/ppa.12879

Chen, J., Zhu, S., and Zhao, G. (2017). Rapid determination of total protein and wet gluten in commercial wheat flour using siSVR-NIR. *Food Chem.* 221, 1939–1946. doi: 10.1016/j.foodchem.2016.11.155

Curci, P. L., Aiese-Cigliano, R., Zuluaga, D. L., Janni, M., Sanseverino, W., and Sonnante, G. (2017). Transcriptomic response of durum wheat to nitrogen starvation. *Sci. Rep.* 7:1176. doi: 10.1038/s41598-017-01377-0

D'Amico, F., Candela, M., Turroni, S., Biagi, E., Brigidi, P., Bega, A., et al. (2018). The rootstock regulates microbiome diversity in root and rhizosphere compartments of *Vitis vinifera* cultivar Lambrusco. *Front. Microbiol.* 9:2240. doi: 10.3389/fmicb.2018.02240

Debbi, A., Boureghda, H., Monte, E., and Hermosa, R. (2018). Distribution and genetic variability of *Fusarium oxysporum* associated with tomato diseases in Argelia and a biocontrol strategy with indigenous *Trichoderma* spp. *Front. Microbiol.* 9:282. doi: 10.3389/fmicb.2018.00282

Delgado-Vaquerizo, M., Oliverio, A. M., Brewer, T. E., Benavent-González, A., Eldridge, D. J., Bardgett, R. D., et al. (2018). A global atlas of the dominant bacteria found in soil. *Sciences* 359, 320–325. doi: 10.1126/science.aap9516

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Ding, Y., Jin, Y., He, K., Yi, Z., Tan, L., Liu, L., et al. (2020). Low nitrogen fertilization alter rhizosphere microorganism community and improve sweetpotato yield in a nitrogen-deficient rocky soil. *Front. Microbiol.* 11:678. doi: 10.3389/fmicb.2020.00678

Donn, S., Kirkegaard, J. A., Perera, G., Richardson, A. E., and Watt, M. (2015). Evolution of bacterial communities in the wheat crop rhizosphere. *Environ. Microbiol.* 17, 610–621. doi: 10.1111/1462-2920.12452

Dumas, J. B. A. (1831). Procedes de l'Analyse Organique. *Ann. Chim. Phys.* 247, 198–213.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than blast. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C. (2013). UPARSE: highly accurate otu sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C. (2016). SINTAX: a simple non-bayesian taxonomy classifier for 16S and its sequences. *bioRxiv* [Preprint]. doi: 10.1101/074161

FAOSTAT, (2020). *Food and Agriculture Organization of the United Nations (2018) Roma, Italy*. Available at: www.fao.org/faostat/en/#data (accessed April 20, 2020).

Fernandes, A. D., Macklaim, J. M., Linn, T. G., Reid, G., and Gloor, G. B. (2013). ANOVA-like differential expression (aldex) analysis for mixed population RNA-Seq. *PLoS One* 8:e67019. doi: 10.1371/journal.pone.0067019

Friberg, H., Persson, P., Funck, D., and Bergkvist, G. (2019). Preceding crop and tillage systems affect winter survival of wheat and the fungal communities on young wheat roots and in soil. *FEMS Microbiol. Lett.* 366:fnz189. doi: 10.1093/femsle/fnz189

Fricker, A. M., Podlesny, D., and Fricke, W. F. (2019). What is new and relevant for sequencing-based microbiome research? A mini-review. *J. Adv. Res.* 19, 105–112. doi: 10.1016/j.jare.2019.03.006

Frossard, R., Fokkema, N. J., and Tietema, T. (1983). Influence of *Sporobolomyces roseus* and *Cladosporium cladosporioides* on leaching of 14C-labelled assimilates from wheat leaves. *Trans. Br. Mycol. Soc.* 80, 289–296. doi: 10.1016/S0007-1536(83)80012-6

Ganuza, M., Pastor, N., Boccolini, M., Erazo, J., Palacios, S., and Oddino, C. (2019). Evaluating the impact of the biocontrol agent *Trichoderma harzianum* ITEM 3636 on indigenous microbial commnunities from field soils. *J. Appl. Microbiol.* 126, 608–623. doi: 10.1111/jam.14147

Hassani, M. A., Durán, P., and Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome* 6:58. doi: 10.1186/s40168-018-0445-0

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of Trichoderma and of its genes. *Microbiology* 158, 17–25. doi: 10.1099/mic.0.052274-0

Hirsch, P. R., and Mauchline, T. H. (2012). Who's who in the plant root microbiome? *Nat. Biotechnol.* 30, 961–962. doi: 10.1038/nbt.2387

International Wheat Genome Sequencing Consortium [IWGSC], (2018). Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science* 361:eaar7191. doi: 10.1126/science.aar7191

Jiménez, J. J., Igual, J. M., Villar, L., Benito-Alonso, J. L., and Abadia-Ullod, J. (2019). Hierarchical drivers of soil microbial community structure variability in "Monte Perdido" Massif (Central Pyrenees). *Sci. Rep.* 9:8768. doi: 10.1038/s41598-019-45372-z

Jung, B., Park, S.-Y., Lee, Y.-W., and Lee, J. (2013). Biological efficacy of *Streptomyces* sp. strain BN1 against the cereal head blight pathogen *Fusarium graminearum*. *Plant Pathol. J.* 29, 52–58. doi: 10.5423/PPJ.OA.07.2012.0113

Kavamura, V. N., Hayat, R., Clark, I. A., Rossmann, M., Mendes, R., Hirsch, P. R., et al. (2018). Inorganic nitrogen application affects both taxonomical and predicted functional structure of wheat rhizosphere bacterial communities. *Front. Microbiol.* 9:1074. doi: 10.3389/fmicb.2018.01074

Kavamura, V. N., Robinson, R., Hughes, D., Clark, I., Rossmann, M., and Soares de Melo, I. (2020). Wheat dwarfing influences selection of the rhizosphere microbiome. *Sci. Rep.* 10:1452. doi: 10.1038/s41598-020-58402-y

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi: 10.1111/mec.12481

Lê Van, A., Quaiser, A., Duhamel, M., Michon-Coudouel, S., Dufresne, A., and Vandenkoornhuyse, P. (2017). Ecophylogeny of the endospheric root fungal microbiome of co-occurring *Agrostis stolonifera*. *PeerJ* 5:e3454. doi: 10.7717/peerj.3454

Liu, X., Zhang, S., Jiang, Q., Bai, Y., Shen, G., Li, S., et al. (2016). Using community analysis to explore bacterial indicators for disease suppression of tobacco bacterial wilt. *Sci. Rep.* 6:36773. doi: 10.1038/srep36773

Mahato, S., Bhuju, S., and Shrestha, J. (2018). Effect of Trichoderma viride as biofertilizer on growth and yield of wheat. *Malays. J. Sustain. Agric.* 2, 1–5. doi: 10.26480/mjsa.02.2018.01.05

Martinez-Arbizu, P. (2017). *Pairwise-Adonis: Pairwise multilevel comparison using adonis. R Package Version 0.0.1*. Available at: github.com/pmartinezarbizu/pairwiseAdonis (accessed November 16, 2019).

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Medeiros, H. A., Araújo Filho, J. V., Freitas, L. G., Castillo, P., Rubio, M. B., Hermosa, R., et al. (2017). Tomato progeny inherit resistance to the nematode *Meloidogyne javanica* linked to plant growth induced by the biocontrol fungus *Trichoderma atroviride*. *Sci. Rep.* 6:40216. doi: 10.1038/srep40216

Meena, S. K., Rakshit, A., and Meena, V. S. (2016). Effect of seed bio-riming and N doses under varied soil type on nitrogen use efficiency (NUE) of wheat (*Triticum aestivun* L.) under greenhouse conditions. *Biocatal. Agric. Biotechnol.* 6, 68–75. doi: 10.1016/j.bcab.2016.02.010

Mehrabi, Z., McMillan, V., Clark, I. M., and Mauchline, T. (2016). *Pseudomonas* spp. diversity is negatively associated with suppression of the wheat take-all pathogen. Sci. Rep. 6:29905. doi: 10.1038/srep29905

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R., et al. (2015). *Vegan: Community Ecology Package, version 2.3-2*. Available at: CRAN.R-project.org/package=vegan (accessed November 12 2019).

Olsen, S., Cole, C., Watanabe, F., and Dean, L. (1954). *Estimation of Available Phosphorus in Soils by Extraction with Sodium Bicarbonate. USDA Circular No* 939. Washington, DC: US Gov. Print. Office.

Pang, G., Cai, F., Li, R., Zhao, Z., Li, R., Gu, X., et al. (2017). Trichoderma-enriched organic fertilizer can mitigate microbiome degeneration of monocropped soil to maintain better plant growth. *Plant Soil* 416, 181–192. doi: 10.1007/s11104-017-3178-0

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Porras-Alfaro, A., and Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Annu. Rev. Phytopathol.* 49, 291–315. doi: 10.1146/annurev-phyto-080508-081831

Poveda, J., Hermosa, R., Monte, E., and Nicolás, C. (2019). *Trichoderma harzianum* favours the access of arbuscular mycorrhizal fungi to non-host Brassicaceae roots and increases plant productivity. *Sci. Rep.* 9:11650. doi: 10.1038/s41598-019-48269-z

Qiao, C., Penton, C., Xiong, W., Liu, C., Wang, R., Liu, Z., et al. (2019). Reshaping the rhizosphere microbiome by bio-organic amendment to enhance crop yield in a maize-cabbage rotation system. *Appl. Soil Ecol.* 142, 136–146. doi: 10.1016/j.apsoil.2019.04.014

R Core Team, (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rascovan, N., Carbonetto, B., Perrig, D., Díaz, M., Canciani, W., and Abalo, M. (2016). Integrated analysis of root microbiomes of soybean and wheat from agricultural fields. *Sci. Rep.* 6:28084. doi: 10.1038/srep28084

Robinson, R. J., Fraaije, B. A., Clark, I. M., Jackson, R. W., Hirsch, P. R., and Mauchline, T. H. (2015). Endophytic bacterial community composition in wheat (*Triticum aestivum*) is determined by plant tissue type, developmental stage and soil nutrient availability. *Plant Soil* 405, 381–396. doi: 10.1007/s11104-015-2495-4

Rockström, J., Steffen, W., Noone, K., Persson, A., Stuart-Chapin, F., Lambin, E. F., et al. (2009). A safe operating space for humanity. *Nature* 461, 472–475. doi: 10.1038/climate.2009.92

Ros, M., Raut, I., Santisima-Trinidad, A. B., and Pascual, J. A. (2017). Relationship of microbial communities and suppressiveness of Trichoderma fortified composts for pepper seedlings infected by Phytophthora nicotianae. *PLoS One* 12:e0174069. doi: 10.1371/journal.pone.0174069

Rossmann, M., Pérez-Jaramillo, J. E., Kavamura, V. N., Chiaramonte, J. B., Dumack, K., Fiore-Donno, A. M., et al. (2020). Multitropic interactions in the rhizosphere microbiome of wheat: from bacteria and fungi to protists. *FEMS Microbiol. Ecol.* 96:fiaa032. doi: 10.1093/femsec/fiaa032

Rubio, M. B., Hermosa, R., Vicente, R., Gómez-Acosta, F. A., Morcuende, R., Monte, E., et al. (2017). The combination of *Trichoderma harzianum* and chemical fertilization leads to the deregulation of phytohormone networking, preventing the adaptative responses of tomato plants to salt stress. *Front. Plant Sci.* 8:294. doi: 10.3389/fpls.2017.00294

Rubio, M. B., Martínez de Alba, A. E., Nicolás, N., Monte, E., and Hermosa, R. (2019). Early root transcriptomic changes in wheat seedlings colonized by *Trichoderma harzianum* under different inorganic nitrogen supplies. *Front. Microbiol.* 10:2444. doi: 10.3389/fmicb.2019.02444

Samolski, I., Rincón, A. M., Pinzón, L. M., Viterbo, A., and Monte, E. (2012). The qid74 gene from *Trichoderma harzianum* has a role in root architecture and plant biofertilization. *Microbiology* 158, 129–138. doi: 10.1099/mic.0.053140-0

Saravanakumar, K., Li, Y., Yu, C., Wang, Q., Wang, M., Sun, J., et al. (2017). Effect of *Trichoderma harzianum* on maize rhizosphere microbiome and biocontrol of Fusarium Stalk rot. *Sci. Rep.* 7:1771. doi: 10.1038/s41598-017-01680-w

Schlatter, D. C., Hansen, J. C., Schillinger, W. F., Sullivan, T. S., and Paulitz, T. C. (2019). Common and unique rhizosphere microbial communities of wheat and canola in a semiarid Mediterranean environment. *Appl. Soil Ecol.* 144, 170–181. doi: 10.1016/j.apsoil.2019.07.010

Schöps, R., Goldmann, K., Herz, K., Lentendu, G., Schöning, I., Bruelheide, H., et al. (2018). Land-use intensity rather than plant functional identity shapes bacterial and fungal rhizosphere communities. *Front. Microbiol.* 9:2711. doi: 10.3389/fmicb.2018.02711

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Singh, A., Lasek-Nesselquist, E., Chaturvedi, V., and Chaturvedi, S. (2018). *Trichoderma polysporum* selectively inhibits white-nose syndrome fungal pathogen *Pseudogymmnoascus destructans* amidst soil microbes. *Microbiome* 6:319. doi: 10.1186/s40168-018-0512-6

Sommermann, L., Geistlinger, J., Wibberg, D., Deubel, A., Zwanzig, J., Babin, D., et al. (2018). Fungal community profiles in agricultural soils of a long-term field trial under different tillage, fertilization and crop rotation conditions analyzed by high-throughput ITS-amplicon sequencing. *PLoS One* 13:e0195345. doi: 10.1371/journal.pone.0195345

Turner, T. R., James, E. K., and Poole, P. S. (2013). The plant microbiome. *Genome Biol.* 14:209. doi: 10.1186/gb-2013-14-6-209

Umadevi, P., Anandarai, M., Srivastav, V., and Benjamin, S. (2017). *Trichoderma harzianum* MTCC 5179 impacts the population and functional dynamics of microbial community in the rhizosphere of black pepper (*Piper nigrum* L.). *Braz. J. Microbiol.* 49, 463–470. doi: 10.1016/j.bjm.2017.05.011

Vandenkoornhuyse, P., Baldauf, S. L., Leyval, C., Straczek, J., and Young, J. P. W. (2002). Extensive fungal diversity in plant roots. *Science* 295:2051. doi: 10.1126/science.295.5562.2051

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Wachowska, U., Irzykowski, W., Jędryczka, M., Stasiulewicz-Paluch, A. D., and Glowacka, K. (2013). Biological control of winter wheat pathogens with the use of antagonistic Sphingomonas bacteria under greenhouse conditions. *Biocontrol Sci. Technol.* 23, 1110–1122. doi: 10.1080/09583157.2013.812185

Wang, Y., Wang, H., Cheng, H. Y., Chang, F., Wan, Y., and She, X. (2020). Niche differentiation in the rhizosphere and endosphere fungal microbiome of wild Paris polyphylla Sm. *PeerJ* 8:e8510. doi: 10.7717/peerj.8510

Wang, Z., Li, Y., Zhuang, L., Yu, Y., Liu, J., and Zhang, L. (2019). A rhizosphere-derived consortium of *Bacillus subtilis* and *Trichoderma harzianum* suppresses common scab of potato and increases yield. *Comput. Struct. Biotechnol. J.* 17, 645–653. doi: 10.1016/j.csbj.2019.05.003

Wani, Z. A., Kumar, A., Sultan, P., Bindu, K., Riyaz-Ul-Hassan, S. R., and Ashraf, N. (2017). *Mortierella alpina* CS10E4, and oleaginous fungal endophyte of *Crocus sativus* L. enhances apocarotenoid biosynthesis and stress tolerance in the host plant. *Sci. Rep.* 7:8598. doi: 10.1038/s41598-017-08974-z

Yamamoto, K., Shiwa, Y., Ishige, T., Sakamoto, H., Tanaka, K., Uchino, M., et al. (2018). Bacterial diversity associated with the rhizosphere and endosphere of two halophytes: *Glaux maritima* and *Salicornia europaea*. *Front. Microbiol.* 9:2878. doi: 10.3389/fmicb.2018.02878

Zhang, F., Huo, Y., Cobb, A. B., Luo, G., Zhou, J., Yang, G., et al. (2018). *Trichoderma biofertilizer* links to altered soil chemistry, altered microbial communities, and improved grasslandbiomass. *Front. Microbiol.* 9:848. doi: 10.3389/fmicb.2018.00848

Zhang, F. G., Zhu, Z., Yang, X. M., Ran, W., and Shen, Q. R. (2013). *Trichoderma harzianum* T-E5 significantly affects cucumber root exudates and fungal community in the cucumber rhizosphere. *Appl. Soil Ecol.* 72, 41–48. doi: 10.1016/j.apsoil.2013.05.016

Zhou, Y., Coventry, D. R., Gupta, V. V. S. R., Fuentes, D., Merchant, A., Kaiser, B. N., et al. (2020). The preding root system drives the composition and function of the rhizosphere microbiome. *Genome Biol.* 21:89. doi: 10.1186/s13059-020-01999-0

Zörb, C., Ludewig, U., and Hawkesford, M. J. (2018). Perspective on wheat yield and quality with reduced nitrogen supply. *Trends Plant Sci.* 23, 1029–1037. doi: 10.1016/j.tplants.2018.08.012

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Different Age-Induced Changes in Rhizosphere Microbial Composition and Function of *Panax ginseng* in Transplantation Mode

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Transplantation is a cultivation mode widely applied in perennial plant growing. This method might be an effective way to alleviate problems associated with continuous cultivation (4–6 years) in ginseng production, but the alleviating mechanism and effects on soil microbial community is unclear. To study this issue, non-transplanted 2-year-old, and 5-year-old (transplantation mode: 2 + 3) and 9-year-old (transplantation mode: 3 + 3 + 3) ginseng rhizosphere soils were analyzed *via* MiSeq sequencing. The results showed that 9-year-old ginseng rhizosphere soil had lower available nitrogen and the lowest pH, available phosphorus, observed species and community diversity and richness (Chao1, and ACE) among all samples ($p < 0.05$). The abundances of some bacterial classes (Thermoleophilia, Bacilli, and Nitrospira) and fungal genera (*Mortierella*, *Epicoccum*, and *Penicillium* spp.) and functional richness associated with nutrient element cycles and antifungal activity decreased, while abundances of some fungal genera (*Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp.) increased with increasing age of ginseng plants ($p < 0.05$ or $p < 0.01$). However, there was greater similarity between soil samples of 2-year-old and transplanted 5-year-old ginseng plants and the increase in cultivation time from 2 to 5 years did not significantly influence the microbial community, suggesting that transplantation is a viable strategy for suppressing soil-borne diseases in *Panax ginseng* plants over long growth periods.

Keywords: transplantation mode, cultivation years, microbial community, microbial functions, rhizosphere soils

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INTRODUCTION

Soil sickness causes a reduction in crop yield and a prevalence of soil borne diseases, and it is a negative plant-soil feedback with the same crop growing on the same soil successively (Huang et al., 2013). Soil sickness is a common phenomenon not only for many crops such as maize (Gentry et al., 2013), peanut (Li et al., 2018a), cucumber (Jin et al., 2019), but also for many perennial medicinal plants (Xiao et al., 2016a; Tan et al., 2017a). For example, *Panax ginseng* (Araliaceae family) is a perennial herbaceous plant that is widely cultivated in northeast China, Korea, and Japan for its highly valued root, which possesses multifunctional properties (Yang et al., 2014; Shin et al., 2015; Xu et al., 2017). Moreover, the potency of pharmacological/

bioactive constituents in ginseng root tends to increase with cultivation age (Shi et al., 2007; Li et al., 2014a). Generally, it takes at least 5–6 years before ginseng is ready to be harvested (Ying et al., 2012). However, the risks for soil-borne diseases increase over longer periods of cultivation, and these factors have severe negative effects on the yield and quality of ginseng (Ying et al., 2012; Li et al., 2014b).

The causal agents of soil sickness have also been proved to be a combination of biotic and abiotic factors (Huang et al., 2013). Regarding the biotic factors, rhizosphere microorganisms are considered to be the important indicators of soil function that significantly affect the growth, nutrition, and health of plants (Franke-Whittle et al., 2015; López-Carmona et al., 2019). An imbalance in these communities is responsible for the soil sickness, as the abundances of some microorganisms decrease, including *Pseudomonas*, *Bacillus*, and arbuscular mycorrhizal fungal species, which can prevent plant disease and improve growth (Li et al., 2012, 2014b; Kil et al., 2014). In contrast, pathogenic fungi such as *Cylindrocarpon*/*Ilyonectria*, unclassified genera *Leotiomycetes*, and *Fusarium*, which are associated with soil-borne diseases, tend to pose greater risks to plants with increasingly long growth periods of 4–6 years (Xiao et al., 2016a; Tan et al., 2017a; Dong et al., 2018). In addition, abiotic factors such as the soil pH and fertility play vital roles in ginseng growth, which requires slightly acidic and nutrient-rich soil. The soil pH and fertility have been shown to be strongly linked with soil microbial communities (Shen et al., 2013; Siciliano et al., 2014). In the rhizosphere of ginseng plants, the soil pH and nutrient concentrations decline with increasing years of cultivation, ultimately leading to decreased microbial diversity, which in turn is responsible for the development of soil-borne ginseng diseases (Nguyen et al., 2016a; Xiao et al., 2016a; Dong et al., 2017). It has also been demonstrated that allelochemicals (root exudates) of *P. ginseng* significantly decrease the genetic diversity and carbon-metabolic activity of microorganisms and cause chemotaxis responses of ginseng pathogenic microorganisms (Li et al., 2014c, 2016; Lei et al., 2017). Consequently, negative plant-soil feedbacks *via* pathogenic activity, deteriorated soil conditions, or allelopathy, played an important role in soil sickness (Huang et al., 2013; Zhou et al., 2018).

A transplantation mode involves transplantation of ginseng to a new location after growing in one place for 2–3 years (Li et al., 2014c). It is an effective way to avoid the excessive accumulation of allelochemicals and soil deterioration that occurs when the same plants are cultivated in one field for years. This practice is common in the cultivation of the perennial herbaceous plant widely used in traditional Chinese medicines for their highly valued root (Xiao et al., 2016a). For example, 5-year-old ginseng in transplantation mode requires direct sowing and growing in one field for 2–3 years; it is then transplanted to a new field with no recent ginseng plantation history, where it grows for another 2–3 years. Many studies have proved that microbial community became unbalanced, and phytopathogens would gradually be the predominant in the rhizosphere soil of *P. ginseng* (Li et al., 2012; Ying et al., 2012). In addition, ginseng soil samples without transplantation have lower microbial diversity than ginseng soil samples with transplantation (Nguyen et al., 2016a; Xiao et al., 2016a). Because of the importance of transplantation mode, it is necessary to explore the changes in soil abiotic factors and microbial communities associated with transplantation mode. This study will increase our understanding of the status of rhizosphere microbial communities in relation to years of the perennial herbaceous plant growing in transplantation mode and will help in field management with respect to perennial plant cultivation. However, the composition and function of rhizosphere microbial community following different numbers of years in transplantation mode remain poorly understood and is due to complicated metabolic pathways of microbial community and methodological limitations (Xiao et al., 2016a; Wang et al., 2018). Amplicon-based studies involving 16S rRNA genes or internal transcribed spacer (ITS) sequences have been an effective way to achieve high sample-throughput and a deeper insight into soil microbial communities (Miao et al., 2016; Sun et al., 2017; Xiong et al., 2017). Recently, FAPROTAX and FUNGuild were developed to predict the functions, lifestyles, or guilds of bacterial and fungal communities following the data from high-throughput sequencing, respectively (Louca et al., 2016; Nguyen et al., 2016b). These two bioinformatic tools were employed to analyze bacterial functional diversity and fungal trophic mode in soil (Bao et al., 2018; Tayyab et al., 2019); thus, they can provide the potential ways to decipher function succession of rhizosphere microbial community associated with the transplantation mode.

In our study, rhizosphere microbial composition and functional potential of *P. ginseng* at various ages in transplantation mode were investigated by MiSeq sequencing of the 16S rRNA gene and ITS1 region, and FAPROTAX and FUNGuild tools. The objectives of the present study were (1) to characterize the rhizosphere microbial community composition and functional profiling of *P. ginseng* at various ages in transplantation mode, and (2) to assess the relationships between soil properties and the rhizosphere microbial communities of *P. ginseng*. We demonstrated that (1) microbial community diversity decreased, and the soil microbial community composition and function changed with increasing number of years of cultivation, especially with 9-year-old transplanted ginseng plants and (2) there would not be significant difference in microbial communities between 2-year-old and 5-year-old ginseng rhizosphere soils in transplantation mode. This study will increase our understanding of the status of rhizosphere microbial communities in relation to years of the perennial herbaceous plant growing in transplantation mode and will help in soil amelioration after ginseng cultivation.

MATERIALS AND METHODS

Soil Sampling

The main production region of *P. ginseng* is Fusong in the Changbai Mountains of China. A mixture of local soils, humus and albic horizons (1:1), one of the main soil types in Fusong, were used to create raised ginseng bed soils for ginseng cultivation (You et al., 2015; Wang et al., 2019a). In September 2014, soil samples of *P. ginseng* cultivated in transplantation

mode for increasing times were obtained from five ginseng fields (named A–E) from three locations (Manjiang, Donggang, and Wanliang Town) in Fusong (**Table 1**; Wang et al., 2016). Because ginseng is usually harvested at the age of 5 years, soil samples of 5-year old ginseng plants from three fields (A, C, and D; **Table 1**) in transplantation mode were selected. Disease occurrence and mortality rates in ginseng seedlings generally increase after 2 years of consecutive growth (Dong et al., 2018); therefore, soil samples from non-transplanted 2-year-old ginseng plants were chosen as the control in this study (soil E in **Table 1**). Soil samples from ginseng plants in transplantation mode with a high age (9 years old) were also studied (soil B in **Table 1**). G2, G5, and G9 represent soil samples from 2-year-old, 5-year-old, and 9-year-old ginseng plants, respectively, which were obtained as follows:

1. G2 ginseng plants: direct sowing and growing for 2 years without transplantation;
2. G5 ginseng plants: direct sowing and growing in one field for 2 years with subsequent transplantation to a second field, where they grew for 3 years before sample collection;
3. G9 ginseng plants: direct sowing and growing in one field for 3 years, followed by transplantation to another field for 3 years, and final transplantation to a third field, where they grew for another 3 years before sampling.

In our study, ginseng seeds were sowed and all transplanted plants were replanted in soils with no recent history of ginseng cultivation. Forty to sixty rhizosphere soil samples were randomly collected by gently scraping the soil directly attached to the ginseng roots from each ginseng field. Ten soil samples from 10 ginseng roots with the same rust area were uniformly mixed after removal of visible plant materials and were considered one replicate (Wang et al., 2019a). All soil samples were placed on ice for 1–2 days, after which they were passed through a 2-mm sieve and homogenized. Soil samples were stored at −80°C until DNA extraction. For soil properties, soil samples were air-dried and passed through a 0.15 mm sieve for organic matter (OM) content analysis, and a 2 mm sieve for pH, available nitrogen (AN), available phosphorus (AP), and available potassium (AK) content, respectively. Soil properties, including the pH, OM, AN, AP, and AK, were determined as previously described (Wang et al., 2016).

Soil DNA Extraction, PCR Amplification, and Illumina Miseq Sequencing

Total DNA from 0.50 g of each soil sample was extracted using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, CA, USA) according to the manufacturer's instructions and was quantified using a NanoDrop2000 device (Thermo Scientific, Pittsburgh, PA, USA). Each soil sample was extracted in triplicate, and the three DNA solutions were combined together.

DNA was amplified by the PCR using the primer set 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-barcode-GACTACHVGGGTATCTAATCC-3') for the V3–V4 regions of 16S rRNAs, or ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS1R (5'-barcode-ATGAGCGCTGCGTTCTTCATCGA TGC-3') for the fungal ITS1 sequences. Sample-specific barcodes were incorporated into the primers. The PCR conditions used were as follows: 95°C for 2 min; 27 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; final extension at 72°C for 10 min; and a hold at 10°C. Only one PCR was performed for each soil sample with 20-μl reaction mixtures containing 4 μl of 5× FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.4 μl of each primer (5 μM), 0.4 μl of TransStart FastPfu DNA Polymerase (TransGen Biotech, Beijing, China), and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), following the manufacturer's instructions and then quantified using QuantiFluor™-ST (Promega, Madison, WI, USA). The normalized PCR products were subjected to paired-end sequencing using MiSeq platform (Illumina, San Diego, CA, USA) at SinoGenoMax (Beijing) according to the standard protocol.

Sequence Data Processing

After removing the barcode and primer sequences using MOTHUR (Schloss et al., 2009), the remaining reads were merged and quality filtered using FLASH (Magoč and Salzberg, 2011) and QIIME (Caporaso et al., 2010). Following the removal of chimeric sequences by UCHIME (Edgar et al., 2011), operational taxonomic units (OTUs) were clustered at 97% sequence similarity using the UPARSE pipeline (Edgar, 2013). Finally, the taxonomic affiliation of each OTU was calculated using the RDP classifier (version 2.2; Wang et al., 2007) against the Silva128 Database for bacteria (confidence coefficient = 0.8–1) and the UNITE_INSD

TABLE 1 | Soil samples used in the present work.

Fields	Transplantation mode[a]	Start time	Transplanting time	Harvesting time	Cultivation years[b]	Location
A	2 + 3	2009	2011	2014	5 (G5)	Manjiang Town, Fusong
B	3 + 3 + 3	2005	2008, 2011	2014	9 (G9)	Manjiang Town, Fusong
C	2 + 3	2009	2011	2014	5 (G5)	Donggang Town, Fusong
D	2 + 3	2009	2011	2014	5 (G5)	Wanliang Town, Fusong
E	2	2012	/	2014	2 (G2)	Wanliang Town, Fusong

[a]*In the transplantation mode column, the number presented (i.e., 2 + 3) indicates that Panax ginseng plants were grown in one field for 2 years and then transplanted to the indicated sampling field and grown for 3 years. Similarly, 3 + 3 + 3 indicates cases, where plants were directly seeded and grown in one field for 3 years, then transplanted to another field for 3 years, and finally transplanted to the indicated sampling field and grown for 3 years.*

[b]*G2, G5, and G9 indicate soil samples from 2-year, 5-year, and 9-year-old ginseng plants, respectively.*

v7.0 Fungal ITS database for fungi (E value = 1e–05; Kõljalg et al., 2013). The taxon abundances in each sample were generated at the phylum, class, order, family, and genus levels. Sequences were deposited in the NCBI Short Read Archive under accession numbers SRP131809 and SRP129584.

Statistical Analysis

After removing singletons, the alpha diversity was calculated with QIIME (version 1.7.0) based on the Shannon, Simpson, Chao1, and ACE diversity indices. Statistical analysis was carried out using the SAS 9.1 software package (SAS institute Inc., Cary, NC, USA). One-way ANOVA with least significant difference (LSD) test was used to compare the means of samples with 4–6 replicates, and variability in the data was expressed as the standard error. Differences at $p < 0.05$ and $p < 0.01$ were considered significant and highly significant, respectively.

Microbial community similarities among the different samples were determined by performing UniFrac analyses (Lozupone et al., 2011). Principal coordinates analysis (PCoA) based on the weighted UniFrac distance and analysis of similarities (ANOSIM) were used to depict differences in the microbial community compositions. A heatmap was generated using the gplots package in R (version 2.15.3) to compare the top 35 bacterial classes and fungal genera in soil samples with different years of cultivation. Redundancy analysis (RDA) has been performed to measure the linkage between variables of soil microbial community and soil factors by CANOCO5.0 (Biometrics Wageningen, the Netherlands; Xiao et al., 2016b). Spearman's correlation analyses were also performed to assess the relationships among the soil properties, plant age, and microbial community, using the Vegan package of R software (Oksanen et al., 2019). The functions, lifestyles, or guilds of the bacterial and fungal OTUs were identified using FAPROTAX (Louca et al., 2016) and FUNGuild (Nguyen et al., 2016b), respectively. Differences in functional groups between different soils were determined using ANOVA.

RESULTS

Rhizosphere Soil Properties of *P. ginseng*

The rhizosphere soil properties (pH, AN, AP, AK, and OM) were strongly influenced by the plant age (**Table 2**). Compared with the G2 soil samples, ANOVA showed that the pH and AN were significantly lower in G5 by 13.90 and 30.32%, respectively, and in G9 by 27.32 and 48.21%, respectively, ($p < 0.05$), whereas the OM was markedly higher in G5 by 249.87% and in G9 by 181.87% ($p < 0.05$), respectively. The level of AP did not notably differ between G2 and G5 soils. However, the AP levels of G9 were notably lower than those in G2 by 61.32%, and G5 by 72.74% ($p < 0.05$), respectively. Furthermore, no significant difference was found in AK across all soil samples with different transplantation treatments.

Microbial Diversity and Structure

The alpha-diversity indices, including the number of observed species and diversity and richness indices of the 16S rRNA bacterial and ITS fungal libraries, were different across all soil samples. For bacteria, G9 soil (B) had the lowest number of observed species (882) and community diversity and richness (Shannon = 8.451, Simpson = 0.994, Chao1 = 1,271, and ACE = 1,325) among all samples ($p < 0.05$); for fungi, the lowest observed species number (705) and richness (Chao1 = 997 and ACE = 1,022) were also observed in G9 (B; $p < 0.05$; **Table 3**). However, no significant differences in any of the bacterial and fungal alpha-diversity indices were found between the G2 and G5 groups.

The beta diversities of the soil microbial communities in *P. ginseng* rhizosphere soil at varying ages were evaluated using PCoA (**Figure 1**) and ANOSIM analysis (**Table 4**).

TABLE 2 | The basic properties of soil samples used in the present work.

Fields	Cultivation years	pH	OM (g/kg)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)
E	2 (G2)	6.26 ± 0.18^{a}	34.37 ± 2.40^{b}	565.84 ± 41.32^{a}	32.73 ± 8.16^{a}	325.00 ± 78.01^{a}
A, C, and D	5 (G5)	5.39 ± 0.07^{b}	120.25 ± 7.95^{a}	394.25 ± 30.14^{b}	46.44 ± 3.54^{a}	252.72 ± 18.80^{a}
B	9 (G9)	4.55 ± 0.08^{c}	96.88 ± 7.98^{a}	293.05 ± 26.11^{c}	12.66 ± 1.50^{b}	327.75 ± 27.61^{a}

The OM, AN, AP, and AK represented, respectively, the contents of organic matter, available nitrogen, available phosphorus, and available potassium; means followed by different letters (a-c) within a column are significantly different as determined by the LSD test (p < 0.05).

TABLE 3 | Number of observed species, and diversity and richness indices of the 16S rRNA bacterial and ITS fungal libraries obtained by clustering at 97% identity.

	Cultivation years	Observed species	Shannon	Simpson	Chao1	ACE
Bacteria	2 (G2)	1,220 ± 112^{a}	9.351 ± 0.181^{a}	0.997 ± 0.0002^{a}	1,583 ± 186^{a,b}	1,676 ± 207^{a}
	5 (G5)	1,300 ± 24^{a}	9.381 ± 0.053^{a}	0.997 ± 0.0002^{a}	1866 ± 43^{a}	1973 ± 43^{a}
	9 (G9)	882 ± 67^{b}	8.451 ± 0.175^{b}	0.994 ± 0.0009^{b}	1,271 ± 145^{b}	1,325 ± 129^{b}
Fungi	2 (G2)	883 ± 83^{a}	6.645 ± 0.526^{a}	0.949 ± 0.022^{a}	1,344 ± 198^{a}	1,207 ± 80^{a}
	5 (G5)	829 ± 23^{a}	6.598 ± 0.127^{a}	0.966 ± 0.005^{a}	1,119 ± 44^{a,b}	1,171 ± 41^{a}
	9 (G9)	705 ± 41^{b}	6.194 ± 0.240^{a}	0.954 ± 0.010^{a}	997 ± 52^{b}	1,022 ± 50^{a}

Means followed by the different letters (a,b) within a column represent significant differences, as determined by the LSD test (p < 0.05).

TABLE 4 | Dissimilarities in the microbial community composition across different groups as determined by analysis of similarities (ANOSIM).

Cultivation years	Bacteria		Fungi	
	R	*p*	*R*	*p*
G2–G5	0.278	0.046	0.300	0.047
G5–G9	0.834	0.001	0.626	0.001
G2–G9	0.888	0.007	0.980	0.011

An R-value near +1 indicates that dissimilarity was observed between the groups, whereas an R-value near 0 indicates that no significant dissimilarity was observed between the groups. $p < 0.05$ indicates significant dissimilarity; G2, G5, and G9 indicate soil samples from 2-year, 5-year, and 9-year-old ginseng plants, respectively.

Differences in the microbial communities were observed, especially between the G9 group and the other two groups (G2 and G5); however, most of the soil microbial communities in the G2 and G5 samples clustered together (**Figure 1**). In the pairwise ANOSIM test, the lowest *R* values were observed between G2 and G5 ($R \leq 0.300$, $p < 0.05$), whereas a much higher R value was observed between G9 and the other two samples (G2 and G5; $R \geq 0.626$, $p < 0.05$) for both bacteria and fungi (**Table 4**). Thus, the PCoA analysis and ANOSIM test suggested that the closest similarity existed between the non-transplanted G2 samples and the transplanted G5 samples.

Bacterial Community Composition and Function Analysis

The effective bacterial sequences were all assigned to 39 phyla using QIIME with the default settings, and the three predominant phyla across all samples were Proteobacteria (25.59–32.38%), Actinobacteria (12.56–15.20%), and Acidobacteria (11.15–16.51%) across the samples with different years (**Figure 2A**). Different cultivation years significantly changed the relative abundances of the major bacterial phyla. The relative abundances of Proteobacteria (32.38%) and Saccharibacteria (3.44%) phyla were markedly higher in the rhizospheres of older ginseng plants (G9), whereas Nitrospirae (0.97 and 1.14%), Chloroflexi (6.94 and 6.54%), and Elusimicrobia (0.20 and 0.16%) phyla were higher in those of younger ginseng plants (G2 soil and G5 soils; $p < 0.05$). The Bacteroidetes phylum (3.82%) showed the highest abundance in the G2 samples ($p < 0.05$).

Furthermore, a comparison of the top 35 classes from the predominant phyla revealed that the G2 and G5 samples clustered together and had similar microbial community structures at the class level with most classes being found in soils samples from both groups. However, only nine predominant classes were found in the G9 soil samples. Specifically, Thermoleophilia (4.25 and 3.97%), Deltaproteobacteria (2.54 and 2.58%), TK10 (0.46 and 0.43%), KD4.96 (0.82 and 0.68%), and Nitrospira (1.13 and 1.30%) classes were more abundant, respectively, in G2 and G5 samples, whereas Gammaproteobacteria (7.66%) and Alphaproteobacteria (19.05%) classes were more abundant in G9 samples ($p < 0.05$ or $p < 0.01$). Betaproteobacteria (6.17%) and Bacilli (1.16%) classes were only detected in high abundance in the G2 group ($p < 0.05$; **Figure 3A**). Differences in bacterial functions between the G2, G5, and G9 groups were investigated using FAPROTAX (**Figure 3B**). G9 had the lowest functional richness in terms of only three functions. Long cultivation years (>5 years) significantly increased functional groups of cellulolysis (5.79%), chemoheterotrophy (15.50%), and aerobic chemoheterotrophy (14.97%; $p < 0.05$ or $p < 0.01$), and decreased functional groups of aerobic ammonia oxidation (0.39%), nitrification (0.57%), respiration of sulfur compounds (0.05%), sulfur respiration (0.04%), nitrogen fixation (0.48%), and aerobic nitrite oxidation (0.18%; $p < 0.05$ or $p < 0.01$). Some functional groups associated with nutrient element cycles (e.g., methylotrophy, methanol_oxidation nitrification, respiration of sulfur compounds, sulfur respiration, nitrogen fixation, and aerobic nitrite oxidation) and antifungal activity (chitinolysis) were markedly enriched in G2 or G5, which were 1.48–19.37 times higher than that in G9 ($p < 0.05$ or $p < 0.01$). G2 had the highest functional richness in terms of 14 functions.

Environmental factors, such as pH, AN, AP, OM and plant age appear to be the most important environmental factors (**Figure 4A**). According to the RDA analysis, Deltaproteobacteria, Betaproteobacteria, Thermoleophilia, Bacilli, and Nitrospira classes were positively affected by pH, AN, and AP, and negatively affected by plant age, and even Betaproteobacteria, Thermoleophilia, and Bacilli were negatively affected by OM, whereas Alphaproteobacteria, and unidentified_Acidobacteria classes were negatively affected by pH, AN, and AP, and positively affected by plant age. Moreover, Spearman's rank correlations revealed that approximately 12 classes, including Thermoleophilia, Bacilli, and Nitrospira, showed significant negative correlations with the plant age ($r = -0.44$, $p < 0.05$; $r = -0.61$, $p < 0.01$; $r = -0.47$, $p < 0.05$), or Thermoleophilia negatively correlated with AK ($r = -0.42$, $p < 0.05$), but Thermoleophilia and Bacilli were positively correlated with the pH($r = 0.54$, $p < 0.01$and $r = 0.53$, $p < 0.01$), or Nitrospira was positively correlated with AP ($r = 0.42$, $p < 0.05$) and AN($r = 0.58$, $p < 0.01$). In contrast, three classes (Alphaproteobacteria, unidentified_Actinobacteria, and unidentified_Acidobacteria) were positively correlated with the plant age ($r = 0.70$, $p < 0.01$; $r = 0.47$, $p < 0.05$; $r = 0.40$, $p < 0.05$), but negatively with the pH($r = -0.58$, $p < 0.01$; $r = -0.42$, $p < 0.05$; and $r = -0.48$, $p < 0.05$), or Alphaproteobacteria negatively with AP ($r = -0.47$, $p < 0.05$) and AN ($r = -0.55$, $p < 0.01$; **Figure 5A**). At the OTU levels, six OTUs (OTU11, OTU13, OTU15, OTU29, OTU45 and OTU46) belonging to Alphaproteobacteria and Gammaproteobacteria classes were also more abundant in G9 samples (1.38, 1.14, 1.22, 1.30, 0.92, and 0.58%; $p < 0.05$ or $p < 0.01$); and two OTUs (OTU663 and OTU1212) belonging to Bacilli class were also only detected in high abundance in the G2 group (0.32 and 0.28%; $p < 0.05$).

Fungal Community Composition and Function Analysis

Fungal sequences were only assigned to six phyla according to QIIME using default settings. Fungal sequences were predominantly associated with the phyla Ascomycota (47.04–70.25%), Zygomycota (17.93–32.18%), and Basidiomycota (7.67–34.40%), whereas Glomeromycota (or Glomeromycotina;

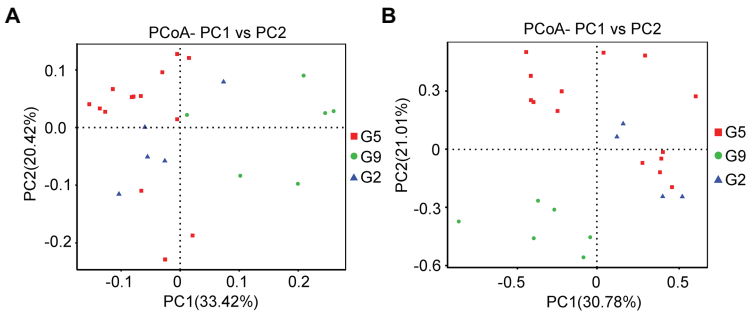

FIGURE 1 | Different analyses of the microbial community structures based on operational taxonomic units (OTUs). **(A)** Principal coordinates analysis (PCoA) graph based on the 16S rDNA sequence. **(B)** PCoA graph based on the ITS1 sequence.

0.34–1.07%), Chytridiomycota (0.27–0.57%), and Neocallimastigomycota (0.02–0.23%) were present in most soils, but at relatively low abundance (**Figure 2B**). The abundance of some phyla varied in different soil samples. For example, Ascomycota and Zygomycota were most prevalent in G2 (70.25 and 20.41%, respectively) and G5 (54.95 and 32.18%, respectively) but had low abundances in G9 (47.04 and 17.93%, respectively; $p < 0.05$). In contrast, Basidiomycota was most abundant in G9 (34.40%; $p < 0.05$).

At the genus level, a heatmap analysis of the top 35 fungal genera also revealed significant differences in the fungal community compositions of ginseng soils at various ages (**Figure 3C**). G2 was enriched with *Penicillium* and *Epicoccum* spp., which respectively were 4.22–7.94 and 4.69–7.16 times higher than in G5 and G9 soils ($p < 0.05$). G2 and G5 were enriched with *Mortierella* spp. 2.59–2.65 higher than in G9 soil. G9 showed 3.04–6.52, 2.50–3.02, and 9.81–27.90 times higher abundances of *Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp. than in G2 and G5, respectively ($p < 0.05$). There were seven major fungal trophic modes detected in all samples (**Figure 3D**). These modes were varied among different samples, and most pathotrophic relationships (pathotroph-saprotroph, pathotroph-saprotroph-symbiotroph, and pathotroph-symbiotroph) were enriched in G9 soil, which were 4.13–4.19, 2.12–3.21, 2.26–3.17 times higher than in G2 and G5 soils, respectively ($p < 0.05$).

According to the RDA analysis, *Epicoccum*, *Mortierella* and *Penicillium* spp. were negatively affected by plant age, positively affected by pH and AP, and even *Epicoccum* and *Penicillium* spp. were positively affected by AN, whereas *Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp. were negatively affected by pH, AN, and AP, and positively affected by plant age (**Figure 4B**). Furthermore, Spearman's rank correlations were also employed to evaluate the relationships between soil properties and the top 35 fungal genera (**Figure 5B**). Twelve genera, including *Mortierella*, *Epicoccum*, and *Penicillium* spp., were negatively correlated with the plant age ($r = -0.59$, $p < 0.01$; $r = -0.42$, $p < 0.05$; and $r = -0.57$, $p < 0.01$), and even *Epicoccum* spp. were negatively correlated with OM ($r = -0.44$, $p < 0.05$) and AK ($r = -0.50$, $p < 0.05$). However, *Mortierella* and *Epicoccum* spp., etc. displayed a significant positive correlation with the pH ($r = 0.52$, $p < 0.01$ and $r = 0.46$, $p < 0.05$), and *Mortierella* and *Penicillium* spp., were positively correlated with AP ($r = 0.52$, $p < 0.01$) or AN ($r = 0.51$, $p < 0.05$), respectively. In contrast, eight genera, including *Ilyonectria*, *Leptodontidium*, *Tetracladium*, and *Sclerotrichum* spp., were positively correlated with the plant age ($r = 0.72$, $p < 0.01$; $r = 0.75$, $p < 0.01$; $r = 0.47$, $p < 0.05$ and $r = 0.58$, $p < 0.01$) and AK ($r = 0.66$, $p < 0.01$; $r = 0.50$, $p < 0.05$; $r = 0.53$, $p < 0.01$ and $r = 0.49$, $p < 0.05$) but were negatively correlated with the pH ($r = -0.79$, $p < 0.01$; $r = -0.86$, $p < 0.01$; $r = -0.46$, $p < 0.05$ and $r = -0.66$, $p < 0.01$). In addition, *Leptodontidium* and *Sclerotrichum* spp. were negatively correlated with AP ($r = -0.47$, $p < 0.05$ and $r = -0.41$, $p < 0.05$). At the OTU levels, two OTUs (OTU1 and OTU4) belonging to *Mortierella* spp. were also more abundant in G2 (8.24 and 5.16%) and G5 (8.77 and 4.90%) samples ($p < 0.05$).

DISCUSSION

Rhizosphere Soil Characteristic Response to Cultivation Years in Transplantation Mode

Soil pH is the primary factor involved in ginseng growth and development. It has been found in previous studies that the soils with ginseng cultivation had a much lower pH values than those without ginseng (You et al., 2015; Xiao et al., 2016a). The soil pH in transplantation mode also decreased with increasing cultivation years in our study (**Table 2**).

The soil pH can influence the environmental AN, AP, and OM contents by controlling the transformation of N, P, and OM (Šantrůčková et al., 2004; Yao et al., 2011; Sommer et al., 2017). It has been demonstrated that changes in the soil pH significantly affect the rate of soil N cycling, and the absence of nitrification (a crucial N cycling process) in some highly acidic soils resulted in low concentrations of AN (nitrate; De Boer and Kowalchuk, 2001; Kemmitt et al., 2006). Nguyen et al. (2016a) suggested that long-term ginseng cultivation may decrease the soil pH and that the acidic soil can cause P absorption *via* Fe–Al and Ca^{2+} interactions, leading to low soil P concentrations. Owing to the lowest pH, the concentrations

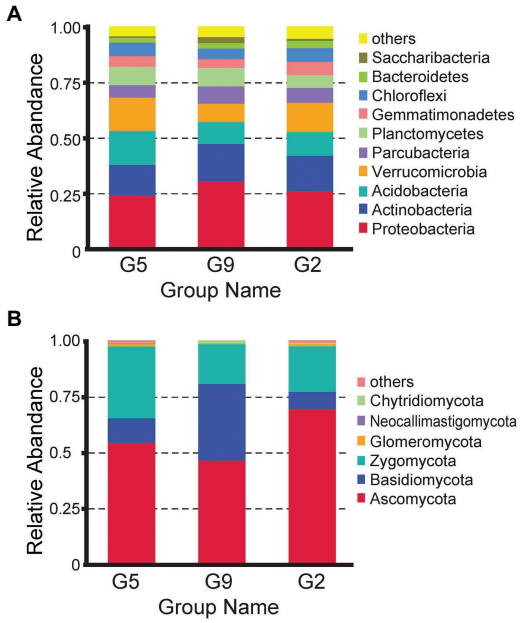

FIGURE 2 | Comparison of the bacterial **(A)** and fungal **(B)** communities from G2, G5, and G9 soil samples at the phylum level.

of AN and AP deceased in 9-year old ginseng soil (**Table 2**). Previously, it has been shown that an increased pH drove OM solubilization and transformation, and lowered the soil OM contents (Grybos et al., 2009); conversely, our current findings showed that OM accumulation in soils with older ginseng plants was attributable to a lower soil pH, which prevented OM transformation.

Rhizosphere Bacterial Community Composition and Functions in Transplantation Mode

The increase of cultivation years (>5 years) had a negative effect on microbial diversity (**Table 3**). This effect has also been reported previously (Vendan et al., 2012; Li et al., 2014b; Xiao et al., 2016a). However, the lack of differences in microbial diversity between the non-transplanted G2 samples and the transplanted G5 samples suggested that increased cultivation years (≤5 years) did not significantly influence the bacterial diversity of ginseng soil because of transplantation.

Growing ginseng plants for increasing years negatively affected the bacterial composition. Proteobacteria was the major bacterial phylum associated with *P. ginseng* soils (Xiao et al., 2016a; Sun et al., 2017), and different classes of Proteobacteria were found to be associated with *P. ginseng* plants of different ages (**Figure 3**). The abundances of some bacterial classes (including members of Thermoleophilia, Bacilli, and Nitrospira) that negatively correlated with the plant age (**Figures 4A, 5A**) proved to be beneficial for plant growth (Kumar et al., 2012; Guan et al., 2013; Zhou et al., 2015). However, some of them (Thermoleophilia and Nitrospira) were enriched in the soil of ginseng plants with lower cultivation years (up to 5 years) in transplantation mode.

Bacterial community composition is very closely related to microbial function. Nitrospirae and Chloroflexi classes have been shown to play key roles in nitration-anammox reactors (Shu et al., 2016; Gu et al., 2019). Some families of Desulfobacterales in Deltaproteobacteria class are closely associated with sulfur (S) cycling (Zhang et al., 2018). In addition, many species of *Bacillus* in Bacilli class can reduce the incidence of soil-borne disease by producing chitinases and acting as biocontrol agents (Kishore and Pande, 2007; Li et al., 2018b). The reduction in bacterial diversity and the abundance of some microbes resulted in a decrease of functional groups involved in nutrient element cycling and soil resistance with higher cultivation years (9-year-old ginseng plants) In contrast, in our study, the functional groups of N and S cycling were higher and might be contributing to the enrichment of Nitrospira, TK10, KD4.96 and Deltaproteobacteria classes in soil of ginseng with lower cultivation years (up to 5 years) in transplantation mode.

Rhizosphere Fungal Community Composition and Functions in Transplantation Mode

Our result suggested that increasing cultivation years decreased fungal diversity. The decreased fungal diversity could lead to plant disease and increased death rate, whereas increased soil fungal diversity might involve the amelioration of soil sickness (Dong et al., 2016; Tan et al., 2017b). The fungal community compositions of ginseng rhizosphere soil changed with increasing cultivation years, which has also been found in previous study (Dong et al., 2018). The *Mortierella*, *Epicoccum*, and *Penicillium* spp., which were beneficial for plant growth, were negatively correlated with the plant age (**Figures 4B, 5B**). It was inferred that *Mortierella* spp., which can produce antibiotics and potential antagonistic agents against various plant pathogens and exists in the rhizosphere soil of *P. notoginseng*, potentially plays a role in maintaining the microecological balance, as protective microbes, by suppressing soil-borne pathogens (Tagawa et al., 2010; Miao et al., 2016; Xiong et al., 2017). Similarly, *Epicoccum* and *Penicillium* spp. can produce numerous biologically active compounds that act as bacterial antagonists and plant growth promoters (Fávaro et al., 2012; Franke-Whittle et al., 2015). As potential pathogens, *Ilyonectria* spp. mainly caused root-rot disease (Farh et al., 2018). *Tetracladium* and *Leptodontidium* spp., which belong to the Leotiomycetes class, also were responded to soil sickness (Tan et al., 2017a). Some pathogenic fungi including *Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp., as indicators of soil sickness (Xiao et al., 2016a; Tan et al., 2017a), markedly increased with increasing cultivation years (**Figure 5B**). The accumulation of allelochemicals (root exudates) from *P. ginseng* plants, such as phenolic compounds, had an important effect on the fungal community as the years of cultivation increased and could promote the growth

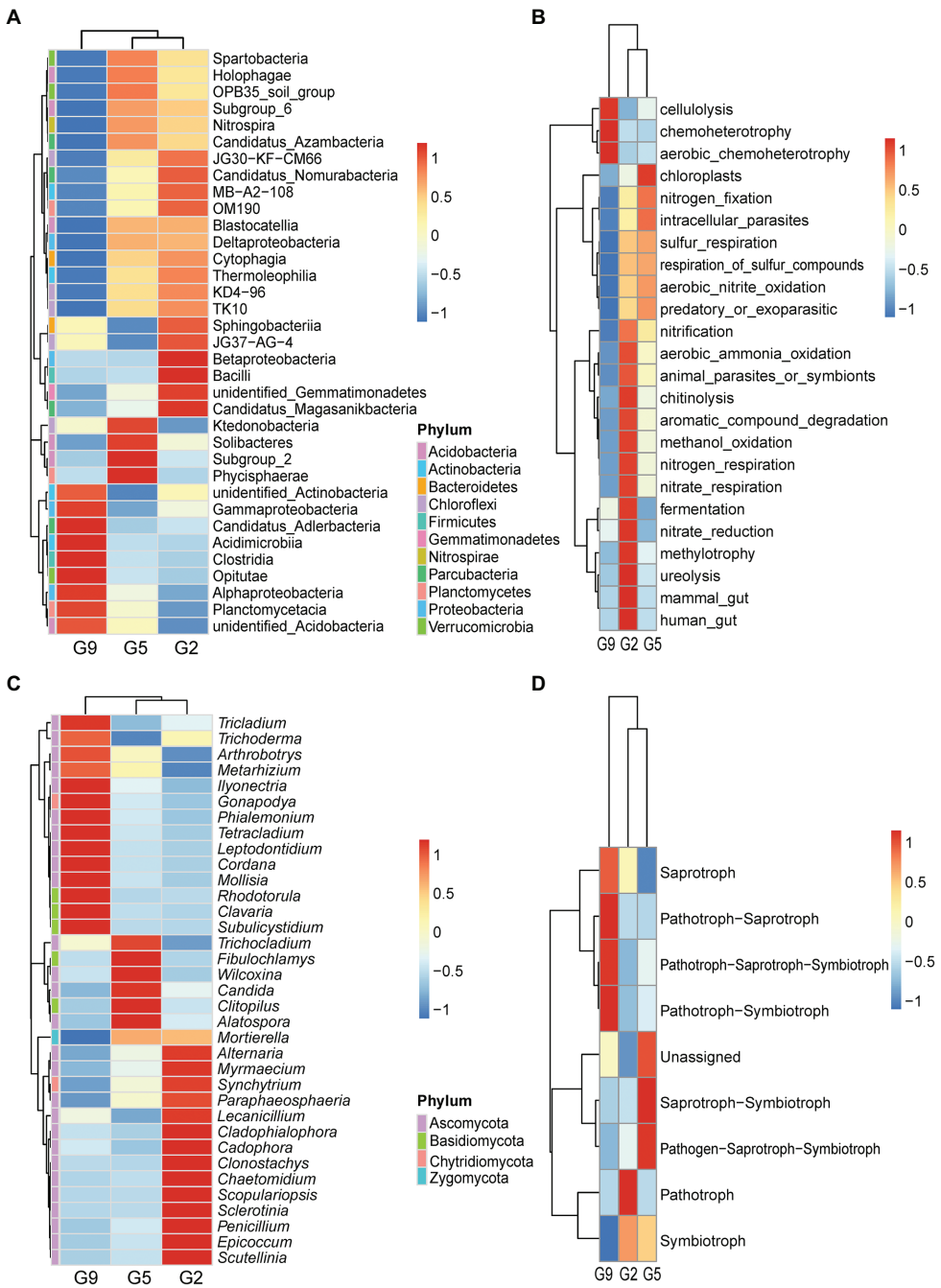

FIGURE 3 | Variations in microbial community compositions and functional characteristics in G2, G5, and G9 soil samples. Heatmap analyses of the abundances of the top 35 bacterial classes **(A)** and fungal genera **(C)**. Functional analysis was performed using FAPROTAX for bacteria **(B)** and FUNGuild for fungi **(D)**, respectively.

of some pathogenic microorganism species (*Cylindrocarpon destructans*;Li et al., 2016; Zhang et al., 2017). Moreover, the increased abundance of pathogenic fungal genera (*Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp.) in ginseng rhizosphere soil promoted the development of soil-borne diseases and increased death rates (Tan et al., 2017a; Dong et al., 2018). The decreased abundance of antagonistic fungi and increased abundance of pathogenic fungi could lead to an enrichment of pathotrophs, enhancing the rate of soil-borne disease with higher cultivation years (9-year-old ginseng plants). However, some fungal genera (*Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp.) acting as indicators of soil sickness (Xiao et al., 2016a;

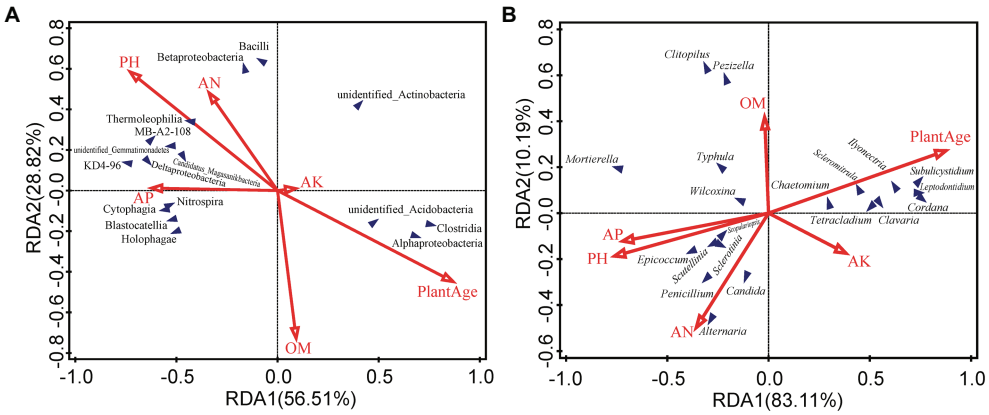

FIGURE 4 | Redundancy analysis (RDA) to measure the linkage between variables of soil microbial community and soil factors, based on the relative abundance of bacterial classes **(A)** and fungal genera **(B)**. OM, organic matter; AN, available nitrogen; AP, available phosphorous; and AK, available potassium.

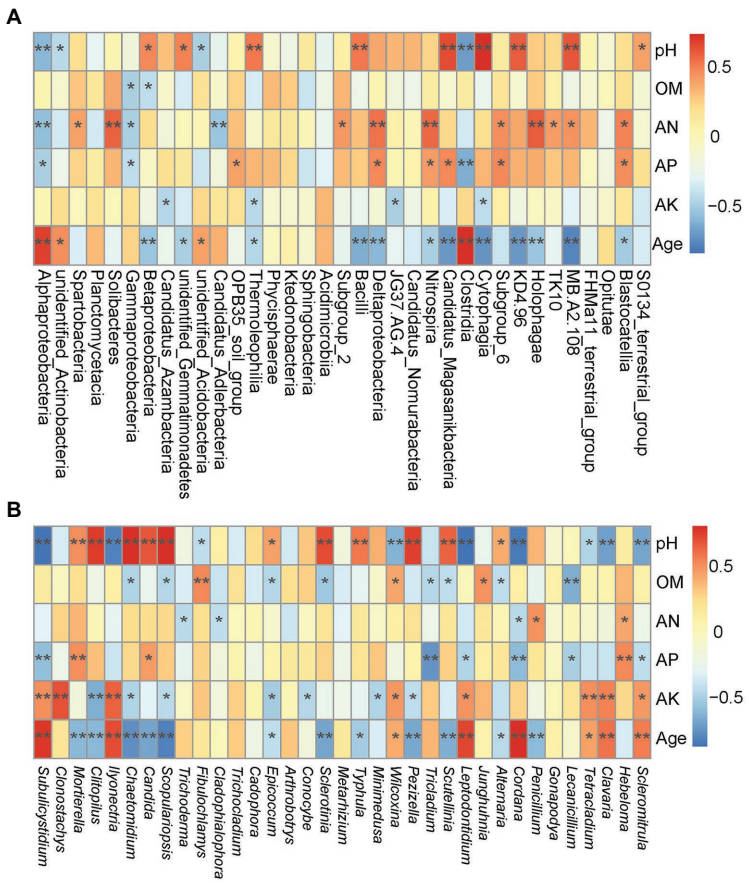

FIGURE 5 | Spearman's correlation analyses between the soil properties and plant age, and abundance of the top 35 bacterial classes **(A)** and fungal genera **(B)**. $^{**}p < 0.01$; $^{*}p < 0.05$. OM, organic matter; AN, available nitrogen; AP, available phosphorous; and AK, available potassium.

Tan et al., 2017a) were not enriched, whereas some beneficial fungal genera (*Mortierella* spp.) were even enriched in the soil of ginseng plants with lower cultivation years (up to 5 years) in transplantation mode. This result revealed that the increase in cultivation years (up to 5 years) did not significantly influence the fungal community and function in the soil of ginseng plants in transplantation mode. Because of an accumulation of root exudates (even after transplantation),

increased fungal pathogens were still observed with the 9-year-old transplanted ginsengs. Future studies including the effect of root exudates on soil microbial community are necessary to better characterize the alleviating mechanism of transplantation mode for *P. ginseng*.

Relationship Between the Microbial Community and Soil Properties

The composition of soil bacterial and fungal communities was closely correlated with the soil properties (**Figures 4, 5**). The soil pH is a key determinant of the microbial community composition (Shen et al., 2013; Ren et al., 2018). Although ginseng plants grow better when cultivated in slightly acidic soils, a highly acidic soil could restrain ginseng growth by shaping the microbial community (You et al., 2015; Zhou et al., 2015). For instance, plant growth-promoting and yield-enhancing Bacilli, which were positively correlated with the soil pH, have been found in moderately acidic soil (Yadav et al., 2011; Kumar et al., 2012). The relative abundances of some fungal genera (*Mortierella* and *Epicoccum* spp.; which preferentially grow in slightly acidic soils) decreased, and other fungal genera (*Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp.), preferring highly acidic soils, increased after the soil pH decreased, which could affect the growth of ginseng plants.

Microbes have been shown to be dominant drivers of biogeochemical cycles including nutrient cycling in soils (Wang et al., 2019b). Shifts in the relative abundances of some microbes with differing pH values can influence the nutrient availability in soils (Rubenecia et al., 2015; Tan et al., 2017b). Thermoleophilia class, a predominant Actinobacteria phylum subgroup that was negatively correlated with OM, is known to be involved in the degradation of OM in soils (Zhou et al., 2015). *Nitrospira* spp., belonging to Nitrospira class, was positively correlated with AN. These are nitrite-oxidizing bacteria that play pivotal roles in the N cycle (Pii et al., 2016). Betaproteobacteria class positively correlated with the soil pH and negatively correlated with OM levels have also been found by Zhou et al. (2015). However, our results showed that Alphaproteobacteria class was negatively correlated with AP and that Gammaproteobacteria class was negatively correlated with AP and OM; the opposite trend to that which has been previously reported (Zhou et al., 2015). Thus, Betaproteobacteria and Gammaproteobacteria classes may also be involved in the degradation of OM in soils. We also found that soil fungal communities were also closely correlated with soil properties (**Figures 4B, 5B**). Furthermore, *Mortierella* spp. were positively correlated with AP and has been suggested to be an important component of phosphorus cycling by Curlevski et al. (2010); in contrast, some potential genera of pathogens (*Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp., among others) were positively correlated with the plant age and AK (**Figures 4B, 5B**). Our results indicated that low pH may inhibit the growth of some microbes involving nutrient element cycles and antifungal activity, leading to low AP or AN level and high abundances of some potential pathogens with an increasing age of *P. ginseng* plants.

CONCLUSION

During prolonged growth (up to 9 years) of *P. ginseng* plants, the rhizosphere microbial diversity, abundance of some microbes and functional richness associated with nutrient element cycles and antifungal activity decreased, while the abundances of some potential pathotroph fungi increased. The abundance of some bacterial classes (Thermoleophilia, Bacilli, and Nitrospira) and fungal genera (*Mortierella*, *Epicoccum*, and *Penicillium* spp.) decreased with increasing age of *P. ginseng* plants. Conversely, the abundance of some fungal genera (*Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp.) increased under the same conditions. Although microbial community diversity decreased, and the microbial composition and function changed with increasing number of years of cultivation, compared with cultivation for 2 years, cultivating ginseng plants for up to 5 years did not significantly influence the microbial communities of the ginseng rhizosphere soil in transplantation mode. Some fungal genera (*Ilyonectria*, *Tetracladium*, and *Leptodontidium* spp.) were not enriched in response to continuous cropping and some microbes (Thermoleophilia, Nitrospira, and *Mortierella* spp.) were enriched even in the soil of ginseng plants cultivated for up to 5 years in the transplantation mode.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: sequences were deposited in the NCBI short read archive under accession numbers SRP131809 and SRP129584.

AUTHOR CONTRIBUTIONS

QW and YZ conceived and designed the study. QW, HS, and CX contributed to all experiments in this study. QW wrote the manuscript. QW and ML collected all the samples. ML helped with the data analysis of the Miseq sequencing. YZ helped to draft the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Bao, M., He, H., Ma, X., Wang, Z., and Qiu, W. (2018). Effects of chemical nitrogen fertilizer and green manure on diversity and functions of soil bacteria in wheat field. *Acta Pedol. Sin.* 55, 734–743. doi: 10.11766/trxb201710270425

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Curlevski, N. J. A., Xu, Z. H., Anderson, I. C., and Cairney, J. W. G. (2010). Diversity of soil and rhizosphere fungi under *Araucaria bidwillii* (bunya pine) at an Australian tropical montane rainforest site. *Fungal Divers.* 40, 12–22. doi: 10.1007/s13225-009-0001-0

De Boer, W., and Kowalchuk, G. A. (2001). Nitrification in acid soils: micro-organisms and mechanisms. *Soil Biol. Biochem.* 33, 853–866. doi: 10.1016/S0038-0717(00)00247-9

Dong, L., Xu, J., Feng, G., Li, X., and Chen, S. (2016). Soil bacterial and fungal community dynamics in relation to *Panax notoginseng* death rate in a continuous cropping system. *Sci. Rep.* 6:31802. doi: 10.1038/srep31802

Dong, L., Xu, J., Zhang, L., Cheng, R., Wei, G., Su, H., et al. (2018). Rhizospheric microbial communities are driven by *Panax ginseng* at different growth stages and biocontrol bacteria alleviates replanting mortality. *Acta Pharm. Sin. B* 8, 272–282. doi: 10.1016/j.apsb.2017.12.011

Dong, L., Xu, J., Zhang, L., Yang, J., Liao, B., Li, X., et al. (2017). High-throughput sequencing technology reveals that continuous cropping of American ginseng results in changes in the microbial community in arable soil. *Chin. Med.* 12, 1–11. doi: 10.1186/s13020-017-0139-8

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/Nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Farh, E. A., Kim, Y. J., Kim, Y. J., and Yang, D. C. (2018). Mini review: *Cylindrocarpon destructans*/*Ilyonectria radicicola*-species complex: causative agent of ginseng root-rot disease and rusty symptoms. *J. Ginseng Res.* 42, 9–15. doi: 10.1016/j.jgr.2017.01.004

Fávaro, L., Sebastianes, F., and Araújo, W. (2012). *Epicoccum nigrum* P16, a sugarcane endophyte, produces antifungal compounds and induces root growth. *PLoS One* 7:e36826. doi: 10.1371/journal.pone.0036826

Franke-Whittle, I. H., Manici, L. M., Insam, H., and Stres, B. (2015). Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted apple orchards. *Plant Soil* 395, 317–333. doi: 10.1007/s11104-015-2562-x

Gentry, L. F., Ruffo, M. L., and Below, F. E. (2013). Identifying factors controlling the continuous corn yield penalty. *Agron. J.* 105, 295–303. doi: 10.2134/agronj2012.0246

Grybos, M., Davranche, M., Gruau, G., Petitjean, P., and Pédrot, M. (2009). Increasing pH drives organic matter solubilization from wetland soils under reducing conditions. *Geoderma* 154, 13–19. doi: 10.1016/j.geoderma.2009.09.001

Gu, S., Hu, Q., Cheng, Y., Bai, L., Liu, Z., Xiao, W., et al. (2019). Application of organic fertilizer improves microbial community diversity and alters microbial network structure in tea (*Camellia sinensis*) plantation soils. *Soil Tillage Res.* 195:104356. doi: 10.1016/j.still.2019.104356

Guan, X., Wang, J., Zhao, H., Wang, J., Luo, X., Liu, F., et al. (2013). Soil bacterial communities shaped by geochemical factors and land use in a less-explored area, Tibetan plateau. *BMC Genomics* 14:820. doi: 10.1186/1471-2164-14-820

Huang, L. F., Song, L. X., Xia, X. J., Mao, W. H., Shi, K., Zhou, Y. H., et al. (2013). Plant-soil feedbacks and soil sickness: from mechanisms to application in agriculture. *J. Chem. Ecol.* 39, 232–242. doi: 10.1007/s10886-013-0244-9

Jin, X., Zhang, J., Shi, Y., Wu, F., and Zhou, X. (2019). Green manures of indian mustard and wild rocket enhance cucumber resistance to *Fusarium* wilt through modulating rhizosphere bacterial community composition. *Plant Soil* 441, 283–300. doi: 10.1007/s11104-019-04118-6

Kemmitt, S. J., Wright, D., Goulding, K., and Jones, D. (2006). pH regulation of carbon and nitrogen cdynamics in two agricultural soils. *Soil Biol. Biochem.* 38, 898–911. doi: 10.1016/j.soilbio.2005.08.006

Kil, Y. J., Eo, J. K., Lee, E. H., and Eom, A. H. (2014). Root age-dependent changes in arbuscular mycorrhizal fungal communities colonizing roots of *Panax ginseng*. *Mycobiology* 42, 416–421. doi: 10.5941/MYCO.2014.42.4.416

Kishore, G., and Pande, S. (2007). Chitin-supplemented foliar application of chitinolytic *Bacillus cereus* reduces severity of *Botrytis* gray mold disease in chickpea under controlled conditions. *Lett. Appl. Microbiol.* 44, 98–105. doi: 10.1111/j.1472-765X.2006.02022.x

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi: 10.1111/mec.12481

Kumar, P., Khare, S., and Dubey, R. C. (2012). Diversity of bacilli from disease suppressive soil and their role in plant growth promotion and yield enhancement. *New York Sci. J.* 5, 90–111.

Lei, F., Fu, J., Zhou, R., Wang, D., Zhang, A., Ma, W., et al. (2017). Chemotactic response of ginseng bacterial soft-rot to ginseng root exudates. *Saudi J. Biol. Sci.* 24, 1620–1625. doi: 10.1016/j.sjbs.2017.05.006

Li, X., de Boer, W., Zhang, Y., Ding, C., Zhang, T., and Wang, X. (2018a). Suppression of soil-borne *Fusarium* pathogens of peanut by intercropping with the medicinal herb *Atractylodes lancea*. *Soil Biol. Biochem.* 116, 120–130. doi: 10.1016/j.soilbio.2017.09.029

Li, W., Liu, Q., and Chen, P. (2018b). Effect of long-term continuous cropping of strawberry on soil bacterial community structure and diversity. *J. Integr. Agric.* 17, 2570–2582. doi: 10.1016/S2095-3119(18)61944-6

Li, C. T., Yan, Z. F., Zhang, L. X., and Li, Y. (2014a). Research and implementation of good agricultural practice for traditional Chinese medicinal materials in Jilin province. *China. J. Ginseng Res.* 38, 227–232. doi: 10.1016/j.jgr.2014.05.007

Li, Y., Ying, Y., and Ding, W. (2014c). Dynamics of *Panax ginseng* rhizospheric soil microbial community and their metabolic function. *Evid. Based Complement. Alternat. Med.* 2014:160373. doi: 10.1155/2014/160373

Li, Y., Ying, Y., Zhao, D., and Ding, W. (2012). Microbial community diversity analysis of *Panax ginseng* rhizosphere and non-rhizosphere soil using randomly amplified polymorphic DNA method. *Open J. Genet.* 2, 95–102. doi: 10.4236/ojgen.2012.22014

Li, Y., Ying, Y., Zhao, D., and Ding, W. (2014b). Influence of allelochemicals on microbial community in ginseng cultivating soil. *Chin. Herb. Med.* 6, 313–318. doi: 10.1016/S1674-6384(14)60047-2

Li, Z., Zhou, R., Xie, Y., and Fu, J. (2016). Allelopathic effects of phenolic compounds of ginseng root rhizosphere on *Cylindrocarpon destructans*. *Chin. J. Appl. Ecol.* 27, 3616–3622. doi: 10.13287/j.1001-9332.201611.002

López-Carmona, D., Alarcón, A., Martínez-Romero, E., Peña Cabriales, J., and Larsen, J. (2019). Maize plant growth response to whole rhizosphere microbial communities in different mineral N and P fertilization scenarios. *Rhizosphere* 9, 38–46. doi: 10.1016/j.rhisph.2018.11.004

Louca, S., Parfrey, L., and Doebeli, M. (2016). Decoupling function and taxonomy in the global ocean microbiome. *Science* 353, 1272–1277. doi: 10.1126/science.aaf4507

Lozupone, C., Lladser, M., Knights, D., Stombaugh, J., and Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *ISME J.* 5, 169–172. doi: 10.1038/ismej.2010.133

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Miao, C. P., Mi, Q. L., Qiao, X. G., Zheng, Y. K., Chen, Y. W., Xu, L. H., et al. (2016). Rhizospheric fungi of *Panax notoginseng*: diversity and antagonism to host phytopathogens. *J. Ginseng Res.* 40, 127–134. doi: 10.1016/j.jgr.2015.06.004

Nguyen, N. L., Kim, Y. J., Hoang, V. A., Subramaniyam, S., Kang, J. P., Kang, C. H., et al. (2016a). Bacterial diversity and community structure in Korean ginseng field soil are schifted by cultivation time. *PLoS One* 11:e0155055. doi: 10.1371/journal.pone.0155055

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., et al. (2016b). FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* 20, 241–248. doi: 10.1016/j.funeco.2015.06.006

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., and McGlinn, D. (2019). Vegan: com-munity ecology package. R package version 2.5–4. Available at: https://github.com/vegandevs/vegan (Accessed 20 Nov, 2019).

Pii, Y., Borruso, L., Brusetti, L., Crecchio, C., Cesco, S., and Mimmo, T. (2016). The interaction between iron nutrition, plant species and soil type shapes the rhizosphere microbiome. *Plant Physiol. Biochem.* 99, 39–48. doi: 10.1016/j.plaphy.2015.12.002

Ren, B., Hu, Y., Chen, B., Zhang, Y., Thiele, J., Shi, R., et al. (2018). Soil pH and plant diversity shape soil bacterial community structure in the active layer across the latitudinal gradients in continuous permafrost region of northeastern China. *Sci. Rep.* 8, 1–10. doi: 10.1038/s41598-018-24040-8

Rubenecia, M. R., Ultra, V., Woo, C., Ahn, Y., and Lee, S. C. (2015). Soil chemical and microbial properties and its relationship with the root growth of *Panax ginseng*. *Int. J. Agric. Biol.* 17, 1157–1164. doi: 10.17957/IJAB/15.0058

Šantrůčková, H., Vrba, J., Picek, T., and Kopáček, J. (2004). Soil biochemical activity and phosphorus transformations and losses from acidified forest soils. *Soil Biol. Biochem.* 36, 1569–1576. doi: 10.1016/j.soilbio.2004.07.015

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Shen, C., Xiong, J., Zhang, H., Feng, Y., Lin, X., Li, X., et al. (2013). Soil pH drives the spatial distribution of bacterial communities along elevation on Changbai Mountain. *Soil Biol. Biochem.* 57, 204–211. doi: 10.1016/j.soilbio.2012.07.013

Shi, W., Wang, Y., Li, J., Zhang, H., and Ding, L. (2007). Investigation of ginsenosides in different parts and ages of *Panax ginseng*. *Food Chem.* 102, 664–668. doi: 10.1016/j.foodchem.2006.05.053

Shin, B. K., Kwon, S. W., and Park, J. H. (2015). Chemical diversity of ginseng saponins from *Panax ginseng*. *J. Ginseng Res.* 39, 287–298. doi: 10.1016/j.jgr.2014.12.005

Shu, D., He, Y., Yue, H., and Yang, S. (2016). Effects of Fe (II) on microbial communities and nitrogen transformation pathway of nitrogen and iron cycling in the anammox process: kinetic, quantitative molecular mechanism and metagenomic analysis. *RSC Adv.* 6, 68005–68016. doi: 10.1039/C6RA09209H

Siciliano, S. D., Palmer, A. S., Winsley, T., Lamb, E. G., Bissett, A., Brown, M. V., et al. (2014). Soil fertility is associated with fungal and bacterial richness, whereas pH is associated with community composition in polar soil microbial communities. *Soil Biol. Biochem.* 78, 10–20. doi: 10.1016/j.soilbio.2014.07.005

Sommer, S. G., Clough, T., Balaine, N., Hafner, S., and Cameron, K. (2017). Transformation of organic matter and the emissions of methane and ammonia during storage of liquid manure as affected by acidification. *J. Environ. Qual.* 46, 514–521. doi: 10.2134/jeq2016.10.0409

Sun, H., Wang, Q., Liu, N., Li, L., Zhang, C. G., Liu, Z., et al. (2017). Effects of different leaf litters on the physicochemical properties and bacterial communities in *Panax ginseng*-growing soil. *Appl. Soil Ecol.* 111, 17–24. doi: 10.1016/j.apsoil.2016.11.008

Tagawa, M., Tamaki, H., Manome, A., Koyama, O., and Kamagata, Y. (2010). Isolation and characterization of antagonistic fungi against potato scab pathogens from potato field soils. *FEMS Microbiol. Lett.* 305, 136–142. doi: 10.1111/j.1574-6968.2010.01928.x

Tan, Y., Cui, Y., Li, H., Kuang, A., Li, X., Wei, Y., et al. (2017a). Rhizospheric soil and root endogenous fungal diversity and composition in response to continuous *Panax notoginseng* cropping practices. *Microbiol. Res.* 194, 10–19. doi: 10.1016/j.micres.2016.09.009

Tan, Y., Cui, Y., Li, H., Kuang, A., Li, X., Wei, Y., et al. (2017b). Diversity and composition of rhizospheric soil and root endogenous bacteria in *Panax notoginseng* during continuous cropping practices. *J. Basic Microbiol.* 57, 337–344. doi: 10.1002/jobm.201600464

Tayyab, M., Islam, W., Lee, C. G., Pang, Z., Khalil, F., Lin, S., et al. (2019). Short-term effects of different organic amendments on soil fungal composition. *Sustainability* 11:198. doi: 10.3390/su11010198

Vendan, R., Lee, S. H., Yu, Y., and Rhee, Y. (2012). Analysis of bacterial community in the ginseng soil using denaturing gradient gel electrophoresis (DGGE). *Indian J. Microbiol.* 52, 286–288. doi: 10.1007/s12088-011-0193-3

Wang, Q., Garrity, G., Tiedje, J., and Cole's, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wang, K., Mao, H. L., and Li, X. K. (2018). Functional characteristics and influence factors of microbial community in sewage sludge composting with inorganic bulking agent. *Bioresour. Technol.* 249, 527–535. doi: 10.1016/j.biortech.2017.10.034

Wang, Q., Sun, H., Xu, C., Ma, L., Li, M., Shao, C., et al. (2019a). Analysis of rhizosphere bacterial and fungal communities associated with rusty root disease of *Panax ginseng*. *Appl. Soil Ecol.* 138, 245–252. doi: 10.1016/j.apsoil.2019.03.012

Wang, Q., Xu, C., Sun, H., Ma, L., Li, L., Zhang, D., et al. (2016). Analysis of the relationship between rusty root incidences and soil properties in *Panax ginseng*. *IOP Conf. Ser. Earth Environ. Sci.* 41:012001. doi: 10.1088/1755-1315/41/1/012001

Wang, Z., Zhang, Q., Staley, C., Gao, H., Ishii, S., Wei, X., et al. (2019b). Impact of long-term grazing exclusion on soil microbial community composition and nutrient availability. *Biol. Fertil. Soils* 55, 121–134. doi: 10.1007/s00374-018-01336-5

Xiao, C., Yang, L., Zhang, L., Liu, C., and Han, M. (2016a). Effects of cultivation ages and modes on microbial diversity in the rhizosphere soil of *Panax ginseng*. *J. Ginseng Res.* 40, 28–37. doi: 10.1016/j.jgr.2015.04.004

Xiao, K., Li, B., Ma, L., Bao, P., Zhou, X., Zhang, T., et al. (2016b). Metagenomic profiles of antibiotic resistance genes in paddy soils from South China. *FEMS Microbiol. Ecol.* 92:fiw023. doi: 10.1093/femsec/fiw023

Xiong, W., Li, R., Ren, Y., Liu, C., Zhao, Q., Wu, H., et al. (2017). Distinct roles for soil fungal and bacterial communities associated with the suppression of vanilla *Fusarium* wilt disease. *Soil Biol. Biochem.* 107, 198–207. doi: 10.1016/j.soilbio.2017.01.010

Xu, J., Chu, Y., Liao, B., Xiao, S., Yin, Q., Bai, R., et al. (2017). *Panax ginseng* genome examination for ginsenoside biosynthesis. *GigaScience* 6, 1–15. doi: 10.1093/gigascience/gix093

Yadav, S., Kaushik, R., Saxena, A., and Arora, D. (2011). Diversity and phylogeny of plant growth-promoting bacilli from moderately acidic soil. *J. Basic Microbiol.* 51, 98–106. doi: 10.1002/jobm.201000098

Yang, W., Hu, Y., Wu, W., Ye, M., and Guo, D. (2014). Saponins in the genus *Panax* L. (Araliaceae): a systematic review of their chemical diversity. *Phytochemistry* 106, 7–24. doi: 10.1016/j.phytochem.2014.07.012

Yao, H., Campbell, C., and Qiao, X. (2011). Soil pH controls nitrification and carbon substrate utilization more than urea or charcoal in some highly acidic soils. *Biol. Fertil. Soils* 47, 515–522. doi: 10.1007/s00374-011-0554-4

Ying, Y., Ding, W., and Li, Y. (2012). Characterization of soil bacterial communities in rhizospheric and nonrhizospheric soil of *Panax ginseng*. *Biochem. Genet.* 50, 848–859. doi: 10.1007/s10528-012-9525-1

You, J., Liu, X., Zhang, B., Xie, Z., Hou, Z., and Yang, Z. (2015). Seasonal changes in soil acidity and related properties in ginseng artificial bed soils under a plastic shade. *J. Ginseng Res.* 39, 81–88. doi: 10.1016/j.jgr.2014.08.002

Zhang, X., Hu, B. X., Ren, H., and Zhang, J. (2018). Composition and functional diversity of microbial community across a mangrove-inhabited mudflat as revealed by 16S rRNA gene sequences. *Sci. Total Environ.* 633, 518–528. doi: 10.1016/j.scitotenv.2018.03.158

Zhang, A. H., Lei, F. J., Fu, J. F., Zhou, R. J., and Zhang, L. X. (2017). Influence of exogenous ginsenosides on new forest soil microbial communities. *China J. Chin Mater Med.* 42, 4756–4761. doi: 10.19540/j.cnki.cjcmm.2017.0213

Zhou, J., Guan, D., Zhou, B., Zhao, B., Ma, M., Qin, J., et al. (2015). Influence of 34-years of fertilization on bacterial communities in an intensively cultivated black soil in northeast China. *Soil Biol. Biochem.* 90, 42–51. doi: 10.1016/j.soilbio.2015.07.005

Zhou, X., Zhang, J., Pan, D., Ge, X., Jin, X., Chen, S., et al. (2018). P-coumaric can alter the composition of cucumber rhizosphere microbial communities

and induce negative plant-microbial interactions. *Biol. Fertil. Soils* 54, 363–372. doi: 10.1007/s00374-018-1265-x

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Soil Disturbance Affects Plant Productivity via Soil Microbial Community Shifts

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Recent advances in climate research have discovered that permafrost is particularly vulnerable to the changes occurring in the atmosphere and climate, especially in Alaska where 85% of the land is underlain by mostly discontinuous permafrost. As permafrost thaws, research has shown that natural and anthropogenic soil disturbance causes microbial communities to undergo shifts in membership composition and biomass, as well as in functional diversity. Boreal forests are home to many plants that are integral to the subsistence diets of many Alaska Native communities. Yet, it is unclear how the observed shifts in soil microbes can affect above ground plant communities that are relied on as a major source of food. In this study, we tested the hypothesis that microbial communities associated with permafrost thaw affect plant productivity by growing five plant species found in Boreal forests and Tundra ecosystems, including low-bush cranberry and bog blueberry, with microbial communities from the active layer soils of a permafrost thaw gradient. We found that plant productivity was significantly affected by the microbial soil inoculants. Plants inoculated with communities from above thawing permafrost showed decreased productivity compared to plants inoculated with microbes from undisturbed soils. We used metagenomic sequencing to determine that microbial communities from disturbed soils above thawing permafrost differ in taxonomy from microbial communities in undisturbed soils above intact permafrost. The combination of these results indicates that a decrease in plant productivity can be linked to soil disturbance driven changes in microbial community membership and abundance. These data contribute to an understanding of how microbial communities can be affected by soil disturbance and climate change, and how those community shifts can further influence plant productivity in Boreal forests and more broadly, ecosystem health.

Keywords: permafrost thaw, microbial communities, boreal forest, metagenomics, plant growth

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INTRODUCTION

With nearly 85% of the land in Alaska underlain with discontinuous permafrost, Alaska is particularly vulnerable to large-scale ecosystem changes due to climate change-driven permafrost thaw and resulting soil disturbance (Department of Natural Resources: Alaska Division of Geological and Geophysical Surveys, 2020). As foundational abiotic factors such as nutrient availability and soil hydrology begin to change, they in turn affect ecosystem processes including succession and productivity, that regulate plant and microbial

communities present in the disturbed soils (Schuur and Mack, 2018). Recent research has turned to exploring the effects of climate change on microbial communities residing in soil and permafrost (Jansson and Hofmockel, 2018). As permafrost thaws and soil disturbance events occur, microbial communities undergo shifts in membership composition and biomass, as well as in functional diversity (Mackelprang et al., 2011; Coolen and Orsi, 2015; Schuur et al., 2015; Schütte et al., 2019). As these smaller scale changes develop, it is important that we build a better understanding of how microbial communities in northern latitude soils affect larger scale processes such as plant growth.

Permafrost is frozen ground (rock, ice, soil) that remains at less than 0°C for more than 2 years. Permafrost underlies approximately 26% of terrestrial ecosystems globally and is a critical structural element that holds large potential for altering ecosystem composition through thawing and melting of ice wedges (Schuur and Mack, 2018). It has been estimated that the carbon stored in permafrost accounts for more than 2.5 times as much as the atmospheric carbon pool (Schuur et al., 2007, 2015). As permafrost thaws, previously frozen organic matter, nutrients, water, and microbes are exposed and reintroduced into actively cycling pools of carbon and nutrients (Chapin et al., 2006). Plant roots interact within the active layer of soil which sits above the permafrost and undergoes seasonal cycles of freezing and thawing (Christensen et al., 2004; Schuur et al., 2015). The depth of the active layer defines what nutrients, rooting space, and water are available to plants which can in turn affect community succession and composition.

Previous research has found evidence that the composition, diversity, and biomass of microbial communities can become altered in locations that are undergoing permafrost thaw and disturbance events such as thermokarst formation (Schuur et al., 2007; Mackelprang et al., 2011; Monteux et al., 2019). The atmospheric release of carbon trapped in soils following permafrost thaw is mediated by soil bacterial and fungal respiration. These organisms have been surviving in the active layer and frozen soil below for millennia, either frozen or respiring at extremely low rates, using the cold organic matter as an energy source (Tripathi et al., 2018). As the permafrost thaws, soil microbes exit dormancy and microbial communities gain access to large pools of previously frozen carbon and nutrients (Burkert et al., 2019). As the activity of the microbial communities increases with a warming climate, microbial effects may be more complicated than merely releasing greenhouse gases into the environment through respiration. These microbes can also cause alterations and feedback to ecosystem nutrient cycling and may in turn drive alterations to the plant community in associated soils (Natali et al., 2012; Du Toit, 2018).

Plant-microbe interactions within the rhizosphere are known determinants of plant health and productivity, and consequently, plant growth (Reynolds et al., 2003; Bever et al., 2010; Mangan et al., 2010). Interactions between microbes and plants are critical for the acquisition and cycling of nutrients such as biological nitrogen fixation and phosphate solubilization (de Souza et al., 2015). Highly studied environments such as agricultural production have shown evidence that small changes to soil microbiomes largely determine the success of plant growth and development (Van Wees et al., 2008; Lugtenberg and Kamilova, 2009). Wei et al. (2019) showed that within an agricultural monoculture system, the composition of the initial soil microbial inoculant predetermined whether a crop succumbed to disease or survived. Previous studies focused in the arctic and sub-arctic have demonstrated that soil microbes are important drivers of plant succession, relative abundance, and productivity (Schuur et al., 2007; Natali et al., 2012). As physical and chemical properties of soil such as temperature and pH are changing, the composition of microbial communities present in the active layer changes as well. Permafrost thaw could induce now active microbes to transform newly available organic matter into gaseous products such as CO_2 and CH_4, and metabolites that are now accessible to plants and other organisms living in and interacting with active layer soils (Graham et al., 2012). With the availability of newly released nutrients and the production of metabolites, soil microbes then have the potential to shape above ground plant communities by mediating and partitioning soil resources (Ho et al., 2017). Not only do beneficial microbes assist in nutrient accessibility, but they can also improve plant performance in a variety of systems by conferring resistance to pathogens.

Here we investigate how active layer soil microbial communities affect above ground plant communities, indicating that microbes play a role in climate change driven alterations to Alaskan plant communities. We hypothesized that plants inoculated with microbial communities from disturbed soils associated with greater permafrost thaw will experience lower levels of plant productivity compared to plants inoculated with microbes from less disturbed soils. We tested this hypothesis by conducting a plant growth experiment on boreal plant species inoculated with microbes from soils with different degrees of permafrost thaw and assessed soil microbial community composition.

MATERIALS AND METHODS

Sample Site Description and Soil Collection

The Fairbanks Permafrost Experiment Station (FPES) is located in interior Alaska (64.877°N, 147.670°W) and is part of the US Army Corps of Engineers Cold Regions Research and Engineering Laboratory (CRREL). FPES is divided into three Linell plots (Linell, 1973; Douglas et al., 2008), each 3,721 m^2: undisturbed (UD), semi disturbed (SD), and most disturbed (MD) (**Supplementary Figure 1**). When these three plots were created in 1946, the first plot, UD, was left untouched. The second plot (SD) was cleared of trees and above ground vegetation by hand, but the roots and other organic material were left intact. The third plot (MD) was stripped of all vegetation and surface level organic material down to mineral soil. Over the next 25 years, total stripping (MD) led to permafrost thaw and degradation to 6.7 m below surface level, and partial stripping (SD) led to thaw 4.7 m below surface level. These plots were created to simulate soil disturbance events and to then identify potential ecological effects.

Each plot is representative of the subarctic taiga forest. The undisturbed plot is covered by a dense black spruce stand (*Picea mariana*) with intermittent white spruce (*Picea glauca*). Its understory is dominated by Labrador tea (*Rhododendron groenlandicum*) and low-bush cranberry (*Vaccinium vitis-idaea*) with a ground cover of continuous feather and *Sphagnum* moss and lichen. The surface soils are moderately moist and can be classified as mesic, with an average soil organic layer thickness of 35 cm and a consistent thaw depth of 85 cm (Douglas et al., 2008; Johnstone et al., 2008). The semi-disturbed plot is covered by a mixed stand of Alaskan birch (*Betula neoalaskana*), willow (*Salix* sp.), black spruce, and white spruce, with a developing understory of moss and shrubs. The trees at the semi-disturbed plot are taller than those in the most disturbed. The most disturbed plot is covered by a mixed stand of Alaskan birch, willow, and young black spruce. The understory is a mixture of moss and grass (Douglas et al., 2008).

We collected 48 soil cores from FPES on 28 May 2018, 16 individual cores at each FPES treatment level. Using an established grid layout of FPES, we took cores from four selected quadrats per plot to demonstrate the fine-scale heterogeneity of the sample site. At each quadrat we took four samples at the corners, approximately 1 m apart. We utilized a sterile technique to sample soil and at each sampling point, the top layer of moss and vegetation was removed. We used a soil corer (4.5 cm diameter by 10 cm height) to collect the top 10 cm of soil, which were stored in a cooler throughout the duration of sample collection. We then transported the cores back to the lab where each was homogenized and stored at 4°C to be used 10 days later for soil inoculants and between 7 and 45 days later for DNA extractions.

Greenhouse Experimental Design and Sampling

To test how the sampled microbial communities affect plant growth, we grew all plants in a climate-controlled greenhouse, within autoclaved, nutrient-poor soils containing soil microbial inoculum. The soil microbial inoculants were obtained from FPES for the three treatment levels: UD, SD, and MD. We also included a sterile inoculant (ST), an autoclaved mixture of UD, SD, and MD soils.

We conducted the growth experiment in the Institute of Arctic Biology Greenhouse at the University of Alaska Fairbanks (UAF), consisting of five plant species: *Vaccinium vitis-idaea* (low-bush cranberry), *Vaccinium uliginosum* (bog blueberry); *Picea mariana* (black spruce); *Ledum groenlandicum* (Labrador tea); and *Chamerion angustifolium* (fireweed), hereafter all plants will be referred to by their colloquial names. We obtained all seeds from Alaskan sources, and we surface-sterilized them prior to planting. On 11 June 2018, we placed 5–10 seeds of one plant type into a pot (SC10 Cone-tainers, Ray Leach; United States) filled with a mixture of densely packed sterile vermiculite and Canadian peat (ProMix), with 1.5 g of soil containing the treatment microbial soil inoculant (**Figure 1**). Sixty-four pots were planted for each plant type, with 16 unique soil inoculants per each of four treatments, for a total of 320 pots. We randomized all pots across seven RL98 trays (Ray Leach, United States) to reduce possible bias from temperature or light gradients within the greenhouse. Plants were maintained under 12 h light cycles and watered once daily.

Following the first month of growth past germination, we measured the plants biweekly for the first 3 months, recording their leaf count and height to the nearest millimeter. We stopped black spruce needle counts in November 2018, when needle counts exceeded 200 and we were unable to accurately measure. By October 2018, all the fireweed (n = 61) had started to decline in height measurements, so on 10 October 2018, we clipped above ground living plant material at the soil surface and then oven dried plants 55°C to a constant mass before determining biomass produced. The bog blueberry, black spruce, Labrador tea, and low-bush cranberry continued to grow and be measured monthly. We then harvested above ground biomass per plant type in March 2020, and below ground biomass in April 2020. We oven dried plants at 55°C to a constant mass before weighing.

Statistical Analysis

All statistical analyses were performed in R version 3.5.2 (R Core Team, 2020). In order to test the effects of the microbial inoculant treatments during the plant growth experiment, we used the Shapiro-Wilk test and Levene's test to check ANOVA assumptions. We log-transformed growth measurements (leaf count, height, biomass) per plant type if it improved normality and reduced heteroscedasticity. We used a one-way ANOVA (Car R package, v3.0.3) to evaluate the significance of soil inoculant on each plant growth measurement within each plant type. We used a Tukey HSD (Agricolae R package, Mendiburu and Yaseen, 2009, v1.3.2) *post hoc* test to identify significant effects of specific inoculant treatments on growth within each plant type. We visualized differences using the R package ggplot2 v3.2.1).

DNA Extractions and Metagenomic Sequencing

In order to analyze the microbial communities used to inoculate plants in the growth experiment, we performed shotgun metagenomic sequencing on each individual core. To do this, we extracted and purified total genomic DNA from approximately 250 mg of soil per homogenized soil core using the DNeasy PowerSoil kit (Qiagen; Germany) following manufacturer instructions. We quantified the yield and the quality of the DNA extracted using a NanoDrop One spectrophotometer (Thermo Fisher Scientific; United States) and a Qubit (Thermo Fisher Scientific; United States). Following DNA extractions, we randomly divided the 48 cores into four sequencing runs. We prepared the DNA sequencing library using the Oxford Nanopore Technologies Ligation Kit with Native barcodes to multiplex 12 samples (SQK-LSK109, ONT; United Kingdom). We diluted sample DNA to 400 ng for input and followed the kit according to the manufacturer instructions. We then sequenced DNA using a MinION and R9.4.1 and R9.5 flow-cells (FLO-MIN106) (**Supplementary Table S1**). The sequencing runs each lasted 48 h.

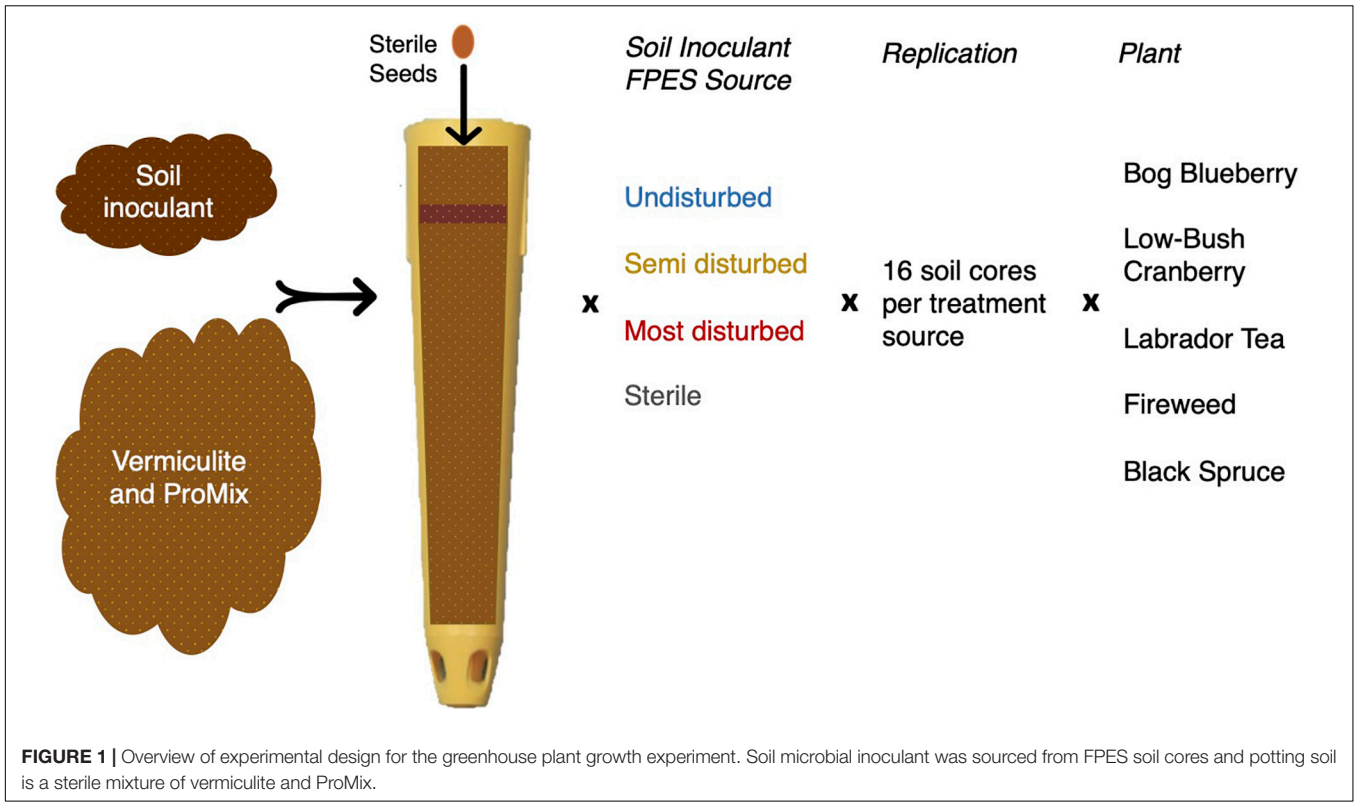

FIGURE 1 | Overview of experimental design for the greenhouse plant growth experiment. Soil microbial inoculant was sourced from FPES soil cores and potting soil is a sterile mixture of vermiculite and ProMix.

Metagenomic Analyses

We base called the raw data using Guppy v3.6.1 (ONT) specifying the high accuracy model (**Supplementary Table S1**). We then demultiplexed samples with the Guppy barcoder using parameters to discard sequences with middle adapters and to trim barcodes. We used Filtlong v0.2.0[1] to control for length (≥50 bp) and quality (Q) score (≥10). Following quality control, we detected taxa with a *k*-mer based approach using Kraken 2 (v.2.0.9-beta; Wood et al., 2019) and subsequently estimated abundance using Bracken (v.2.6.0; Lu et al., 2017). We used a reference database prepared with archaeal, bacterial, fungal, protozoal, and viral sequences from RefSeq on 22 May 2020 (Nicholls et al., 2019). We merged sample Bracken reports and then generated heat maps based on the most prevalent bacterial phyla, families, and genera with gplots v.3.0.3 in R. We excluded reads that were classified as fungal for any further analyses, due to the lack of breadth in our database.

In order to identify biomarkers within the microbial communities we used the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011). This method compares across all taxonomic levels and identifies differentially abundant features between biological classes using a non-parametric factorial Kruskal-Wallis sum-rank test. LEfSe then uses LDA to estimate the effect sizes of each feature. To accomplish this, we uploaded our Bracken results to the Galaxy web platform[2] (Afgan et al., 2018) where we then used the Huttenhower Lab workflow[3] for LEfSe analysis. We used an LDA threshold of 4.0 and significance α of 0.01 to detect biomarkers. Following the LEfSe analysis, we estimated Pearson's correlation values (stats R package v3.6.1), r, between plant productivity measures and the significant biomarkers that LEfSe identified.

RESULTS

Plant Growth Experiment

Our results indicate that soil microbial communities from across the thaw gradient differentially affected plant productivity (**Figures 2–6** and **Supplementary Tables S3–S8**). Most plant types responded negatively when grown in soils inoculated with microbial communities from the most disturbed FPES soil compared to either inoculants from semi disturbed or undisturbed soils, or the sterile treatment (**Supplementary Figure S2** and **Supplementary Table S8**).

Bog Blueberry

Bog blueberry had reduced growth in height and leaf count when grown in soils inoculated with microbial communities from the MD treatment site compared to plants growth with inoculants from UD, SD, or ST soil (**Figure 2**). This trend was consistent across height and leaf count starting at the third through the final measurement point (**Supplementary Figure S2**). While the mean height and leaf count of bog blueberry grown in MD treatment

[1]https://github.com/rrwick/Filtlong
[2]https://galaxyproject.org/
[3]https://huttenhower.sph.harvard.edu/galaxy/

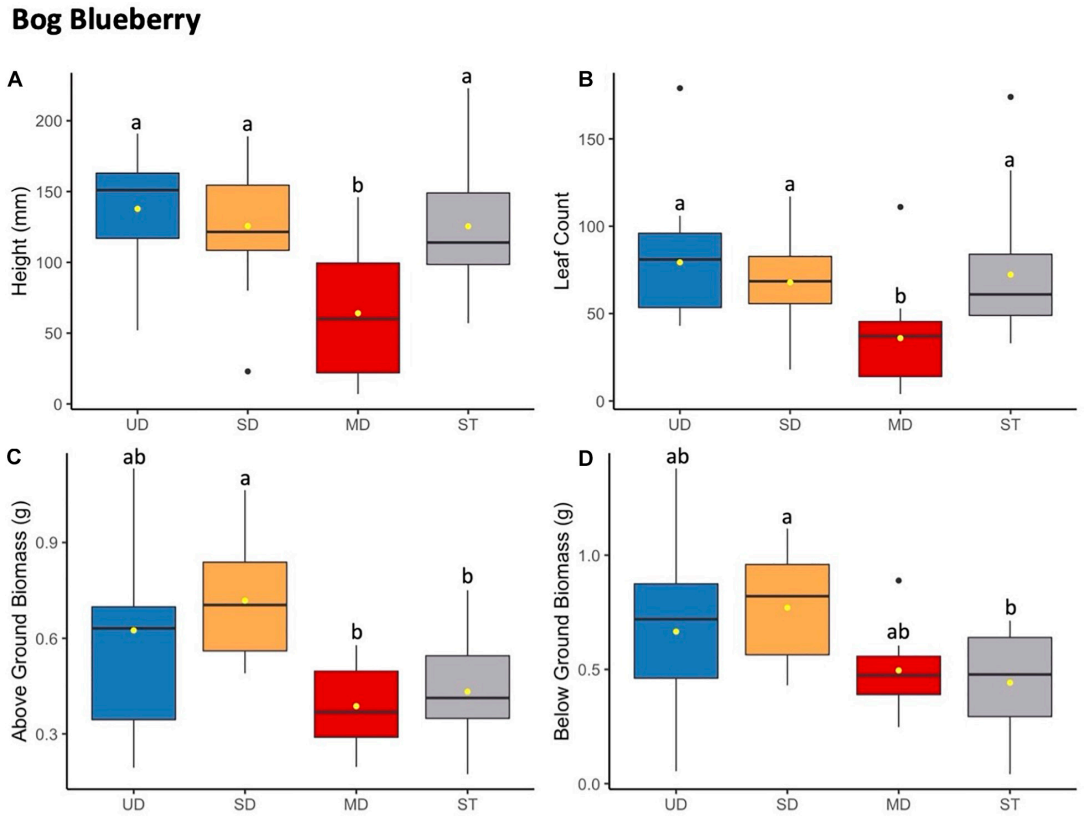

FIGURE 2 | Bog blueberry plant height (mm) **(A)** and leaf count **(B)** at 184 days since planting. Above ground **(C)** and below ground **(D)** biomass after 1.5 years since planting. Boxplots represent median, and the upper and lower quartiles. Yellow circles represent mean value for each group. Tukey HSD *post hoc* comparisons are denoted with lowercase letters above boxplots.

was significantly smaller compared to bog blueberry grown under the SD, UD, or ST treatments (**Supplementary Table S3**), there was no significant difference between height or leaf number for bog blueberry plants grown in UD, SD, or ST treatments. While the source of inoculant was a significant factor in above ground and below ground biomass, the responses did not follow the same trends as height and leaf count. For above ground biomass the *post hoc* Tukey HSD test showed that SD plants had more mass on average and differed significantly from MD and ST, SD plants did not differ significantly in biomass production (**Supplementary Table S8**).

Low-Bush Cranberry

Low-bush cranberry showed the same trends to bog blueberry when grown in soils from the most disturbed treatment site (**Figure 3**). Low-bush cranberry height and leaf count significantly decreased when grown with inoculant from the MD site compared to when grown with inoculant from the SD or UD sites, or with ST inoculant (**Supplementary Tables S4, S8**). There was no significant difference in either height or leaf count between low-bush cranberry grown in UD, SD, or ST treatments. Above ground biomass increased when plants were grown with soil from the SD compared to plants inoculated with UD, MD, and ST treatments. Low-bush cranberry below ground biomass did not differ significantly depending on the soil inoculant treatment.

Labrador Tea

Labrador tea plants displayed nearly identical trends to both bog blueberry and low-bush cranberry when grown in soils inoculated with microbial communities from the most disturbed treatment site (**Figure 4**). At the time of final measurements, the mean height and leaf count of Labrador tea grown in the MD treatment was significantly smaller compared to low-bush cranberry grown SD, UD, or ST treatments (**Supplementary Table S5**). There was no observed difference between Labrador tea grown in UD, SD, or ST treatments. Labrador tea above ground biomass showed that MD plants weighed in at a significantly smaller amount than plants grown in soils inoculated with UD or SD treatments. No differences were observed between below ground biomass measures (**Supplementary Table S8**).

Fireweed

In contrast to cranberry, bog blueberry, and Labrador tea, fireweed did not show a significant difference in mean leaf count or height (**Figure 5**); however, fireweed plants grown in inoculant from MD site showed a significant decrease in above ground

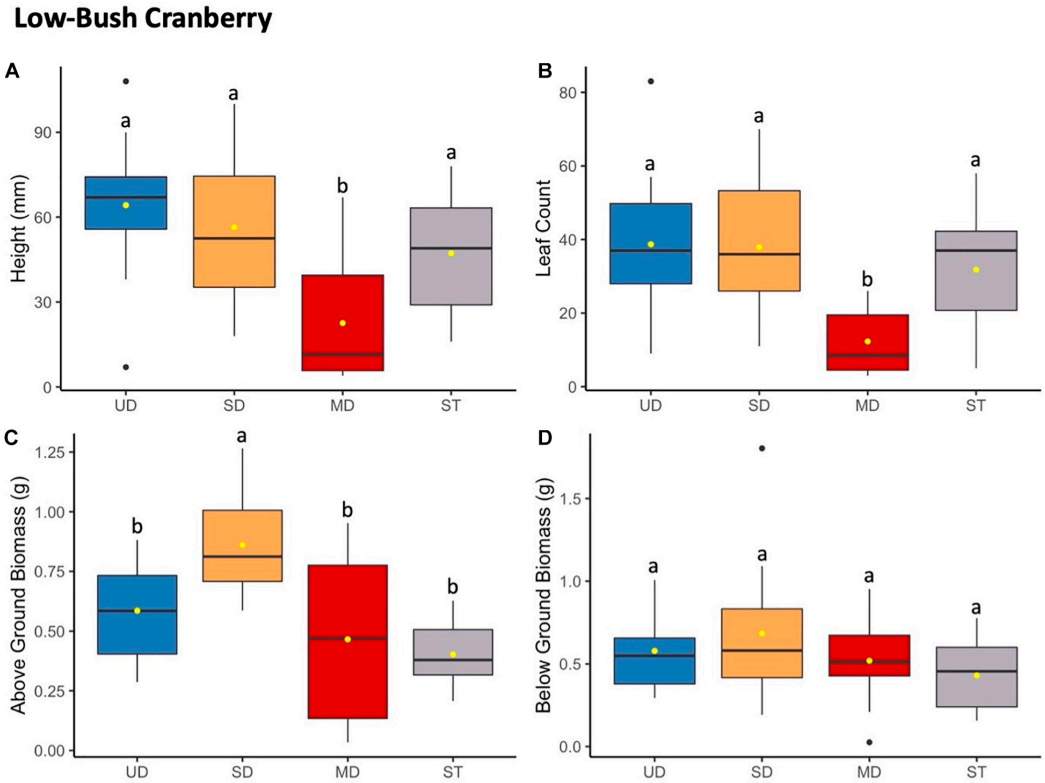

FIGURE 3 | Low-bush cranberry plant height (mm) **(A)** and leaf count **(B)** at 184 days since planting. Above ground **(C)** and below ground **(D)** biomass after 1.5 years since planting. Boxplots represent median, and the upper and lower quartiles. Yellow circles represent mean value for each group. Tukey HSD *post hoc* comparisons are denoted with lowercase letters above boxplots.

biomass compared to those grown in UD, SD, or ST treatment soils (**Figure 5** and **Supplementary Tables S6, S8**).

Black Spruce

Black spruce showed no consistent response patterns to microbial inoculant for growth and biomass measures (**Figure 6** and **Supplementary Table S7**). Black spruce plants grew significantly taller when grown with the sterile inoculant (ST) compared to UD, SD, or MD inoculants. Below ground and above ground biomass showed both showed a similar pattern of ST plants measuring at a significantly lower biomass compared to SD plants, and with MD and UD plants showing no significant differences in mass. For leaf (needle) count, no significant differences were observed across all treatment inoculant groups. Leaf count was discontinued and excluded from analysis after needle numbers exceeded 200 for all plants and was not able to be accurately measured.

Microbial Community Analysis

We sequenced 48 metagenomes through four multiplexed MinION runs, and after demultiplexing and quality control, total sample reads showed a mean read length of 2,594 bp, a N50 of 5,531 bp, and a mean quality score of 13 (**Supplementary Table S2**). All samples had an average yield of 373 Mbase (range: 182–737 Mbase) with an average read count of 143 K reads (range: 53–299 K). The analysis using Kraken 2 and then Bracken resulted in a mean of 41% (range: 30–60%) of reads being classified as bacterial (60,034 reads). The percent classified did not show a correlation to the read depth of the sample (**Supplementary Figure S3**).

Following classification via Kraken 2 and Bracken, we identified 24 bacterial phyla within our microbial communities. A majority of samples across the three levels of disturbance were largely dominated by Proteobacteria and Actinobacteria with mean relative abundances of 62.9 and 19.9%, respectively. Of the 24 bacterial phyla identified, eight were present at relative abundances higher than 1% (**Figure 7A**). We observed a general shift in community membership and abundance across the disturbance gradient, with both the UD and MD soil cores showing variation in the dominant taxonomic groups present, all the way down to the genus level (**Figures 7B,C**). Among the 59 genera present at more than 1% relative abundance, *Bradyrhizobium* and *Streptomyces* were present at the highest relative abundances, with means of 10.2 and 6.9%, respectively.

To further analyze the taxonomic shifts, we utilized the LEfSe method to identify any biomarkers across samples. Out of only the bacterial reads, we identified 13 differentially abundant biomarkers across the phylum to family level within our dataset (**Table 1**). Four of the biomarkers, that all fall under the same taxonomic branch of the phylum Acidobacteria and down to

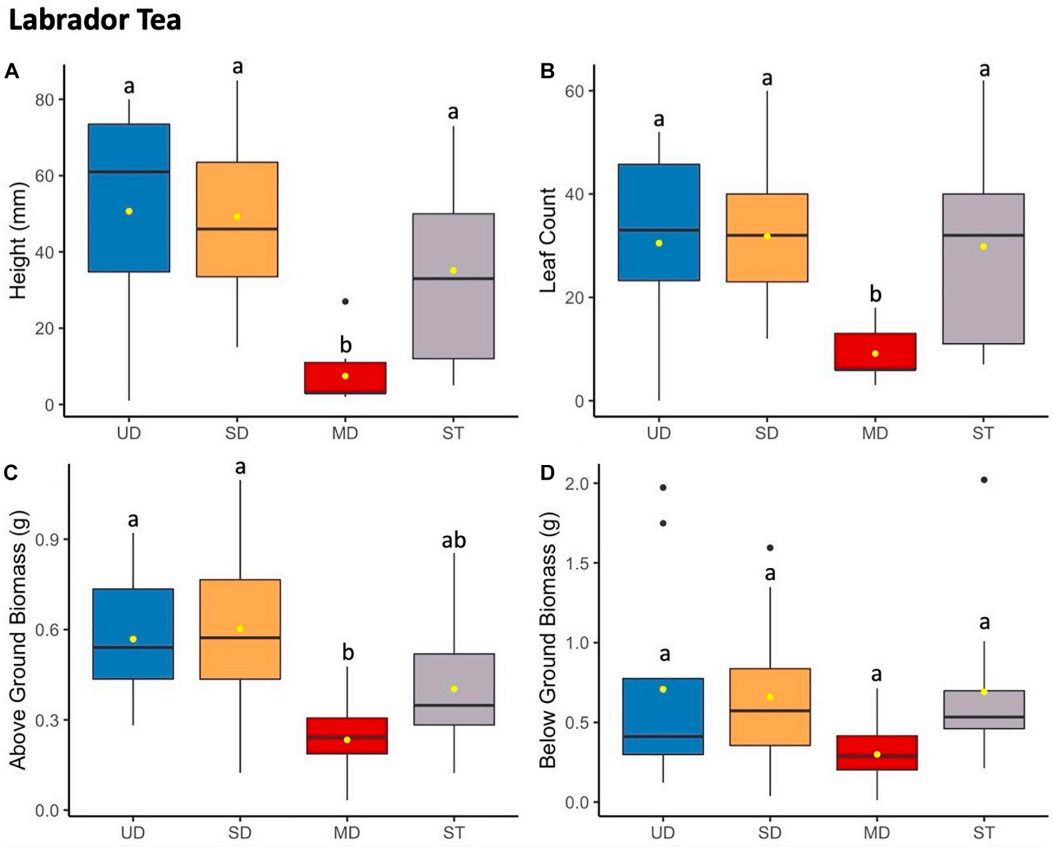

FIGURE 4 | Labrador tea plant height (mm) **(A)** and leaf count **(B)** at 184 days since planting. Above ground **(C)** and below ground **(D)** biomass after 1.5 years since planting. Boxplots represent median, and the upper and lower quartiles. Yellow circles represent mean value for each group. Tukey HSD *post hoc* comparisons are denoted with lowercase letters above boxplots.

the family *Acidobacteriaceae* were found to be overrepresented in UD soil communities. Nine biomarkers were found to be overrepresented in MD soil communities, including members of the phyla Proteobacteria and Actinobacteria (**Table 1**).

Using the 13 biomarkers that we identified through LEfSe, we completed correlation analyses (Pearson's correlation coefficient) between the taxonomic biomarkers and plant productivity measures. We found the relative abundance of UD biomarkers to be positively correlated with the height and leaf count for blueberry and cranberry, and with the leaf count of black spruce. One of the UD biomarkers, *Acidobacteriaceae,* showed the strongest positive correlation between growth measures, and was present at a mean relative abundance of 18% in UD communities compared to the SD (5%) and MD (>5%) soil communities (**Table 1** and **Figure 8A**). Of the MD biomarkers, all nine showed significant negative correlations between relative abundance and the productivity measures for blueberry, cranberry, and Labrador tea (**Table 1**). Looking at the family level, the most negative correlations occurred between plant growth and *Comamonadaceae,* a family that was found to be present at significantly higher relative abundances within the MD soil microbial communities with a mean of 16%, compared to in UD (5%) and SD (8%) soils (**Table 1** and **Figure 8B**). We observed no significant correlations between fireweed plant productivity measures and biomarkers (**Table 1**).

DISCUSSION

Our results indicate that there is a strong relationship between microbial community inoculant and total plant growth. We have evidence that microbes associated with highly disturbed soil negatively affect plant productivity within several boreal plant species. Within our study we see similar growth response patterns across low-bush cranberry, bog blueberry, Labrador tea, and fireweed plants. Our analyses also revealed that microbial community membership and abundance shifts across active layer soils above a permafrost thaw gradient. Several studies have also observed soil microbial community responses to permafrost thaw and soil disturbance events (Mackelprang et al., 2011; Coolen and Orsi, 2015; Mondav et al., 2017), and previous research completed by Schütte et al. (2019) has identified links between fungal microbial communities associated with permafrost thaw and plant growth.

Our research showed that plants including bog blueberry, low-bush cranberry, and Labrador tea demonstrated decreased height

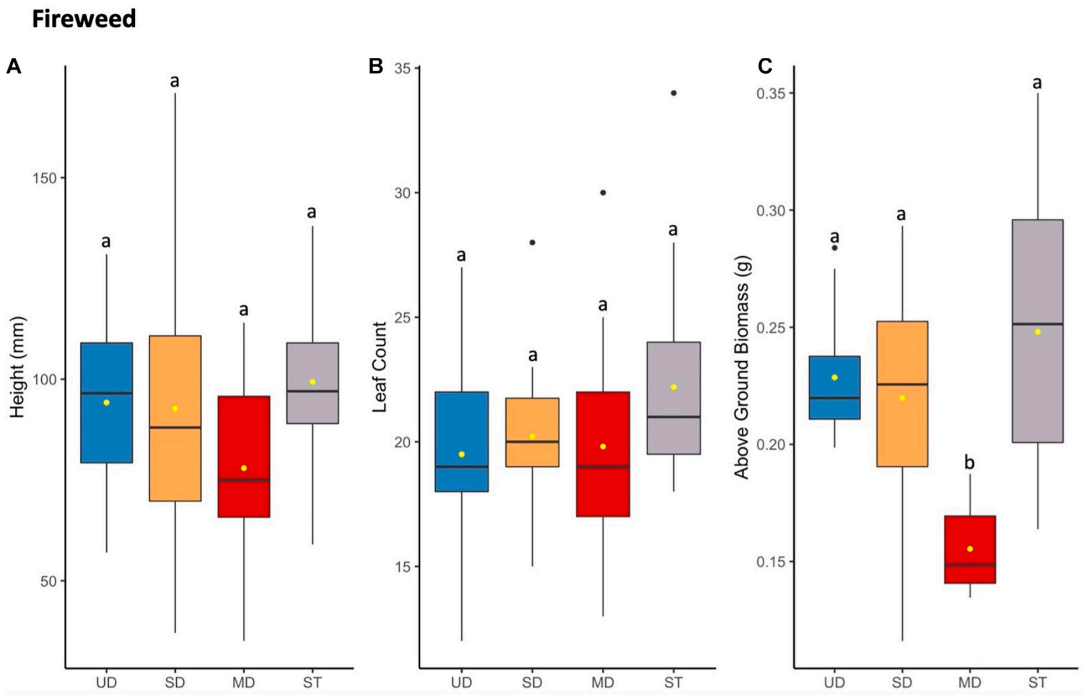

FIGURE 5 | Fireweed plant height (mm) **(A)** and leaf count **(B)** at 121 days since planting. Above ground **(C)** biomass of plants after 165 days since planting. Boxplots represent median, and the upper and lower quartiles. Yellow circles represent mean value for each group. Tukey HSD *post hoc* comparisons are denoted with lowercase letters above boxplots.

and leaf count when grown with microbial communities from highly disturbed soil that was associated with deep permafrost thaw compared to undisturbed soil associated with little to no permafrost thaw. Fireweed plants did not show any changes in growth dependent on soil inoculant when looking at leaf count and height. However, when looking at above-ground biomass, plants inoculated with MD soil communities did exhibit lower levels of growth compared to plants inoculated with UD or SD soils. The evidence of this pattern when looking at biomass could potentially be due to the large variation in leaf area and stem thickness that was not present within other plant species that we grew. Additionally, Fireweed is a robust perennial plant, which is known to tolerate a wide range of soil conditions. It is early successional and very commonly following vegetation disturbance (Dyrness, 1973; Pinno et al., 2014). Due to these factors, we speculated that fireweed would not be affected by the disturbance level of the inoculum.

We can attribute the changes in plant growth to the variation in microbial communities across the active layer of soil above the permafrost thaw gradient. We hypothesized that if the soil microbial communities were to change depending on the level of associated permafrost thaw, so would the growth of plants in that soil. Consistent with our hypothesis, plants (specifically bog blueberry, low-bush cranberry, Labrador tea, and fireweed) grown with microbial inoculum from MD soils exhibited lower growth than plants grown in soils with no added microbes or with inoculants from the UD or SD soils. These results were similar to a study performed by Schütte et al. (2019) on boreal plant species using microbial inoculums from thermokarst bogs (disturbed soils) and permafrost plateaus (undisturbed soil). Schütte et al. (2019) showed that bog blueberry and marsh-cinquefoil (*Potentilla palustris*) grew significantly worse when inoculated with soil microbes from thermokarst bogs than when inoculated by permafrost plateau soil microbes.

We found no apparent relationship between black spruce growth and the initial soil microbial inoculant. While Schütte et al. (2019) found that biomass decreased black spruce when grown with inoculum from a thawed permafrost site, our results are consistent with Sniderhan and Baltzer (2016) analyzing the growth of black spruce across a lateral permafrost thaw gradient in Scotty Creek, Canada. They found that the lateral thaw rate of permafrost did not appear to be a driver of black spruce growth dynamics. Further, controlled warming experiments meant to simulate the quickly warming northern latitudes have shown that black spruce shoot length tends to increase with warming air and soil temperatures (Bronson et al., 2009; Bronson and Gower, 2010). This suggests that while productivity of black spruce may be decreased in some thaw scenarios, overall abiotic factors may be having larger influences on black spruce productivity compared to biotic factors during climate warming events.

Previous studies using both culture-based and culture-independent approaches have been able to identify significant functional and taxonomic shifts in soil microbiomes resulting from various types of disturbance (Axelrood et al., 2002; Mendes et al., 2015; Van Nuland et al., 2020). Variation in

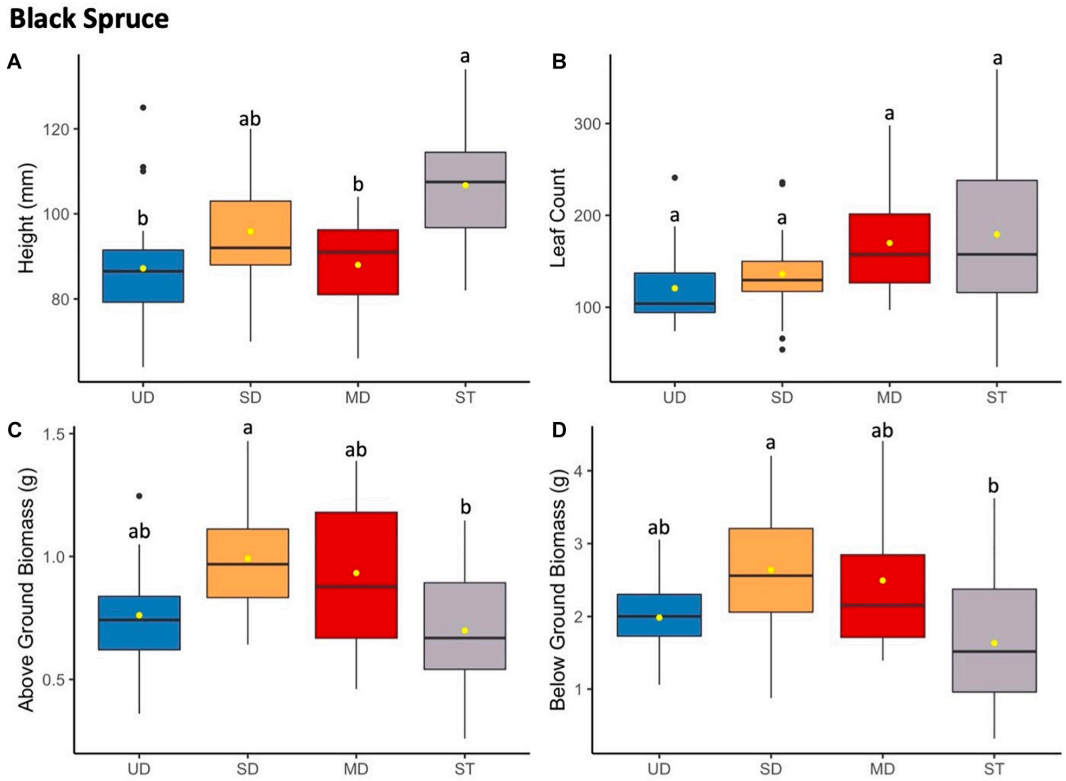

FIGURE 6 | Black spruce height (mm) **(A)** and leaf count **(B)** at 184 and 121 days since planting, respectively. Above ground **(C)** and below ground **(D)** biomass after 1.5 years since planting. Boxplots represent median, and the upper and lower quartiles. Yellow circles represent mean value for each group. Tukey HSD *post hoc* comparisons are denoted with lowercase letters above boxplots.

plant growth across treatments could largely be due to a change in representation from growth-promoting bacterial and fungal species to deleterious, pathogenic ones found in the soil microbiomes between treatment groups. Beneficial microbes in soil are known to enhance nutrient availability to plants, allowing for an increase in plant growth and productivity (Vimal et al., 2017). Common indicators of a neutral or healthy soil microbiome include wide array of bacterial phyla such as *Proteobacteria, Acidobacteria, Actinobacteria,* and *Bacteriodetes,* as well as and fungal phyla such as *Glomeromycota* (Fierer et al., 2005; Ohsowski et al., 2014; Mendes et al., 2015). Consistent with such a roll, and perhaps the most striking difference in microbial taxa between treatment groups, was the bacterial family *Acidobactericae,* which was largely overrepresented in UD soil communities. *Acidobacteriaceae* was also positively correlated with plant productivity measures of both blueberry and cranberry plants, two plants that showed significantly different growth responses between the different inoculant groups. Members of the *Acidobacteriacae* are thought to be plant-promoters and degrade a variety of simple carbon compounds, such as those that are found in root exudates, which contribute to nutrient cycling and the support of a healthy rhizosphere (Campbell et al., 2010; Kielak et al., 2016). Other groups of bacteria that were found to be present at higher relative abundances in the UD soil cores compared to MD soils, including *Bacillales* which can form endospores and provide protection against and *Enterobacterales*, are both ubiquitous in soil microbiomes.

In contrast, the MD soil cores displayed a much higher relative abundance of bacteria from the family *Comamonadaceae,* a member of *Proteobacteria,* than either the UD or SD cores. Additionally, *Comamonadaceae* showed the strongest negative correlation with plant productivity of all identified biomarkers, indicating that members of this family may be driving the decrease in growth that we observed with blueberry, cranberry, and Labrador tea plants. Members of the family *Comamonadaceae* are known to exhibit pathogenic effects on a variety of plants, such as causing bacterial fruit blotch disease in agricultural settings (Willems et al., 1991; Schaad et al., 2008; Burdman and Walcott, 2012). van der Voort et al. (2016) and Yang et al. (2020) have also showed that soils stressed by drought and heat disturbance, respectively, were found to have overrepresented populations of known plant pathogens including members of *Comamonadaceae* and *Erwiniaceae*. Although few studies have looked at the effects of physical disturbances on microbiomes specifically in arctic or subarctic soils, in previous research we cultured and isolated bacterial strains belonging to *Erwiniaceae* from the FPES MD soil plot (Humphrey et al., 2019). It is possible that the higher presence of bacterial groups including known plant-pathogenic bacteria found in the

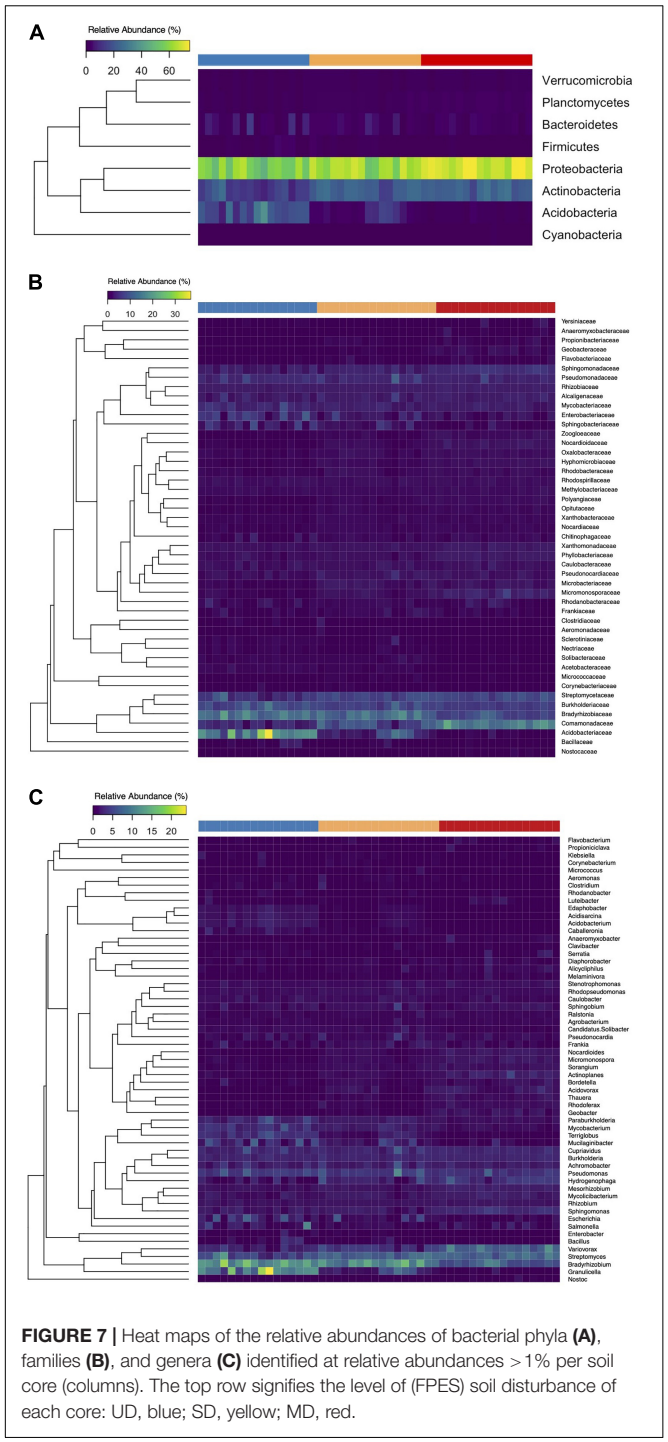

FIGURE 7 | Heat maps of the relative abundances of bacterial phyla **(A)**, families **(B)**, and genera **(C)** identified at relative abundances >1% per soil core (columns). The top row signifies the level of (FPES) soil disturbance of each core: UD, blue; SD, yellow; MD, red.

MD soil microbiomes is leading to the decrease in associated plant growth, caused by a disruption in nutrient cycling and direct alterations to the plant rhizospheres.

Our results suggest that a decrease in plant growth can be linked to changes in the taxonomic makeup of microbial communities. These observed patterns are consistent with Schütte et al. (2019) and underline the importance of understanding plant-soil interactions post disturbance. Data

TABLE 1 | Pearson's correlation estimates (*r*) between plant productivity and microbial community biomarkers.

			Blueberry		Cranberry		Labrador tea		Fireweed		Black spruce	
Taxonomic rank	**Taxa**	**Log_{10} of LDA value**	**Leaf count**	**Height**	**Leaf count**	**Height**	**Leaf count**	**Height**	**Leaf count**	**Height**	**Leaf count**	**Height**
Class	Acidobacteriia	5.28	**0.34**	**0.37**	**0.35**	**0.48**	0.20	0.29	−0.05	0.22	**−0.33**	−0.03
Phylum	Acidobacteria	5.28	**0.33**	**0.37**	0.25	**0.49**	0.20	0.29	−0.05	0.22	**−0.33**	−0.04
Order	Acidobacteriales	5.27	**0.34**	**0.37**	**0.34**	**0.47**	0.19	0.28	−0.05	0.22	**−0.32**	−0.04
Family	*Acidobacteriaceae*	5.27	**0.34**	**0.37**	**0.34**	**0.47**	0.19	0.28	−0.05	0.22	**−0.32**	−0.04
Class	Betaproteobacteria	5.48	**−0.43**	**−0.55**	**−0.53**	**−0.59**	**−0.45**	**−0.58**	0.01	−0.25	0.24	0.02
Phylum	Proteobacteria	5.84	**−0.36**	**−0.41**	**−0.47**	**−0.56**	−0.18	**−0.35**	−0.06	−0.25	0.20	0.04
Family	*Comamonadaceae*	5.20	**−0.48**	**−0.56**	**−0.49**	**−0.54**	**−0.47**	**−0.59**	0.07	−0.23	0.26	0.03
Order	Burkholderiales	5.44	**−0.42**	**−0.54**	**−0.51**	**−0.58**	**−0.43**	**−0.57**	0.04	−0.21	0.23	0.03
Class	Deltaproteobacteria	4.65	**−0.45**	**−0.53**	**−0.55**	**−0.54**	**−0.42**	**−0.54**	−0.10	−0.31	**0.31**	−0.07
Order	Micromonosporales	4.50	**−0.46**	**−0.51**	**−0.57**	**−0.52**	**−0.44**	**−0.48**	0.04	−0.12	**0.50**	−0.09
Family	*Micromonosporaceae*	4.50	**−0.46**	**−0.51**	**−0.57**	**−0.52**	**−0.44**	**−0.48**	0.04	−0.12	**0.50**	−0.09
Order	Propionibacteriales	4.35	**−0.40**	**−0.48**	**−0.53**	**−0.53**	**−0.33**	**−0.41**	0.01	−0.21	**0.35**	0.00
Order	Myxococcales	4.48	**−0.46**	**−0.56**	**−0.51**	**−0.51**	**−0.35**	**−0.49**	−0.04	−0.25	0.26	−0.02

Pearson correlation estimates (r): bold indicates significance with a $P < 0.05$. Log_{10} of LDA values: blue = overrepresented in UD communities, red = overrepresented in MD communities.

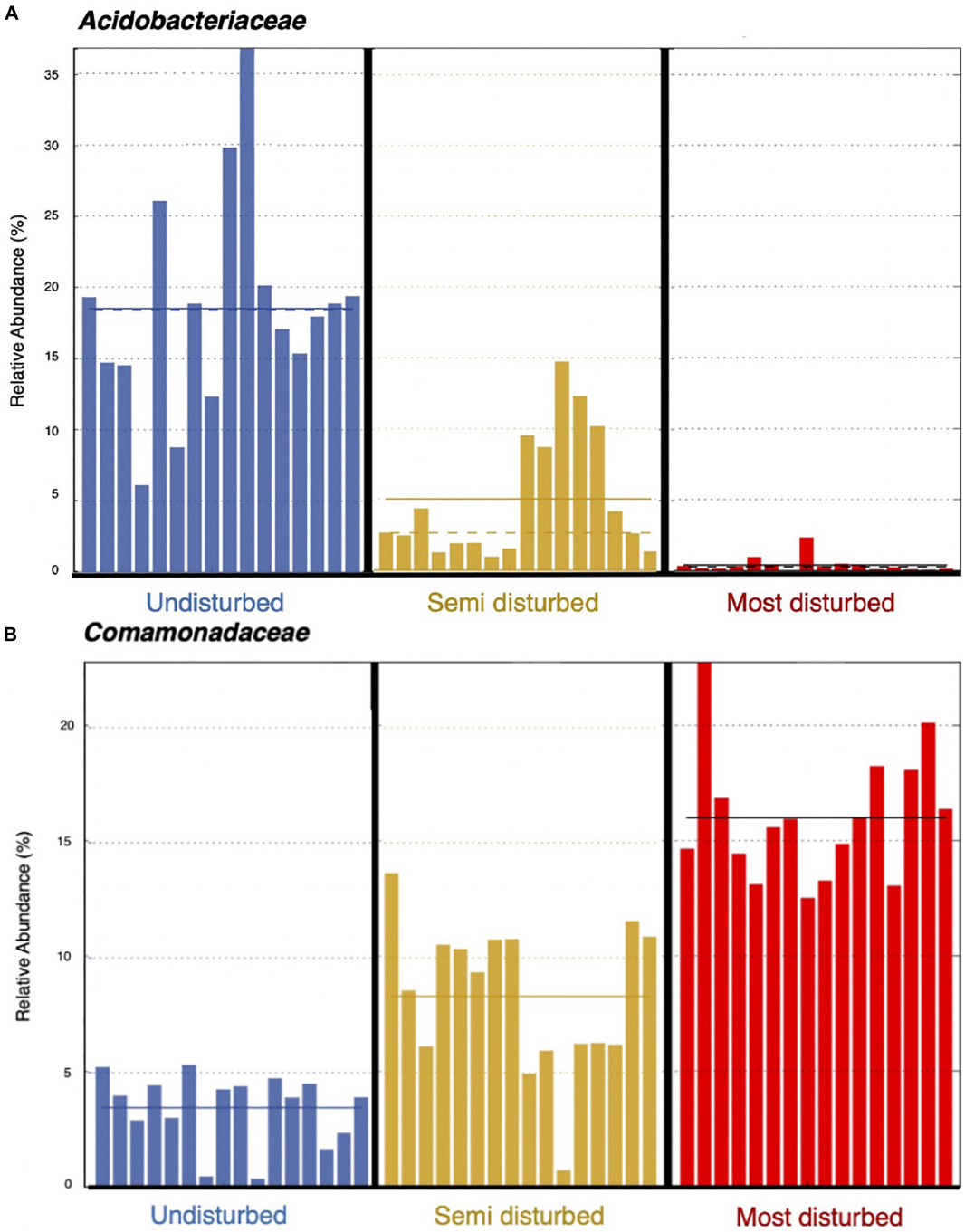

FIGURE 8 | Relative abundances of bacterial families, *Acidobacteriaceae* **(A)** and *Comamonadaceae* **(B)**, per soil core. Solid line represents mean relative abundance per treatment group.

from other disturbance gradients are needed test the generality of these observed patterns. Furthermore, long term studies at sites such as the Alaska Peatland Experiment within the Bonanza Creek LTER demonstrate the effects of permafrost thaw on plant communities, trace gas fluxes, microbial communities, and biogeochemical processes (Mackelprang et al., 2011; Blazewicz et al., 2012; Euskirchen et al., 2014; Klapstein et al., 2014; Hultman et al., 2015; Finger et al., 2016; Neumann et al., 2016; Rupp et al., 2019). It is important that more experimentation integrating microbial mechanisms driving plant growth are needed to understand the patterns we have observed.

Having an increased understanding of how microbial communities that reside above permafrost affect plant growth is important for predicting effects of permafrost thaw on plant

communities and ecosystem health. Specifically, for potentially predicting effects on plants, such as bog blueberry, low-bush cranberry, and Labrador tea, that are relied on as common food sources for both humans and animals throughout the Northern regions. Although the links between soil microbial and plant communities are complex and rarely straight-forward, this study indicates that the variation in active layer soil microbiomes can have a strong effect on plant growth. This data point toward building an understanding of how shifting microbial communities may affect plant growth in the face of climate change causing thawing permafrost; specifically, growth of plants such as bog blueberry and low-bush cranberry, both plants integral to the diets and cultures of many people indigenous to Alaska. The results reported here demonstrate that soil microbes have the capability to alter plant growth in response to climate change, therefore highlighting the need for further research on the taxonomy and functionality of microbial communities residing above thawing permafrost.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: European Nucleotide Archive (ENA) under study accession number PRJEB42020.

AUTHOR CONTRIBUTIONS

US contributed to the concept, design, and analysis of the study. TS performed the data collection and statistical analysis and wrote the first draft of the manuscript. DD was involved in experimental design, data collection, bioinformatic analysis, and drafting the manuscript. All authors contributed to the manuscript revisions and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.619711/full#supplementary-material

REFERENCES

Afgan, E., Baker, D., Batut, B., Van Den Beek, M., Bouvier, D., Ech, M., et al. (2018). The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* 46, W537–W544. doi: 10.1093/nar/gky379

Axelrood, P. E., Chow, M. L., Arnold, C. S., Lu, K., McDermott, J. M., and Davies, J. (2002). Cultivation-dependent characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Can. J. Microbiol.* 48, 643–654. doi: 10.1139/w02-058

Bever, J. D., Dickie, I. A., Facelli, E., Facelli, J. M., Klironomos, J., Moora, M., et al. (2010). Rooting theories of plant community ecology in microbial interactions. *Trends Ecol. Evol.* 25, 468–478. doi: 10.1016/j.tree.2010.05.004

Blazewicz, S. J., Petersen, D. G., Waldrop, M. P., and Firestone, M. K. (2012). Anaerobic oxidation of methane in tropical and boreal soils: ecological significance in terrestrial methane cycling. *J. Geophys. Res.* 117:G02033. doi: 10.1029/2011jg001864

Bronson, D. R., and Gower, S. T. (2010). Ecosystem warming does not affect photosynthesis or aboveground autotrophic respiration for boreal black spruce. *Tree Physiol.* 30, 441–449. doi: 10.1093/treephys/tpq001

Bronson, D. R., Gower, S. T., Tanner, M., and Van Herk, I. (2009). Effect of ecosystem warming on boreal black spruce bud burst and shoot growth. *Glob. Chang. Biol.* 15, 1534–1543. doi: 10.1111/j.1365-2486.2009.01845.x

Burdman, S., and Walcott, R. O. N. (2012). Acidovorax citrulli: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. *Mol. Plant Pathol.* 13, 805–815. doi: 10.1111/j.1364-3703.2012.00810.x

Burkert, A., Douglas, T. A., Waldrop, M. P., and Mackelprang, R. (2019). Changes in the active, dead, and dormant microbial community structure across a pleistocene permafrost chronosequence. *Appl. Environ. Microbiol.* 85:e02646-18. doi: 10.1128/AEM.02646-18

Campbell, B. J., Polson, S. W., Hanson, T. E., Mack, M. C., and Schuur, E. A. G. (2010). The effect of nutrient deposition on bacterial communities in Arctic tundra soil. *Environ. Microbiol.* 12, 1842–1854. doi: 10.1111/j.1462-2920.2010.02189.x

Chapin, F. S., Robards, M. D., Huntington, H. P., Johnstone, J. F., Trainor, S. F., Kofinas, G. P., et al. (2006). Directional changes in ecological communities and social-ecological systems: a framework for prediction based on Alaskan examples. *Am. Nat.* 168, S36–S49. doi: 10.1086/509047

Christensen, T. R., Johansson, T., Åkerman, H. J., Mastepanov, M., Malmer, N., Friborg, T., et al. (2004). Thawing sub-arctic permafrost: effects on vegetation and methane emissions. *Geophys. Res. Lett.* 31:L04501. doi: 10.1029/2003GL018680

Coolen, M. J. L., and Orsi, W. D. (2015). The transcriptional response of microbial communities in thawing Alaskan permafrost soils. *Front. Microbiol.* 6:197. doi: 10.3389/fmicb.2015.00197

Department of Natural Resources: Alaska Division of Geological and Geophysical Surveys (2020). *Permafrost and Periglacial Hazards*. Available online at: https://dggs.alaska.gov/hazards/permafrost.html (accessed July 25, 2020).

de Souza, R., Ambrosini, A., and Passaglia, L. M. P. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genet. Mol. Biol.* 38, 401–419. doi: 10.1590/S1415-475738420150053

Douglas, T., Kanevskiy, M., Romanovsky, V., Shur, Y., and Yoshikawa, K. (2008). "Permafrost dynamics at the Fairbanks permafrost experimental station near Fairbanks, Alaska," in *Proceedings of the 9th International Conference on Permafrost*, Alaska, 373–378.

Du Toit, A. (2018). Permafrost thawing and carbon metabolism. *Nat. Rev. Microbiol.* 16:519. doi: 10.1038/s41579-018-0066-4

Dyrness, C. T. (1973). Early Stages of plant succession following logging and burning in the Western Cascades of Oregon. *Ecology* 54, 57–69. doi: 10.2307/1934374

Euskirchen, E. S., Edgar, C. W., Turetsky, M. R., Waldrop, M. P., and Harden, J. W. (2014). Differential response of carbon fluxes to climate in three peatland ecosystems that vary in the presence and stability of permafrost. *J. Geophys. Res.* 119, 1576–1595. doi: 10.1002/2014jg002683

Fierer, N., Jackson, J. A., Vilgalys, R., and Jackson, R. B. (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* 71, 4117–4120. doi: 10.1128/AEM.71.7.4117-4120.2005

Finger, R. A., Turetsky, M. R., Kielland, K., Ruess, R. W., Mack, M. C., and Euskirchen, E. S. (2016). Effects of permafrost thaw on nitrogen availability and plant–soil interactions in a boreal Alaskan lowland. *J. Ecol.* 104, 1542–1554. doi: 10.1111/1365-2745.12639

Graham, D. E., Wallenstein, M. D., Vishnivetskaya, T. A., Waldrop, M. P., Phelps, T. J., Pfiffner, S. M., et al. (2012). Microbes in thawing permafrost: the unknown variable in the climate change equation. *ISME J.* 6, 709–712. doi: 10.1038/ismej.2011.163

Ho, A., Di Lonardo, D. P., and Bodelier, P. L. E. (2017). Revisiting life strategy concepts in environmental microbial ecology. *FEMS Microbiol. Ecol.* 93:fix006. doi: 10.1093/femsec/fix006

Hultman, J., Waldrop, M. P., Mackelprang, R., David, M. M., Mcfarland, J., Blazewicz, S. J., et al. (2015). Multi-omics of permafrost, active layer and thermokarst bog soil microbiomes. *Nature* 521, 208–212. doi: 10.1038/nature14238

Humphrey, J., Seitz, T., Haan, T., Ducluzeau, A.-L., and Drown, D. M. (2019). Complete Genome Sequence of *Pantoea* agglomerans TH81, isolated from a permafrost thaw gradient. *Microbiol. Resour. Announc.* 8:e01486-18. doi: 10.1128/mra.01486-18

Jansson, J. K., and Hofmockel, K. S. (2018). The soil microbiome — from metagenomics to metaphenomics. *Curr. Opin. Microbiol.* 43, 162–168. doi: 10.1016/j.mib.2018.01.013

Johnstone, J. F., Hollingsworth, T. N., and Chapin, F. S. III (2008). *A Key for Predicting Postfire Successional Trajectories in Black Spruce Stands of Interior Alaska*. General Technical Report – Pacific Northwest Res. Station. USDA For. Serv. 1–44. Available online at: http://www.treesearch.fs.fed.us/pubs/31457 (accessed July 25, 2020).

Kielak, A. M., Cipriano, M. A. P., and Kuramae, E. E. (2016). Acidobacteria strains from subdivision 1 act as plant growth-promoting bacteria. *Arch. Microbiol.* 198, 987–993. doi: 10.1007/s00203-016-1260-2

Klapstein, S. J., Turetsky, M. R., Mcguire, A. D., Harden, J. W., Czimczik, C. I., Xu, X., et al. (2014). Controls on methane released through ebullition in peatlands affected by permafrost degradation. *J. Geophys. Res.* 119, 418–431. doi: 10.1002/2013jg002441

Linell, K. A. (1973). "Long-term effects of vegetative cover on permafrost stability in an area of discontinuous permafrost," in *Proceedings of the 2nd International Conference on Permafrost: North American Contribution*, (Washington, DC: National Academy of Sciences), 688–693.

Lu, J., Breitwieser, F. P., Thielen, P., and Salzberg, S. L. (2017). Bracken: estimating species abundance in metagenomics data. *PeerJ. Comput. Sci.* 2017:e104. doi: 10.7717/peerj-cs.104

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting Rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Mackelprang, R., Waldrop, M. P., Deangelis, K. M., David, M. M., Chavarria, K. L., Blazewicz, S. J., et al. (2011). Metagenomic analysis of a permafrost microbial community reveals a rapid response to thaw. *Nature* 480, 368–371. doi: 10.1038/nature10576

Mangan, S. A., Schnitzer, S. A., Herre, E. A., MacK, K. M. L., Valencia, M. C., Sanchez, E. I., et al. (2010). Negative plant-soil feedback predicts tree-species relative abundance in a tropical forest. *Nature* 466, 752–755. doi: 10.1038/nature09273

Mendes, L. W., Tsai, S. M., Navarrete, A. A., de Hollander, M., van Veen, J. A., and Kuramae, E. E. (2015). Soil-borne microbiome: linking diversity to function. *Microb. Ecol.* 70, 255–265. doi: 10.1007/s00248-014-0559-2

Mendiburu, F., and Yaseen, M. (2009). *agricolae: Statistical Procedures for Agricultural Research*. R package version 1.3.2. Available online at: https://myaseen208.github.io/agricolae/ and https://cran.r-project.org/package=agricolae

Mondav, R., McCalley, C. K., Hodgkins, S. B., Frolking, S., Saleska, S. R., Rich, V. I., et al. (2017). Microbial network, phylogenetic diversity and community membership in the active layer across a permafrost thaw gradient. *Environ. Microbiol.* 19, 3201–3218. doi: 10.1111/1462-2920.13809

Monteux, S., Weedon, J. T., Blume-Werry, G., Gavazov, K., Jassey, V. E. J., Johansson, M., et al. (2019). Correction: long-term in situ permafrost thaw effects on bacterial communities and potential aerobic respiration. *ISME J.* 13, 2140–2142. doi: 10.1038/s41396-019-0384-1

Natali, S. M., Schuur, E. A. G., and Rubin, R. L. (2012). Increased plant productivity in Alaskan tundra as a result of experimental warming of soil and permafrost. *J. Ecol.* 100, 488–498. doi: 10.1111/j.1365-2745.2011.01925.x

Neumann, R. B., Blazewicz, S. J., Conaway, C. H., Turetsky, M. R., and Waldrop, M. P. (2016). Modeling CH4 and CO2 cycling using porewater stable isotopes in a thermokarst bog in Interior Alaska: results from three conceptual reaction networks. *Biogeochemistry* 127, 57–87. doi: 10.1007/s10533-015-0168-2

Nicholls, S. M., Quick, J. C., Tang, S., and Loman, N. J. (2019). Ultra-deep, long-read nanopore sequencing of mock microbial community standards. *Gigascience* 8:5. doi: 10.1093/gigascience/giz043

Ohsowski, B. M., Zaitsoff, P. D., Öpik, M., and Hart, M. M. (2014). Where the wild things are: looking for uncultured Glomeromycota. *New Phytol.* 204, 171–179. doi: 10.1111/nph.12894

Pinno, B. D., Landhäusser, S. M., Chow, P. S., Quideau, S. A., and MacKenzie, M. D. (2014). Nutrient uptake and growth of fireweed (*Chamerion angustifolium*) on reclamation soils. *Can. J. For. Res.* 44, 1–7. doi: 10.1139/cjfr-2013-0091

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Reynolds, H. L., Packer, A., Bever, J. D., and Clay, K. (2003). Grassroots ecology: plant-microbe-soil interactions as drivers of plant community structure and dynamics. *Ecology* 84, 2281–2291. doi: 10.1890/02-0298

Rupp, D., Kane, E. S., Dieleman, C., Keller, J. K., and Turetsky, M. (2019). Plant functional group effects on peat carbon cycling in a boreal rich fen. *Biogeochemistry* 144, 305–327. doi: 10.1007/s10533-019-00590-5

Schaad, N. W., Postnikova, E., Sechler, A., Claflin, L. E., Vidaver, A. K., Jones, J. B., et al. (2008). Reclassification of subspecies of *Acidovorax avenae* as *A. Avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citrulli* Schaad et al., 1978) comb. nov., and proposal of *A. oryzae* sp. nov. *Syst. Appl. Microbiol.* 31, 434–446. doi: 10.1016/j.syapm.2008.09.003

Schütte, U. M. E., Henning, J. A., Ye, Y., Bowling, A., Ford, J., Genet, H., et al. (2019). Effect of permafrost thaw on plant and soil fungal community in a boreal forest: does fungal community change mediate plant productivity response? *J. Ecol.* 107, 1737–1752. doi: 10.1111/1365-2745.13139

Schuur, E. A. G., Crummer, K. G., Vogel, J. G., and Mack, M. C. (2007). Plant Species Composition and Productivity following permafrost thaw and thermokarst in Alaskan Tundra. *Ecosystems* 10, 280–292. doi: 10.1007/s10021-007-9024-0

Schuur, E. A. G., and Mack, M. C. (2018). Ecological response to permafrost thaw and consequences for local and global ecosystem services. *Annu. Rev. Ecol. Evol. Syst.* 49, 279–301. doi: 10.1146/annurev-ecolsys-121415-032349

Schuur, E. A. G., McGuire, A. D., Schädel, C., Grosse, G., Harden, J. W., Hayes, D. J., et al. (2015). Climate change and the permafrost carbon feedback. *Nature* 520, 171–179. doi: 10.1038/nature14338

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Seitz, T. J., Schütte, U. M. E., and Drown, D. M. (2020). Soil disturbance affects plant growth via soil microbial community shifts. *bioRxiv* [Preprint] doi: 10.1101/2020.10.16.343053

Sniderhan, A. E., and Baltzer, J. L. (2016). Growth dynamics of black spruce (*Picea mariana*) in a rapidly thawing discontinuous permafrost peatland. *J. Geophys. Res. Biogeosci.* 121, 2988–3000. doi: 10.1002/2016JG003528

Tripathi, B. M., Kim, M., Kim, Y., Byun, E., Yang, J. W., Ahn, J., et al. (2018). Variations in bacterial and archaeal communities along depth profiles of Alaskan soil cores. *Sci. Rep.* 8:504. doi: 10.1038/s41598-017-18777-x

van der Voort, M., Kempenaar, M., van Driel, M., Raaijmakers, J. M., and Mendes, R. (2016). Impact of soil heat on reassembly of bacterial communities in the rhizosphere microbiome and plant disease suppression. *Ecol. Lett.* 19, 375–382. doi: 10.1111/ele.12567

Van Nuland, M. E., Smith, D. P., Bhatnagar, J. M., Stefanski, A., Hobbie, S. E., Reich, P. B., et al. (2020). Warming and disturbance alter soil microbiome diversity and function in a northern forest ecotone. *FEMS Microbiol. Ecol.* 96:fiaa108. doi: 10.1093/femsec/fiaa108

Van Wees, S. C., Van der Ent, S., and Pieterse, C. M. (2008). Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11, 443–448. doi: 10.1016/j.pbi.2008.05.005

Vimal, S. R., Singh, J. S., Arora, N. K., and Singh, S. (2017). Soil-plant-microbe interactions in stressed agriculture management: a review. *Pedosphere* 27, 177–192. doi: 10.1016/S1002-0160(17)60309-6

Wei, Z., Gu, Y., Friman, V. P., Kowalchuk, G. A., Xu, Y., Shen, Q., et al. (2019). Initial soil microbiome composition and functioning predetermine future plant health. *Sci. Adv.* 5:eaaw0759. doi: 10.1126/sciadv.aaw0759

Willems, A., De ley, J., Gillis, M., and Kersters, K. (1991). *Comamonadaceae*, a new family encompassing the acidovorans rRNA complex, including *Variovorax paradoxus* gen. nov., comb. nov., for *Alcaligenes paradoxus* (Davis) 1969. *Int. J. Syst. Bacteriol.* 41, 445–450. doi: 10.1099/00207713-41-3-445

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biol.* 20:257. doi: 10.1186/s13059-019-1891-0

Yang, L., Schröder, P., Vestergaard, G., Schloter, M., and Radl, V. (2020). Response of barley plants to drought might be associated with the recruiting of soil-borne endophytes. *Microorganisms* 8:1414. doi: 10.3390/microorganisms8091414

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REVIEW
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Linking Plant Secondary Metabolites and Plant Microbiomes: A Review

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Plant secondary metabolites (PSMs) play many roles including defense against pathogens, pests, and herbivores; response to environmental stresses, and mediating organismal interactions. Similarly, plant microbiomes participate in many of the above-mentioned processes directly or indirectly by regulating plant metabolism. Studies have shown that plants can influence their microbiome by secreting various metabolites and, in turn, the microbiome may also impact the metabolome of the host plant. However, not much is known about the communications between the interacting partners to impact their phenotypic changes. In this article, we review the patterns and potential underlying mechanisms of interactions between PSMs and plant microbiomes. We describe the recent developments in analytical approaches and methods in this field. The applications of these new methods and approaches have increased our understanding of the relationships between PSMs and plant microbiomes. Though the current studies have primarily focused on model organisms, the methods and results obtained so far should help future studies of agriculturally important plants and facilitate the development of methods to manipulate PSMs–microbiome interactions with predictive outcomes for sustainable crop productions.

Keywords: root exudates, SynCom, multi-omics, phytohormones, VOCs, rhizobia, endophytes, pathogens

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INTRODUCTION

Plant Secondary Metabolites

Different from primary metabolism, secondary metabolism refers to metabolic pathways and their associated small molecular products that are non-essential for the growth and reproduction of the organism (Yang et al., 2018). In plants, the secondary metabolic pathways produce a diversity of compounds called plant secondary metabolites (PSMs). PSMs contain a large group of structurally diverse compounds originated from either primary metabolites or intermediates in the biosynthetic pathways of these primary metabolites (Piasecka et al., 2015). According to their biosynthetic pathways, PSMs are generally classified into several large molecular families: phenolics, terpenes, steroids, alkaloids, and flavanoids (Kessler and Kalske, 2018).

Plant secondary metabolites play a variety of functions such as in plant growth and developmental processes, innate immunity (Piasecka et al., 2015), defense response signaling (Isah, 2019), and response to environmental stresses (Yang et al., 2018). In addition, PSMs also have

important functions such as repelling pests and pathogens, acting as signals for symbiosis between plants and microbes, and modifying microbial communities associated with hosts (Guerrieri et al., 2019). Many PSMs have positive beneficial effects on human health (Ullrich et al., 2019; Fakhri et al., 2020) and on agriculture production, contributing significantly to the economy. However, the functions of many PSMs remain unknown. For example, while many PSMs and protein-metabolite complexes have been identified, the biological roles of most have not been verified (Kosmacz et al., 2020). There have been several excellent reviews summarizing recent studies reporting the novel roles of PSMs and emphasizing the importance of functional understanding of the plant metabolome (Fang et al., 2019; Kosmacz et al., 2020; Zhou and Pichersky, 2020). The studies presented in those reviews have benefited significantly from recent developments in omics technologies such as high throughput DNA sequencing and high-resolution mass spectrometry.

Technologies for Analyzing Plant Metabolites

Plant metabolomics methods have been used for identifying functional secondary metabolites and metabolic pathways for both basic and applied research. Those methods help provide comprehensive perspectives on how plant metabolic networks are regulated. The most widely used methods include gas chromatography (GC) -mass spectrometry (-MS) (GC-MS), liquid chromatography-MS (LC-MS), capillary electrophoresis-MS (CE-MS), nuclear magnetic resonance spectroscopy (NMR), Fourier transform-near-infrared (FT-NIR) spectroscopy, MS imaging (MSI), and live single-cell -MS (LSC-MS). These methods are often used in combination because they can provide largely complimentary information with each other by analyzing different types of metabolites. A number of excellent technical reviews (Lu et al., 2017; Tahir et al., 2019) and detailed protocols (Zhalnina et al., 2018) regarding the utilization of these analytical tools in metabolomics experiments have been published.

Most traditional studies of PSMs utilize extracts of representative plant tissues as the main materials representing average plant cells in a specific tissue or organ (Masuda et al., 2018). Because of the bulk nature of those samples, it is often difficult to distinguish between PSMs produced by either host plants or their associated microbes. However, at present, there is a growing interest in narrowing PSMs analyses down to the single-cell level, allowing the separation of plant cells from their potentially associated microbial cells. Such separations and individualized analyses can be achieved using approaches such as MSI (Boughton and Thinagaran, 2018), matrix-assisted laser desorption ionization (MALDI) and laser ablation electrospray ionization (LAESI) (Etalo et al., 2018a; Bhattacharjee et al., 2020), live single-cell mass spectrometry (LSC-MS) (Masuda et al., 2018), nanospray desorption electrospray ionization mass spectrometry (Nano-DESI MS) (Battin et al., 2016), and the spatial metabolomics pipeline (metaFISH) (Geier et al., 2020). In combination with MS data alignment and molecular networking software and relevant databases, these tools allow for the detection of a large number (hundreds to thousands) of metabolites acquired from a single plant cell (Brader et al., 2014). These platforms provide significant advancement for the discovery of metabolites produced *in situ* and of the dynamics of interactions between plant and microbial cells at a single-cell level.

Plant Microbiome

The microbial communities of plants, also known as the plant microbiome (or plant microbiota), are found in the rhizosphere, phyllosphere, and endosphere. These plant microbiomes play important roles in helping host plants develop immunity (Stringlis et al., 2018), suppress diseases (Carrion et al., 2019), supply nutrients (Zhang et al., 2019), and protect from biotic and abiotic environmental stresses (de Vries et al., 2020). Over the last 15 years, plant microbiome studies have progressed significantly due to the advent of massive parallel sequencing. These studies have helped define different kinds of plant microbiomes and plant–microbiome interactions, e.g., the epiphytic microbiome, seed microbiome, core microbiome (CM), synthetic community (SynCom), and DefenseBiome (Liu H. et al., 2020). However, these plant microbiomes are not static, they can change in response to environmental stimuli, including both abiotic stresses and biotic factors. Indeed, there is increasing evidence that the structure of plant microbiomes is the result of a series of forward and backward interactions between the plant, the microbes and their environmental physical and chemical conditions. For example, PSMs secreted by roots are important mediators of plant–soil microbiome interactions (Sasse et al., 2018). In maize, secondary metabolites such as benzoxazinoids, were shown to attract bacteria *Chloroflexi* and influence the assembly of the maize microbiomes that subsequently enhance the capacity of maize plants to adapt to their environments (Hu et al., 2018).

Among the plants analyzed so far, model species such as *Arabidopsis thaliana* and *Echinacea purpurea* have been extensively studied to help define their microbiomes and the roles of these microbiomes in enhancing the growth and reproduction of host plants (Kudjordjie et al., 2019; Maggini et al., 2020). However, despite the growing number of studies and reviews demonstrating that different host plants species (Compant et al., 2019), their development stages (Schlechter et al., 2019), their root exudates (Sasse et al., 2018; Vives-Peris et al., 2020), and their rhizodeposits (Tian et al., 2020) can all influence the composition of the plant microbiomes and their functional capacities, relatively few reviews have attempted to integrate the chemical basis and molecular mechanism into the PSMs-microbiome relationship. Complicating the understanding is that the relationship is a dynamic one, involving multiple back-and-forth exchanges of chemical signals and molecular pathways. For example, some microbes can modulate the production of PSMs, including plant bioactive phytometabolites that in turn can influence the microbiome (Mastan et al., 2019). Furthermore, while a number of studies have provided insights into the structure and dynamics of the plant microbiome, relatively little is known about the contribution of plant microbiomes to host PSMs. The latest models of traditional medicinal PSMs–microbiome interactions approaches provide a new framework

for understanding the various types of interactions between PSMs and microbiomes (Maggini et al., 2020). Such an understanding can have significant impacts on several applied fields such as crop cultivation and breeding. During crop breeding, scientists typically select for higher yield and/or better nutrition but only from the crop cultivar perspective with limited consideration of the plant microbiome or the PSMs–microbiome interactions. Understanding of the interactions between PSMs and plant microbiomes could help opening up a new avenue of research in crop production.

Over the past few years, the characterization of the plant microbiomes and their relationships with the host plants using high-throughput techniques including genome and metagenome sequencing has become a hot topic in research. Both the culturome (all microbes that can be cultured in the lab) and metagenome sequencing techniques are providing in-depth information of the plant microbiome. The culturome is an important component of the microbiome. To obtain the culturome, the culturable cells in the microbial community are selected using solid media or liquid medium in high throughput formats. Subsequent shotgun sequencing allows the identification of linkages between taxonomic identity to important functions to the cultured cells, such as biological nitrogen fixation. However, culture-based methods are usually less sensitive than direct amplicon sequencing for identifying rare microbes. High-throughput sequencing of specific gene amplicons is typically more powerful for elucidating the composition, and spatial distribution of microorganisms in their environments and this approach is increasingly used in plant microbiome studies. The metagenome approaches can be combined with other high-throughput methods, such as metabolomics, proteomics, and transcriptomics. There is an excellent review discussing the quality of publicly available genome data, metagenome data, other omics data, and software pipelines for analyzing such data (Lucaciu et al., 2019). In these analyses, it's important to minimize sequence artifacts and reduce noise in data (Davis et al., 2018; Zhou et al., 2019). For processing the bacterial 16S rRNA gene and fungal ITS amplicons, a collection of software, such as QIIME (Caporaso et al., 2010), UPARSE (Edgar, 2013), VSEARCH (Rognes et al., 2016), PIPITS (Gweon et al., 2015), and USEARCH (Edgar and Flyvbjerg, 2015) have been developed. Similarly, for shotgun microbiome sequencing analyses, several recent articles reported specific computational workflow and bioinformatics resources (Liu Y. X. et al., 2020), including Microbiome Helper (Comeau et al., 2017), HmmUFOtu (Zheng et al., 2018), iMicrobe (Youens-Clark et al., 2019), MMinte (Mendes-Soares et al., 2016), MDiNE (McGregor et al., 2020), MicrobiomeAnalyst (Dhariwal et al., 2017), SIMBA (Mariano et al., 2016), and iMAP (Buza et al., 2019). Several in-depth summaries and comparisons of next-generation amplicon sequencing and analyses approaches were published recently (Lucaciu et al., 2019; Nilsson et al., 2019).

Herein, we review the current literature on the bidirectional interactions and effects between PSMs and plant microbiomes. In addition, we review the latest advances in plant metabolome analytical technologies and methods for analyzing the relationships between the plant metabolome and the plant microbiomes. To achieve our objectives, we used the following keywords for database searches: a variety of plants (such as legume plants, medicinal plants), plant microbiome (microbiota), metagenome, metagenomic, amplicon sequencing, PSMs, metabolomics, metabolomic analytical methodology, plant metabolome databases, correlation relationship, metabolomic-microbiome, and omics etc. The main retrieval databases were Web of Science, PubMed, and ResearchGate. **Table 1** shows a few common terms and their definitions used in this review.

Data Analyses Tools for Association Studies Between Plant Metabolome and Microbiome

Due to advances in high-throughput sequencing techniques, direct analyses of microbial communities in their natural environments have become increasingly convenient and cost effective. In recent years, microbiome studies using multi-omics approaches have greatly deepened our understanding of the relationship between microbiomes and hosts. For example, multi-omics studies of the gut microbiome and the human metabolome (Chen M. X. et al., 2019; Ilhan et al., 2020) have provided new understanding in human health and diseases. In order to help the application of multi-omics technologies on plant metabolome and microbiome studies, we reviewed the data integration and

TABLE 1 | Common terms and definitions.

Term	Definition
Secondary metabolism	Metabolic pathways and their associated small molecular products that are non-essential for the growth and reproduction of the organism
Rhizosphere microbiome	All microorganisms found in the narrow region of soil or substrate that is directly influenced by root secretions and associated soil, also called root microbiome
Epiphytic microbiome	All microorganisms found on the surface of aerial parts of plants. These microorganisms use plants for physical support but do not obtain any nutrients from plants nor cause any damage or offer any benefit to host plants.
Endophytic microbiome	All microorganisms found inside the internal tissues of plants, including both aerial and root tissues
Seed microbiome	All microorganisms found on the surface of and inside the seeds
Core microbiome	The group of microorganisms that are found in all individuals of a host species. The persistent association suggests a potentially critical function within the ecological niche of the host in which these microorganisms are found
Synthetic community (SynCom)	Defined systems with reduced complexity for both the host and the microorganisms. SynCom serves as model systems to investigate the performance and stability of microbial communities or to identify the necessary conditions for generating interaction patterns and higher order community structure and function
DefenseBiome	Plant-associated microbes that are positively associated with plant stress resistance
Rhizodeposits	All material transferred from plant roots to the soil. They include dead root tissues and cells, root exudates (both soluble and insoluble materials), and gasses such as CO_2 and ethylene.
Culturome	All microbes in a sample that can be cultured in the lab.

analysis methods for studying human and animal microbiomes and metabolomes; and provided a few suggestions on how they could be used for plant studies. **Table 2** lists the methods and tools for association studies between metabolome and microbiome.

Correlation-based analysis of paired microbiome-metabolite data sets has been a common approach to identify microbial drivers of metabolic variations. A commonly used method to infer the drivers of metabolic variations in a network is correlation analysis, such as Pearson's and Spearman's correlation coefficients among all pairs of operational taxonomic units (OTUs) and the metabolite profiles. An interaction between microbes is inferred when there is a high correlation coefficient

TABLE 2 | Comparison of methods and tools for association studies between metabolites and microbiomes.

Type of analysis	Method	Function	Note (Link)	References
Univariate analysis	Pearson Spearman	Comparatively simple method, but high false positive rate, unable to explain biological mechanism	Multiple calibration tests are required	Mao et al., 2016; Ilhan et al., 2020
Common descending dimension methods	PCA PLS	A multivariable correlation analysis method to describe the relationship between the two data matrices.		van der Kloet et al., 2016
Joint and individual variation explained	JIVE	An extension of PCA, identifying joint variations in multiple data types, reducing the dimensionality of data and determining the unique features of a particular data type.		Lock et al., 2013
Simultaneous component analysis	SCA	DIStinct COmmon SCA (DISCO-SCA) offers new avenues for multi-omics data fusion		Smilde et al., 2017
Canonical correlation	CCA	Samples over variables, Sparse CCA, kernel CCA and RCCA	Multivariable analysis partial least-squares	Kostic et al., 2015
Procrustes analysis	PA	Powerful least-square approach, simplifies omics comparisons, may not be sufficient to draw conclusions		McHardy et al., 2013
Co-inertia analysis	CIA	Suitable approach to determine the relationship, not suitable for large-scale data analysis		Hill et al., 2017
Maximum information coefficient	MIC	MINE statistics for identify associations and characterize such as non-linearity and monotonicity, comes closer than mutual information.	http://exploredata.net	Reshef et al., 2011
Linear regression	LR	Provide more accurate results		Bakker O. B. et al., 2018
Generalized coRrelation analysis	GRaMM	Captures linear/non-linear correlations in datasets and can adjust the influence of confounders, combines LR, MIC et al.	https: //github.com/chentianlu.	Liang et al., 2019
Seed set framework	A TDA	Calculate the symbiosis and competition scores of different microbes and predict the interaction relationship between microbes.		Greenblum et al., 2012
Predicted relative metabolic Turnover	PRMT	Explore metabolite-space inferred from the metagenome, can combine and analyze additional metagenomic and metabolic datasets	http://camera.calit2.net	Larsen et al., 2011
Computational framework	MIMOSA2	Mechanistic interpretation and hypothesis generation. Only analyze metabolites in the specific reference database	www.borensteinlab.com/ software_MIMOSA2.html	Noecker et al., 2016
Genome-scale metabolic models	GEMs	Contains a complete metabolic map of all metabolic reactions of the body and can be used for metabolite. Integration of Histology and Metagenomics Data		Magnúsdóttir et al., 2017
Neural networks	Mmvec	Estimate probability and strength of interaction	https://github.com/ biocore/mmvec)	Morton et al., 2019
A Valid Alternative to Correlation	Proportionality	Present proportionality as a means to analyze related data.		Lovell et al., 2015
Correlation inference for Compositional data through Lasso	CCLasso	An alternating direction algorithm from augmented Lagrangian method. Poorly for the hub model, component fraction estimation	https://github.com/ huayingfang/CCLasso	Fang et al., 2015
Sparse Correlations for Compositional data	SparCC	Not rely on high diversity. Relies on reliable component counts, no considered for the overall property	https://bitbucket.org/ yonatanf/sparcc	Friedman and Alm, 2012
SParse InversE Covariance Estimation	SPIEC-EASI	Making assumptions about the underlying network structure. Scale-free structures elude accurate inference	http://bonneaulab.bio. nyu.edu/	Kurtz et al., 2015
Correlation network	CCREPE	(bioBakery or ReBoot) Provides a similarity measure more appropriate for compositional data analysis, performance is similar to SparCC	http://huttenhower.sph. harvard.edu/ccrepe	Faust et al., 2012; McIver et al., 2018
Multivariate statistical analyses	M^2IA	Integrative data analysis from data preprocessing, univariate and multivariate statistical analyses, advanced functional analysis for biological interpretation, to a summary report.	http://m2ia.met-bioinformatics.cn	Ni et al., 2020

between them (Morton et al., 2019). However, traditional correlation analyses, such as univariate analysis and simultaneous component analysis that treat the observed data as absolute abundances of the microbes, may lead to spurious results. This is because most of the observed data through metagenomic analyses only represent relative abundances (Gevers et al., 2014). For example, concluding that a microbial community showing no signs of microbiome–metabolite interactions based on a single correlation analysis is unlikely correct, as none of the traditional tools can definitively identify actual correlations (Weiss et al., 2016). Consequently, simple correlation analysis alone is not suitable for detecting true microbial contributors to metabolite variations. Thus, special care and appropriate adjustments are required prior to correlation analysis for microbiome and metabolome data (Fang et al., 2015). Recent methods such as MIMOSA2, Correlation inference for Compositional data through Lasso (CCLasso), Neural networks (such as mmvec), Predicted relative metabolic turnover (PRMT), Compositionally Corrected by RElnormalization and PErmutation (CCREPE), and Sparse Correlations for Compositional data (SparCC) (**Table 2**) have been designed to take these compositional biases into account for analyzing microbiome–metabolite interactions. The joint usage of multiple methods can achieve better results. Several tools and resources are described in **Figure 1** and the following subsection.

Parallel approaches can offer new opportunities for analyzing microbiome–metabolite interactions especially if diverse types of information can be integrated. However, such data integration and analysis methods are still in their early stages of development (Lamichhane et al., 2018). In addition, to understand the underlying biological processes for the observed patterns of interaction, it is important to develop computational approaches that include individual organisms' unique biological features (Mallick et al., 2019). With a growing interest in connecting the microbes and metabolites in the context of plant and human health, we also need to bring together researchers from the two domains that traditionally do not interact with each other (Misra, 2020).

Importantly, while there are limitations in the correlation-based analysis to identify key microbiome-metabolite links, such linkages can be found in the current microbiome-metabolome data (Noecker et al., 2019). As is commonly stated, a correlation doesn't mean a causation or a true biological interaction. However, statistically significant correlations do help generate hypotheses and guide experimental efforts. Indeed, appropriately designed and carefully executed experiments are indispensable for confirming the hypotheses about the role of specific metabolites in plant–microbiome interactions. In the sections below, we first describe evidence for and a general model of PSMs–plant microbiome interactions. We then use specific examples to show how PSMs influence plant microbiomes (see section "Evidence for Specific PSMs Modulating the Plant Microbiome") and how plant microbiomes influence PSMs (see section "Plant Microbiomes Contribute to the Productions of PSMs"). We then describe how the PSMs–plant microbiome interactions could be used for crop production (see section "PSMs–Microbiome Interactions Impact Crop Breeding, Abiotic Stress Response, and Plant Invasion"). We finish by discussing potential areas for future research.

EVIDENCE AND MODEL FRAMEWORK FOR INTERACTIONS BETWEEN PLANT SECONDARY METABOLITES AND PLANT MICROBIOMES

As shown by Köberl et al. (2013), the same plants grown in different locations may produce different SMs, with some of the differences attributed to their associated microbes at different sites. Microbes adapted to specific locations and associated with specific plants may produce unique effects on host plants, including the production of SMs (Huang et al., 2018). For example, *Methylobacterium* was found to be involved in modulating the production of phytometabolites associated with flavor and in metabolizing plant host compounds, including volatile organic compounds (VOC) (Brader et al., 2014). Indeed, the induction of PSMs by endophytes may be a very general phenomenon in aromatic and medicinal plants. For example, several studies have shown that root exudates containing compounds such as aromatic organic acids (nicotinic, shikimic, salicylic, cinnamic, and indole-3-acetic acids) could shape the root microbiome (rhizobiome), which subsequently influenced root–microbe interactions (Sasse et al., 2018; Cotton et al., 2019). The combinations of plant exudation and microbial nutrient traits could interact to produce unique microbial community assemblies (Zhalnina et al., 2018). These studies have led to a proposed framework for studying the relationship between microbiome and PSMs, as depicted in **Figure 2**.

Interactions between legume plants and their rhizobia represent among the best studied models of PSMs-microbe interactions. Besides being economically important crops for food and forage, legume plants (such as pea, soybean, peanuts, clover, and alfalfa) and rhizobia have been used for decades for revealing how secondary metabolites from both partners mediate their interactions to establish root nodules for biological nitrogen fixation. Previous studies have observed a higher abundance of symbiotic rhizobia in the root microbiome of legume plants (70% with clover *Trifolium*) than that of bulk soil or the root microbiome of other plants (Hartman et al., 2017; Zhang et al., 2018). Soybean (*Glycine max*) is an example of legume plants that has been studied extensively for its mutualistic relationships with nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi. Soybeans secrete various specialized metabolites such as isoflavones and saponins into the soil (Sugiyama, 2019). Specifically, isoflavones and strigolactones are signal molecules for symbioses between soybean with rhizobia and arbuscular mycorrhizal fungi, respectively. During symbiosis, a hallmark feature of legume plants is that their roots secrete flavonoids/isoflavones [such as condensed tannins (CTs, prodelphinidins and procyanidins), daidzein and genistein)] into the rhizosphere as signal compounds to attract nitrogen-fixing bacteria such as *Ensifer* (formerly *Sinorhizobium*), *Rhizobium*, *Allorhizobium*, *Mesorhizobium*, *Neorhizobium*, *Azorhizobium*,

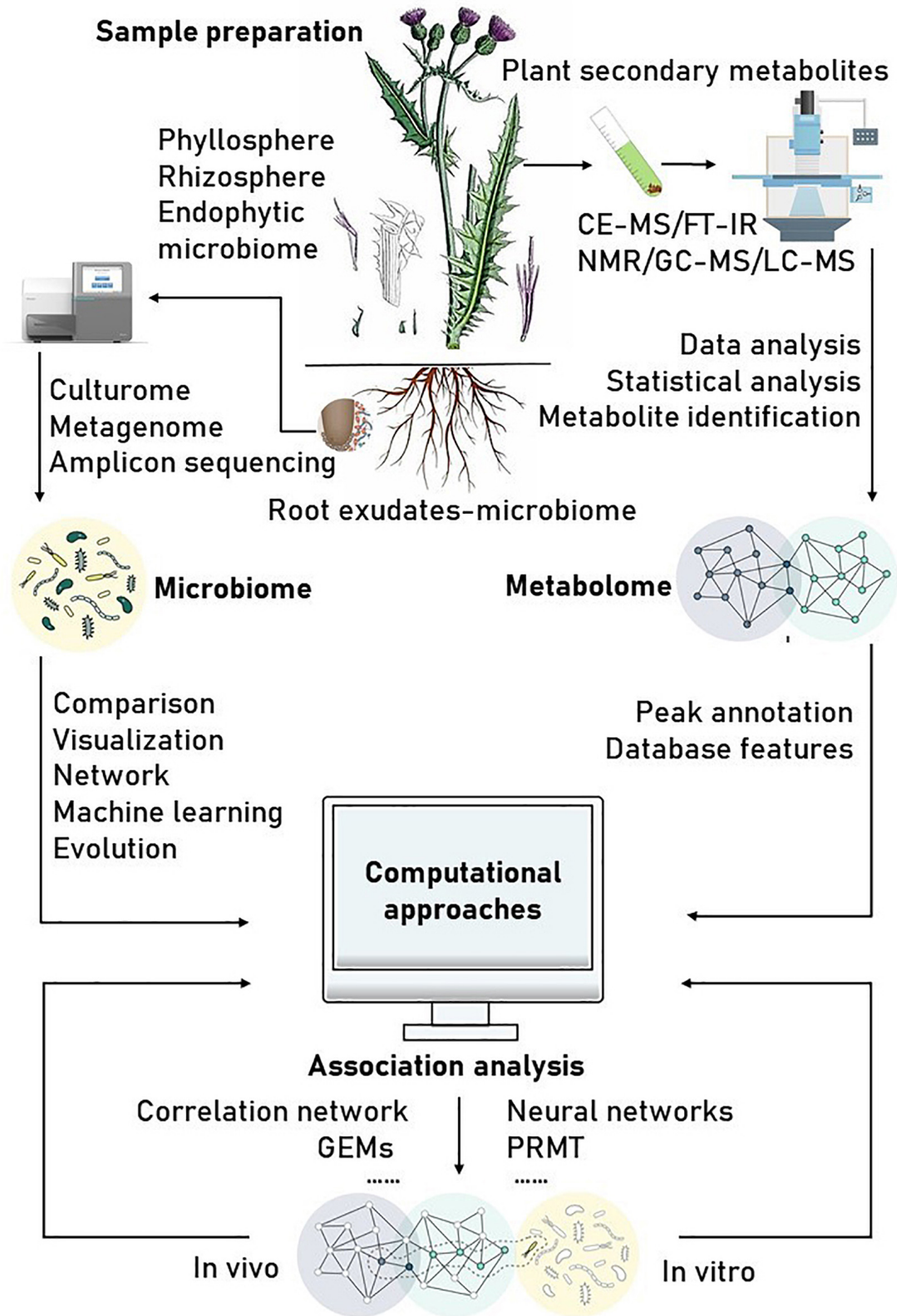

FIGURE 1 | Reference of schematic workflow for plant secondary metabolomics-microbiome discovery projects. Some information and pictures are adapted from Lavelle and Sokol (2020).

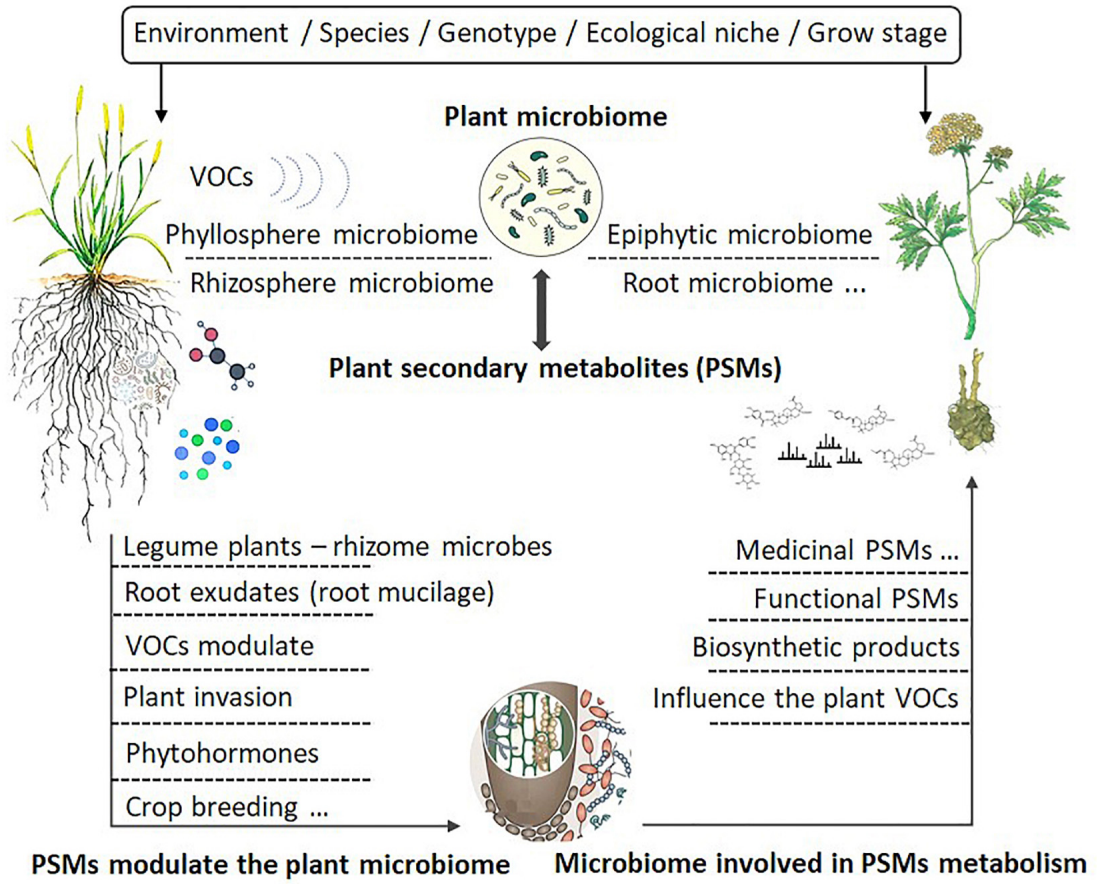

FIGURE 2 | Factors influencing the interactions between plant secondary metabolites and plant microbiomes.

Pararhizobium, and *Bradyrhizobium* (Hartman et al., 2017). Similarly, bacteria in the genera *Cupriavidus*, *Paraburkholderia,* and *Trinickia* also form mutualistic interactions with Papilionoideae and Caesalpinioideae to establish nodulation. The analysis of rhizosphere microbiomes between plants with and without isoflavone synthetase revealed that isoflavones exerted significant influence on the abundance of Xanthomonadaceae and Comamonadaceae (White et al., 2017). In addition, a recent study indicated that daidzein had a significant effect on soybean root microbiome, showing a concentration-specific effect on the bacterial community assemblage (Okutani et al., 2020). Specifically, the results suggested that daidzein functions both as an attractant and a repellent for different groups of bacteria. When the concentration of daidzein is high in the rhizosphere, there is an increased abundance of Comamonadaceae while rhizobia abundance was decreased, causing an overall reduced α-diversity. The overall reduced microbial diversity was probably because daidzein is not a preferred carbon source of rhizobia, different from several other bacteria that were enriched in daidzein-treated soils. In addition, a study showed that root–root interactions between the broad bean (*Vicia faba*) and maize significantly increased both nodulation and symbiotic N_2 fixation in intercropped *V. faba* (Li et al., 2016). However, while root exudates from maize promoted faba bean nodulation (flavonoids), root exudates from wheat and barley did not (Li et al., 2016). Recently, an interesting study suggested that cyanide production by cassava (*Manihot esculenta* Crantz) can trigger ethylene production in adjacent peanut (*Arachis hypogaea* L.) roots (Chen Y. et al., 2020), which subsequently changed the microbial composition and re-assembled the microbial co-occurrence network of peanut plants, causing an increased abundance of *Catenulispora* spp., an actinobacterium. However, the full details of this specific relationship between legume plants and rhizobia remain to be elucidated.

Apart from impacting legume and bacterial interactions, flavanones (such as strigolactones) can positively influence the growth of ectomycorrhizal fungi and increase the colonization of AM fungi. For example, flavanones can enhance the germination of spores of ectomycorrhizal fungi in genera *Pisolithus* and *Suillus* as well as stimulate the production of symbiotic effector protein in the mushroom *Laccaria bicolor* (Garcia et al., 2015; Pei et al., 2020). In contrast, the suppression of flavonoids and phenylpropanoid pathway secretion reduced the endophytes and ectomycorrhizal colonization of the maize and poplar roots, respectively (Mehmood et al., 2020). At present, the mechanisms of their interactions remain unclear.

EVIDENCE FOR SPECIFIC PSMs MODULATING THE PLANT MICROBIOME

Plants exude both low-molecular-weight compounds (such as phenolics, amino acids, nucleotides, sugars, terpenoids, and lipids) and high-molecular-weight compounds (such as nucleic acids, polysaccharides, and proteins). The types of compounds and their relative abundances depend on the species of plants, their growth and developmental stages, and presence of stress (abiotic, biotic) factors (Korenblum et al., 2020). The key classes of PSMs are either non-volatile compounds or VOCs. Plant roots secrete PSMs into the rhizosphere and/or soil environment actively using ATP as the energy source and passively through diffusion. PSMs are also released when root tissues and cells are detached from roots. After entering into the rhizosphere and soil, most PSMs may be quickly utilized by soil microbes, but some can remain in the rhizosphere and mediate interactions among organisms (Sugiyama and Yazaki, 2014). The roles of root exudates in plant–microbe chemical interactions in the rhizosphere is increasingly recognized (Sasse et al., 2018; Yuan et al., 2018; Williams and de Vries, 2020). Furthermore, different rhizodeposits can influence the rhizosphere microbiome composition differently (Pascale et al., 2020). Some root PSMs can affect the assembly of the root microbiome even before microbes reach the root surface (Sasse et al., 2018). Recent studies showed that selected SMs including coumarin, triterpenes, flavonoid, benzoxazinoid, and phytohormones can impact the proliferation or suppression of specific microbes around the root of host plants (Holmer et al., 2017; Hu et al., 2018; Chen Q. et al., 2019; Voges et al., 2019; Chen Q.-L. et al., 2020). These results call for further investigations into how natural habitat variation, crop genetic variation, and plant introduction between locations can potentially affect the PSMs and the recruitment and assembly of plant microbiome.

Coumarin, Benzoxazinoid, Terpenes, and Other Root-Exuded Molecules Modulate Root Microbiome

Plant secondary metabolites capable of changing plant microbiomes belong to diverse classes, including phenolics, benzoxazinoids, terpenes, and alkaloids (Cotton et al., 2019; Voges et al., 2019; Wang and Niu, 2019). Non-volatile compounds such as coumarins and flavonoids are produced by many plant species and are common in the rhizosphere. Coumarins are a family of plant-derived SMs produced via the phenylpropanoid pathway, and are involved in responses of dicotyledonous plants to iron deficiency (Stringlis et al., 2019). Recent studies suggested that coumarins, a sub-group of phenolic compounds, can influence the rhizosphere microbiome composition and exhibit differential toxicity against beneficial and pathogenic microorganisms (Lundberg and Teixeira, 2018; Voges et al., 2019). For example, a coumarin-deficient *Arabidopsis* mutant with beta-glucosidase gene *BGLU42* knocked out showed an increase in the relative abundance of Proteobacteria and a decrease of Firmicutes around its root (Stringlis et al., 2018, 2019). Further experiments showed that one specific coumarin compound called scopoletin inhibited the growth of soilborne pathogens whereas rhizobacteria were not affected. This was further confirmed by evidence showing that coumarins could shape the composition of a SynCom, where the abundance of *Pseudomonas* was significantly higher in coumarin-deficient *Arabidopsis f6'h1* mutants than in wild-type plants (Voges et al., 2019). A recent excellent review described coumarins as the "new kids on the block" in the chemical communications between plant roots and root microbiomes (Stassen et al., 2020).

Recent studies found that benzoxazinoids (BXs), SMs produced by several *Poaceae* species, and several downstream metabolites, could act as allelochemicals and natural pesticides on the root microbiome (Hu et al., 2018; Cotton et al., 2019; Kudjordjie et al., 2019; Schütz et al., 2019; Jacoby et al., 2020). Specifically, Hu et al. (2018) used a benzoxazinoids deficient maize mutant *bx1* and found that different bacterial and fungal communities were assembled in the roots of the mutants compared to wild-type maize. Another research used different maize BX mutant materials (BX knockout mutants, *bx1, bx2*, and *bx6*, and their near-isogenic W22-based controls T43 and a_1-m_3), and found similar results (Kudjordjie et al., 2019). Interestingly, such effects could be detected over several generations of the maize crop, suggesting that these molecules are likely key agents in plant–soil microbe feedback interactions (Jacoby et al., 2020). Overall, benzoxazinoids enriched Methylophilaceae bacteria while repressed Xanthomonadaceae (Cotton et al., 2019), likely due to their differential ability to use benzoxazolinones as carbon and energy sources (Schütz et al., 2019).

Similar to the benzoxazinoids, camalexin, an indolic compound, can also modulate the functionality of root microbiome (Koprivova et al., 2019). Loss of function of *CYP71A27*, a root-specific gene involved in the synthesis of camalexin, affected not only the soil microbiome but also led to the loss of plant growth-promoting effect by *Pseudomonas*. However, loss of the growth-promoting effect in the *CYP71A27* mutants could be complemented chemically by the addition of camalexin. Taken together, these results suggested camalexin's beneficial effects on plants by mediating the interaction between plant roots and microbes (Koprivova et al., 2019).

Terpenoids are a major component of the root-specialized metabolites. They contribute to the assembly of *Arabidopsis*-specific root microbiome by regulating the growth of specific root bacteria (Wang and Niu, 2019). One group of terpenoids is the triterpenes, synthesized via the mevalonate pathway that can accumulate in plant tissues as triterpene glycosides (Pascale et al., 2020). Recent experiments investigated the effects of triterpene and sesterterpene biosynthesis on *Arabidopsis* root microbiome assembly. The results showed that the compositions of the root microbiome communities of the triterpene and sesterterpene biosynthesis mutants were significantly different from those of the wild-type plants (Chen Q. et al., 2019; Huang et al., 2019). The authors further investigated whether triterpenes regulated *Arabidopsis* root bacteria using purified triterpene compounds. Growth assays of selected microbial strains showed that purified triterpenes stimulated the proliferation

of *Arenimonas* while inhibited the growth of *Arthrobacter* (Huang et al., 2019).

Some of the known PSMs have potent antibiotic activities. Plants secreting such compounds may create additional constraints on the groups of bacteria that can grow in the specific plant microbiome (Huang et al., 2018). For example, flavonoids have potent antimicrobial activity against a wide range of pathogenic microorganisms *in vitro* (Górniak et al., 2019). In addition, some PSMs such as flavonoids are not only associated with the regulation of symbiosis between plants and microbes (e.g., arbuscular mycorrhizal, ectomycorrhizal, rhizobial, and actinorhizal symbioses), but also as quorum-sensing (QS) inducers for communications among microbes. Different legumes produce unique flavonoids that bind to specific NodD proteins on the surface of rhizobia strains and species to regulate their symbiosis (Holmer et al., 2017). Application of 7, 4′-dihydroxyflavone, the most abundant flavonoid in the root exudate of *Medicago sativa*, to bulk soil caused significant changes of the relative richness of *Acidobacteria* (increased), *Gaiella*, *Nocardioidaceae*, and *Thermomonosporacea* (reduced). These microbes are known to interact with plant roots (Szoboszlay et al., 2016). Similarly, catecholic coumarins, benzoxazinoid, terpenes, jasmonate, indole glucosinolates, daidzein and others compounds also have antibacterial activity (Maggini et al., 2018; Rajniak et al., 2018; Dubey et al., 2020). Decades of research have demonstrated that a large number of secondary metabolites are involved in plant defense response to pathogens (Wang et al., 2020). Indeed, some of these PSMs have been used in antimicrobial scaffolds (Trda et al., 2019). There is increasing interests in mining bioactive compounds from economic crops such as garlic and ginger who are rich in allicin and curcumin etc. as natural antimicrobial compounds in healthy foods.

Volatile Organic Compounds From Plants Modulate Plant Microbiome

Apart from soluble secondary metabolites mentioned above, plants also release various VOCs which constitute an estimated 1% of PSMs (Venturi and Keel, 2016). Due to their unique physico-chemical properties, VOCs can easily diffuse through gas- and water- filled pores in the soil and can, therefore, have a broad effective range in soil (Schulz-Bohm et al., 2018), including mediating interactions with surrounding soil microorganisms. Examples of major plant VOCs include aldehydes, terpenoids, phenylpropanoids, and common monoterpenes limonene, β-pinene, benzenoids, and β-caryophyllene. Many of these VOCs have antimicrobial properties and can strongly influence plant microbiomes, including that on the plant phyllosphere (Farré-Armengol et al., 2016). A recent study evaluated the antimicrobial and anti-quorum sensing (QS) properties of 29 common essential oil compounds from plants. Twenty-two of these 29 compounds were identified to have QS-inhibitory ability, while seven promoted the QS to a variable extent in populations of two bacteria *Chromobacterium violaceum* and *Pseudomonas aeruginosa* (Peter et al., 2019). These results suggested that QS-inhibitory compounds of natural plant origins could be used to formulate a new generation of antimicrobial agents. In addition, another recent study found that the attraction of certain bacteria with antifungal properties through soil toward roots could be stimulated by VOCs (e.g., propanal, γ-nonalactone, and dimethyl disulfide) produced by *Carex arenaria* roots, with the cell numbers of *Janthinobacterium*, *Collimonas*, and *Paenibacillus* increased by up to three times higher as compared to those in the control soil samples without *C. arenaria* (Schulz-Bohm et al., 2018). However, the soil microbes were not equally attracted by VOCs to colonize plant roots.

Due to their antimicrobial effects and their potential role as carbon sources, plant VOCs can play significant roles in determining the characteristics of the microbiome on the above-ground plant surfaces (including stems, leaves, flowers, and fruits). A recent study found that mutation in *CYP706A3*, a clustered terpene synthase and a cytochrome P450 encoding gene, suppressed sesquiterpene and monoterpene emissions in *Arabidopsis* flowers and changed the floral microbial OTUs in the genus *Pseudomonas* (Boachon et al., 2019a). This study suggested that the *CYP706A3*-generated soluble metabolites played a role in the assembly of specific bacterial taxa colonizing *Arabidopsis* flowers. Furthermore, the floral microbiome may contribute to VOC productions, thereby inducing or reducing the emission of VOCs, and potentially catabolize others. The results suggested that bacteria belonging to the genera *Staphylococcus*, *Bacillus,* and *Sphingomonas* could exploit certain plant VOCs as carbon source, which may reduce their emission rates (Helletsgruber et al., 2017). These bacterial groups contribute to floral scent differences among flowers. One study found that in bee-pollinated *Penstemon digitalis*, the nectar volatile linalool could slow the growth of bacteria across the *P. digitalis* phyllosphere (Burdon et al., 2018). Another recent study showed that β-caryophyllene in tomato leaves can act as a signature VOC, which can lead to the release of a large amount of salicylic acid (SA) from adjacent tomato roots, and contribute to their similar rhizosphere microbial communities (up to 69%) (Kong et al., 2020). Together, these studies show that the plant VOC-microbiome interactions are widespread and are of great ecological interests. A recent review provided an excellent account of the interaction between the phyllosphere or rhizosphere microbiomes and plant VOC emissions (Schenkel et al., 2019).

Phytohormones Modulate the Plant Microbiome

Phytohormones are an integral part of the plant defense system, commonly known as the plant's systemic acquired resistance (SAR) and induced systemic resistance (ISR). Phytohormones are a class of small bioactive molecules (Chen X. et al., 2020). In addition to regulating plant physiological and morphological responses, phytohormones also impact the plant microbiome. Phytohormones known to influence plant microbiomes include SA, jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and strigolactones (SL). Below we briefly review these findings.

The first study on the potential effect of SA on the phyllosphere microbiome examined an SA mutant of *Arabidopsis thaliana* and found limited difference in the phyllosphere microbiome

between the SA mutant and the wild-type (Bodenhausen et al., 2014). However, a different study found SA to be capable of modulating the root microbiome of *A. thaliana* (Lebeis et al., 2015). Specifically, plants with altered SA signaling had root microbiomes that differed from each other in their relative abundance of Proteobacteria, Koribacteraceae, Intrasporangiaceae, Kineosporiaceae, Micromonosporaceae, Nocardioidaceae as well as the core microbiome when compared with those of wild-type plants. The study further showed that different bacterial strains responded to SA in different ways, either as a growth signal or as a carbon source, which in turn affected the root microbiome. While the induction of SA-mediated defenses reduced endophytic bacterial community diversity in *Arabidopsis* (Kniskern et al., 2007), certain members of the endophytic microbiome showed evidence of SA-related dependence for successful colonization. One study showed that in wheat, SA caused changes in microbiome through allelopathy (Kong et al., 2018).

Similarly, the effects of JA on plant microbiome are also evident. One study suggested that JA signaling was involved in controlling the density of *Azoarcus* endophyte, thereby shaping the beneficial microbiome in rice roots (Chen X. et al., 2020). The activation of JA-dependent defense mechanisms led to suppression of the SA-mediated defenses against the hemi biotrophic pathogen *P. syringae pv. tomato* (Wasternack and Hause, 2013). However, the addition of external methyl-JA also affected the root microbiome of *A. thaliana* (Carvalhais et al., 2013) and wheat (Liu et al., 2017). Here, JA acted as a SAR inducer in leaves to impact both the phyllosphere and endophytic microbiomes. Apart from JA, derivatives of JA are also capable of influencing the plant root microbiome (Carvalhais et al., 2017; Sasse et al., 2018). Compared with the wild-type, *Arabidopsis* mutants with JA signaling pathway defects showed lower amounts of asparagine, ornithine, and tryptophan, as well as increased abundance of *Streptomyces*, *Bacillus*, *Enterobacteriaceae,* and *Lysinibacillus* taxa, in the root microbiome (Carvalhais et al., 2015). A study in 2007 showed that plants deficient in JA-mediated defenses had greater epiphytic bacterial diversity (Kniskern et al., 2007).

In addition to JA and SA, ethylene (ET), another phytohormone, can also diffuse through air- and water-filled pores in the soil (Broekgaarden et al., 2015). Similar to SA and JA, ET can modulate arbuscular mycorrhizal colonization and root nodulation in legume-rhizobia symbioses (Nascimento et al., 2018). Therefore, like many VOCs, ET has a wide effective range in soil, including mediating long-distance attraction of bacteria to roots. For example, studies on ET mutants showed that mutations in the *ein2* gene altered rhizosphere microbiome (Doornbos et al., 2011). A recent study also suggested that ET production in peanut roots induced by cyanide could alter the microbiome and re-assembled the microbial co-occurrence network of peanuts by increasing the abundance of *Catenulispora* sp., a keystone actinobacterium, in the intercropped peanut rhizosphere (Chen Y. et al., 2020). While the mechanism of how ET works in mediating the plant microbiome is not known, one study suggested that glucosinolate might be involved (Pangesti et al., 2016).

Different phytohormones induce different effects on plant microbiomes. ABA is a common phytohormone and the exogenous application of ABA caused a preferential selection for microbes in the genera *Limnobacter*, *Massilia,* and *Cellvibrio* in a potting soil mixture (Carvalhais et al., 2014). Strigolactones (SL) are commonly exuded from roots under phosphate or nitrogen starvations to attract AM fungi, and their biosynthesis are downregulated after colonization of AM fungi. In contrast, SA, ET, and gibberellins (GA) can all inhibit both AM and root nodule symbiosis (Rodriguez et al., 2019). A recent study found that SL-mediated metabolic pathways are likely involved in the regulation of root microbiome in rice. In SL deficient mutants, there was a higher bacterial richness and a lower fungal diversity than the wild-type plants (Nasir et al., 2019). In addition, certain beneficial bacteria, including those in Nitrosomonadaceae and Rhodanobacter, were significantly decreased in SL mutants compared to the WT (Nasir et al., 2019). Two recent reviews summarized the relevant research progress of ABA and SL as regulators of plant–microbiome interactions (Shtark et al., 2018; Nasir et al., 2020).

Plant microbiomes contain many beneficial and pathogenic microbes. Overtime, plants have evolved mechanisms to recognize these microbes and correspondingly reprogram their defenses to enable or limit the colonization of specific microbes (Zhou et al., 2020). Apart from the pathways specific for phytohormones, the different phytohormone signaling pathways are interconnected at the molecular and phenotypic levels (Rodriguez et al., 2019). Some of the phytohormones act antagonistically with each other, potentially enabling certain microbes to exploit such antagonism to evade host defense and facilitate their own colonization (Jha et al., 2018). During this dynamic interactions, the plant microbiome may also develop resistance to PSMs (Chen Q.-L. et al., 2020). There is an increasing interest in this expanding field of phytohormone–microbiome interactions for both fundamental and applied research.

Infected Plants Recruit Beneficial Microbes

Interestingly, plants infected by pathogens may change their root exudates which may serve as signals to recruit beneficial root microbes. For example, upon attack by fluorescent pseudomonads (*P. protegens*), *Ordeum vulgar*e L. selectively recruited the Fluorescent pseudomonads carrying antifungal traits to its root microbiome. Such a recruitment leads to a reduced impact by the pathogen on host plants (Dudenhöffer et al., 2016). The same phenomenon was found in citrus root-associated microbiome change upon infection by Huanglongbing (HLB) (Zhang et al., 2017). In *Arabidopsis,* plants challenged by the foliar pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) exudated lower levels of sugars, alcohols, and short-chain organic acids (SCOAs) and higher levels of amino acids, nucleotides, and long-chain organic acids (LCOAs). These changes lead to the recruitment of beneficial rhizosphere microbes, including a few in Proteobacteria (Yuan et al., 2018). Similarly, the

infection of sugar beets by the wilt fungal pathogen *Rhizoctonia solani* caused the enrichment of several endophytic bacteria belonging to *Chitinophaga*, *Flavobacterium*, and *Pseudomonas* species resulting in an activation of their biosynthetic gene clusters to suppress the fungal pathogen (Carrion et al., 2019). These organisms produce antifungal effectors, including cell wall-degrading enzymes, and secondary metabolites such as phenazines, polyketides, and siderophores, that can contribute to their effects on the root mycobiome.

Secreted Root Mucilage Shapes the Nitrogen-Fixation Microbiome

A study on Mexican maize found that the mucilage associated with the aerial roots of Sierra Mixe maize *Z. mays* ssp. *mexicana* (teosinte) can feed a complex diazotrophic microbiome. The diazotrophic microbiome includes microbes containing active nitrogenase, and the fixed nitrogen can be efficiently transported from the nitrogen-fixing microbes to host plants (Van Deynze et al., 2018). Interestingly, this mechanism allows maize to fix up to 82% of its nitrogen needs from the atmosphere. The maize mucilage was rich in monosaccharides such as arabinose, fucose, galactose, xylose, glucuronic acid, and mannose. Unlike most modern maize varieties, the Sierra Mixe maize variety can develop extensive aerial roots and secrete large amounts of mucilage after rain (Bennett et al., 2020). The monosaccharides in mucilage is not commonly found in plant cell walls and may select for specific mutualistic, nitrogen-fixing bacteria that are uniquely capable of degrading and consuming the mucilage mono- and poly- polysaccharide in exchange for fixing atmospheric nitrogen to benefit the plants (Amicucci et al., 2019). The study paves the way toward developing innovative strategies for biological nitrogen fixation in cereal plants. Indeed, a model for plant-microbe association capable of supporting diazotrophic activity was proposed to support nitrogen fixation in cereal crops (Bennett et al., 2020). On the other hand, mucilage may contain proteins and other metabolites with antimicrobials that function in defense against fungal and bacterial pathogens (Sasse et al., 2018).

PLANT MICROBIOMES CONTRIBUTE TO THE PRODUCTIONS OF PSMs

Previous studies have highlighted the capabilities of plant microbiomes to influence important plant traits, such as growth, abiotic stress tolerance, resistance to infectious diseases, and the synthesis of plant growth promoting (PGP) hormones. At present, our understanding of the effects of the microbiome on PSMs, including their mechanism of action remains quite limited. This is different from the large body of literature showing how PSMs can shape the plant–microbiome structure. Improved understanding of PSMs profiles could be achieved by investigating the interaction of the plant (especially medicinal plants and economic crops) with their microbiomes. According to a recent study by Finkel et al. (2020), bacteria in the genus *Variovorax* manipulated *Arabidopsis* root growth and host plant auxin and ethylene levels to influence the development of the *Arabidopsis* root.

Microbiomes Contribute to Productions of PSMs in Medicinal Plants

For medicinal plants, investigations have shown that the plant microbiomes could influence host plants' productivity of important medicinal components such as alkaloids, steroids, terpenoids, etc. For example, two recent studies indicated that plant–microbiome interactions could improve biomass production of *Salvia miltiorrhiza* and influence tanshinone production, which is the major class of bioactive medicinal components from this plant (Chen et al., 2018; Huang et al., 2018). In this study, *S. miltiorrhiza* possesses a distinctive seed-associated microbiome, including *Pantoea*, *Pseudomonas*, *Sphingomonas,* and Dothideomycetes. This microbiome contains a gene reservoir related to the synthesis of forterpenoid backbone and other compounds, thus providing additional metabolic capabilities to host plants (Chen et al., 2018). Another study suggested that *Echinacea purpurea* is an excellent model for studying medicinal plant–microbiome interactions (Maggini et al., 2020). The *E. purpurea* microbiome (bacterial strains isolated from stems and leaves) interaction model showed that microbiome can affect the production of VOCs, phenylpropanoid, and alkamides in the plants (Maggini et al., 2017, 2019a,b).

Microbiome Contributes to Functional PSMs of Economic Crops and Other Plants

Aside from medicinal plants, other economic crops, such as *Cannabis sativa*, are attractive host plants to investigate plant–microbiome interactions. *Cannabis sativa* produces many functional secondary metabolites. Recent research showed that the endophytic bacteria (plant growth-promoting bacteria, PGPR) consortium within *C. sativa* included *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Burkholderia ambifaria*, and *Herbaspirillum seropedicae.* These endophytic microbes facilitated the growth and development of *Cannabis* and the accumulation of Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Pagnani et al., 2018; Taghinasab and Jabaji, 2020).

Similarly, inoculation of *Papaver somniferum* L. with a consortium of *Marmoricola* sp. and *Acinetobacter* sp. increased the morphine yield by enhancing the expression of *COR*, a key gene for morphine biosynthesis (Ray T. et al., 2019). In addition, three fungal endophytes (*Fusarium redolens*, *Phialemoniopsis cornearis*, and *Macrophomina pseudophaseolina*) were found to regulate forskolin biosynthesis in *Coleus forskohlii* (Mastan et al., 2019). Plants can also detect certain molecules released by microbiomes through a chemical recognition system, which can subsequently trigger plants to generate signal transduction networks and make corresponding changes in related gene activities, and leading to the accumulation of certain PSMs (Tidke et al., 2019). Importantly, horizontal gene transfer (HGT) in plants-endophytes may also lead to changes in plant secondary metabolic products (Wang et al., 2019). One recent study showed that local colonization of roots by bacteria in the genus *Bacillus* triggered systemic exudation of acylsugars SMs in tomato (Korenblum et al., 2020). Both

leaf and root metabolomes and transcriptomes changed due to differences in the root microbiome community structure, with different microbiomes inducing specific changes in tomato root exudation, a process called the systemically induced root exudation of PSMs (SIREM) (Korenblum et al., 2020). However, the underlying molecular mechanisms of functional PSMs synthesis regulated by microbiome- have not been completely elucidated.

Microbiomes Influence Plant VOCs

Plant microbiomes can participate in and/or influence the production of plant VOCs. For example, the suppression of phyllospheric microbiome in *Sambucus nigra* by antibiotic fumigation also changed the composition and proportion of terpenes in the volatile mix (Peñuelas et al., 2014). This result was confirmed in a later research showing that the application of antibiotics decreased the concentration of acetyl-CoA, citraconic acid, isoleucine, and several other PSMs (such as terpenes and phenols in the epiphytic extracts) in the same plant *S. nigra* (Gargallo-Garriga et al., 2016). Similar observations were made in *Penstemon digitalis* (Burdon et al., 2018), *Arabidopsis thaliana* (Raza et al., 2020), *Brassica rapa* (Helletsgruber et al., 2017), petunia (Boachon et al., 2019b), and *Atractylodes lancea* (Zhou et al., 2018). A recent review provided an excellent summary on the effects of plant microbiomes on plant VOC emissions (Schenkel et al., 2019).

Are Secondary Metabolites From Plants or Their Microbiomes?

The subsections above discussed how the plant microbiome could contribute to host PSMs. However, it is entirely possible that some of these so-called "PSMs" could be the biosynthetic products of their plant microbiome, including those by their endophytic microbes. Endophytes can produce diverse classes of phytochemicals, including podophyllotoxin, paclitaxel (taxol), deoxypodophyllotoxin, and camptothecin that are also produced by plants (Etalo et al., 2018b; Furtado et al., 2019; Mastan et al., 2019). A previous review discussed endophytic microbiome as potential sources of bioactive compounds (Ray S. et al., 2019). It is necessary to distinguish which of these compounds are produced by host plants and which ones by the plant microbiome. Such knowledge will help with novel developments in the *in situ* analysis of metabolites during the interaction between plants and microbes.

Aside from produce secondary metabolites similar to those produced by plants, endophytes can metabolize secondary compounds produced by host plants. For example, the leaf endophytic mycobiome could metabolize glycosylated flavonoids, the secondary metabolome of *Cephalotaxus harringtonia* (Tian et al., 2014). In another example, deglycosylated flavonoids showed beneficial effects on the hyphal growth of their endophytic fungi. Similarly, the biotransformation of *Huperzine* has also been found in fungal endophytes of *Huperzia serrata* (Zhan et al., 2019). Two recent reviews summarized microbiome-induced metabolic changes in roots and shoots of various crop species (Korenblum and Aharoni, 2019; Ray S. et al., 2019).

Microbial Secondary Metabolites (MSMs) Influence Plant Traits

While the focus of this review is on how PSMs impact plant microbiomes and how the plant microbiomes can influence the production of PSMs, there have been extensive documentations of how microbial secondary metabolites (MSMs) can impact plant growth and development. Here we describe a few examples. On the one hand, many plant pathogenic microbes can secrete toxins that cause diseases and death to plants. For example, sphinganine-analog mycotoxins including fumonisins and AAL-toxins produced by plant pathogenic fungi in the *Fusarium* genus and in *Alternaria alternata* f. sp. Lycopersici respectively have diverse cytotoxicity and phytotoxicity and are a destructive force to crop production worldwide (Chen J. et al., 2020). On the other hand, there are many examples of bacteria and fungi that produce plant growth – promoting SMs. For example, rhizobacterium *Bacillus tequilensis* SSB07 produces several phytohormones including gibberellins (GA1, GA3, GA5, GA8, GA19, GA24, and GA53), indole-3-acetic acid, and ABA. Application of *B. tequilensis* SSB07 enhanced the growth of Chinese cabbage seedlings and increased the shoot length and biomass, leaf development, and photosynthetic pigment contents of soybeans. For *B. tequilensis* SSB07, its plant growth-enhancing effects were further increased under heat stress, by significantly upregulating the endogenous JA and SA contents in the soybean phyllosphere while down-regulating the production of stress-responsive ABA (Kang et al., 2019).

The positive effects of MSMs on plant growths are shown not only for agricultural crops and vegetables but also for trees. For example, two bacterial strains, *Bacillus* sp. s50 and *Paenibacillus* sp. s37, recently showed significant effects on *Abies nordmanniana*, the most common Christmas tree species in the world. Both bacteria produced high quantities of indole-3-acetic acid, with *Bacillus* sp. s50 increased the seed germination rate and systemic resistance to pathogens while *Paenibacillus* sp. s37 increased plant root growth in both greenhouse and field conditions (Garcia-Lemos et al., 2020). Similar to those found in PGP rhizobacteria, several fungal species such as *Trichoderma virens* are also known to produce indole-3-acetic acid and other auxin-related compounds to positively impact the growth and development of plants, including rice, cotton, and *Arabidopsis* (Contreras-Cornejo et al., 2009).

Aside from phytohormones, the plant microbiomes can also produce abundant VOCs that can impact plant phenotypes (Kai et al., 2009). Many VOCs have been reported from the plant microbiome, including alcohols, aldehydes, ammonia, acids, ketones, esters, and terpenes. These microbial VOCs can influence plant communications, participate in defense against pathogens, and promote plant growth and development (Ortíz-Castro et al., 2009). For example, VOCs emitted by the *Bacillus subtilis* GB03 can trigger changes in major hormonal signaling networks in *A. thaliana* and impact

the expressions of over 600 genes related to cell wall modifications, primary and secondary metabolisms, stress responses and auxin homeostasis (Zhang et al., 2007). The VOCs emitted by strain *B. subtilis* GB03 included short-chained alcohols, aldehydes, acids, esters, ketones, hydrocarbons, and sulfur-containing compounds and these VOCs increased photosynthetic efficiency and chlorophyll content in *A. thaliana*. Overall, many microbial VOCs analyzed so far showed evidence of not only impacting plants directly but also indirectly such as by regulating the activities of herbivorous insects and plant parasitic nematodes (Hansen and Moran, 2014; Zhang et al., 2020).

PSMs–MICROBIOME INTERACTIONS IMPACT CROP BREEDING, ABIOTIC STRESS RESPONSE, AND PLANT INVASION

Plant hosts and their microbiome are highly interlinked and may have co-evolved to function as a meta-organism or holobiont with integrated ecologies. In domesticated crops (predominantly selected for yield traits), microbiomes can also be domesticated, causing different cultivars to be preferentially associated with different microbiomes (Escudero-Martinez and Bulgarelli, 2019). A number of studies suggested that crop microbiomes may have been affected by the domestication process in several crops, including barley (Bulgarelli et al., 2015), rice (Edwards et al., 2018), and the common bean (Perez-Jaramillo et al., 2019). These studies revealed the differences in plant microbiomes between modern cultivars and their wild ancestors in these species. Further studies identified that domestication changed root exudates and several secondary metabolites in modern varieties, likely contributing to the recruitment and maintenance of the plant microbiomes (Iannucci et al., 2017). The findings on PSMs–microbiome interactions have provided valuable insights to guide microbiome-based approaches to improve agricultural productivity. Given the large species diversity and enormous number of potential interactions between PSMs and microbes within individual plants, we are far from understanding the biology of the plant system and its microbiome (or PSMs and microbiome). Identifying specific variations in root exudation among plant species and genotypes could suggest the potential for manipulation of root exudation or PSMs in agricultural cultivars, in order to create specific selective effects on the plant microbiome (Bakker et al., 2012).

Despite many studies on abiotic stress tolerance of crop species, responses of roots to such stresses have so far largely been overlooked. A recent review indicated that plant-associated microbiomes can influence several plant traits including growth and abiotic stress tolerance (de Vries et al., 2020). Similarly, host plants also can adapt to changing environments by adjusting their production of PSMs (Bont et al., 2020). Indeed, interests in PSMs have been significantly enhanced with the knowledge of its importance in enhancing abiotic stress tolerance (de Vries et al., 2020), plant nutrient uptake, and the formation of humus in soil (Sokol et al., 2019). Such studies suggest that PSMs may be central to ecosystem responses to abiotic stresses and that we need an integrated approach to enhance the joint plant-microbiome responses to stresses. On the one hand, PSMs through root exudates can be abiotic stress response mediators. For example, changes in root exudates can help recruit microbiome associations to improve nutrient and water retentions (Huang et al., 2017), and to reduce damaging reactive oxygen species (ROS) by increasing the production of root peroxidases (Naylor and Coleman-Derr, 2017). The exudates of *Quercus ilex* under drought stress contained primarily SMs (71% of total metabolites) (Gargallo-Garriga et al., 2018), brought about mainly by regulating the expression of genes involved in secondary metabolite biosynthesis (Xu et al., 2018; Varoquaux et al., 2019). The altered PSMs further influence the structure of root microbiome, including the recruitment of Actinobacteria, *Streptomyces* or Firmicutes, contributing to the plants' DefenseBiome and enhancing the plants' survival under conditions (Bakker P. A. H. M. et al., 2018; Liu and Brettell, 2019; Liu H. et al., 2020; Williams and de Vries, 2020). Together, these studies suggested that root exudates could serve as signals to reshape root microbiome, by acting as chemoattractant or nutrition sources to reconstruct microbiomes to help alleviate abiotic stresses on host plants. At present, the exact chemicals that promote such relationships remain largely unknown. Deciphering this interaction could advance our ability to use microbiome to enhance abiotic stress tolerance in crop plants (Cheng et al., 2019; de Vries et al., 2020).

Invasive plants can change element cycling, soil nutrient pools, and/or soil microbiome that can all potentially accelerate further invasion and prevent re-establishment of native species (Stefanowicz et al., 2017). An example is the invasive plant *Ageratina adenophora* that changed the local soil microbial community and further enhanced *A. adenophora*'s competitive advantage over native plants (Chen L. et al., 2019). The detection and allelopathy of plant neighbors are driven by signal chemicals secreted by roots. There have been many studies on the role of below-ground function of PSMs-microbiome, such as plants releasing SMs (also including VOCs) to communicate with their root microbiome to gain a competitive advantage over other plants (Schandry and Becker, 2020). An example of PSM attracting beneficial microbes was shown in maize with exudate Benzoxazinoids attracting plant-beneficial *Pseudomonas* strains to the maize rhizosphere (Ahmad et al., 2011; Cotton et al., 2019). Another study supported a scenario in which an invasive plant, the Chinese tallow tree (*Triadica sebifera*), enhanced its AMF association and invasion success by changing its root flavonoid metabolism (Pei et al., 2020). Similar findings have been reported from thistle (Verbeek and Kotanen, 2019), *Spartina alterniflora* (Yang et al., 2019), and others (Kamutando et al., 2019; Pei et al., 2020). The recruited microbiome of invasive plants could directly or indirectly interfere their antagonism toward other plants via SMs, enhancing host plant nutrient acquisition (phosphorous and nitrogen) and modulating host root physiology (such as root exudation). Many crop species (including wheat, maize, and rice) are allelopathic, thus, targeted exploitation of allelopathy among

plants to reduce weed invasion coupled with a simultaneous reduction of herbicide application provides an attractive option for sustainable agriculture (Schandry and Becker, 2020). For example, investigating model SynComs with various PSMs-microbiome strategies can help derive methods to suppress parasitic weeds in agricultural field. Such methods hold great promise for developing novel integrated crop management strategies (Masteling et al., 2019). However, although some PSMs such as several VOCs are among the biochemically best-characterized allelochemicals, the extent and the molecular mechanisms by which the release of PSMs influencing the root microbiome requires further investigation.

Phosphate is a limiting nutrient in most crop fields. However, the effects of phosphate on microbiome have not been fully described. At present, there are two opposing types of results. In the first, microbes recruited by the PSMs under phosphate limitation provide the plants an advantage in coping with phosphate limitation (Castrillo et al., 2017; Fabiańska et al., 2019; You et al., 2020). On the other hand, the microbes could extract the limited amount of phosphate from the soil and make the phosphate less available to plants (Finkel et al., 2019). Together, these results suggest that different plant-microbiome combinations likely react to phosphate limitations differently. Further research is needed to understand how PSMs might be involved in mediating the plant–microbiome interactions for individual species during phosphate starvation.

DISCUSSION AND FUTURE PROSPECTS

While studies on the human (gut) microbiome have stolen most of the limelight, botanists have also been making progress toward elucidating the composition and function of plant microbiomes and PSMs over the last few years. In spite of a large number of contributions on plant microbiome, a thorough comprehension on plant microbiome structure, dynamics, and function associated with PSMs still remains largely unexplored. One potential area of research involves understanding the factors influencing plant microbiome assembly, and the signaling crosstalk in plant–microbiome interactions (Sasse et al., 2018). On the one hand, systematic research on the associated microbiomes in model plants, such as *Arabidopsis*, rice and maize, could help clarify the roles microbes may play in modulating the biosynthetic pathways of PSMs. Approaches such as SynCom may provide both functional and mechanistic insights into how plants regulate their microbiomes, and on how the microbiomes influence PSMs. Secondly, the single-cell genomics and specialized plant metabolome analytical tools are opening new possibilities for a diversity of potential research topics. Thirdly, spatial patterns of PSMs–microbiome interactions are largely unexplored. To improve the root exudate analysis, several modern technologies such as microfluidics and bacterial biosensors that respond to selected PSMs have been introduced (Massalha et al., 2017). And last but not the least, the underpinning genetic controls on PSMs and how they are affected by changing microbiomes and environmental conditions require greater focus.

Methods for Detecting and Tracking Plant Secondary Metabolites

The focus of this review is on the interaction between PSMs–microbiome. Thus, it's important to discuss methods for plant metabolome analysis. Metabolome analyses include data acquisition and processing. Data processing includes data normalization, peak alignment, and data scaling (Duan and Qi, 2015; Tahir et al., 2019). Several excellent software and websites are currently available for the processing of NMR and MS data. These include MZmine 2, XCMS, Open MS, Decon2LS, and MS-DIAL, all of which have been extensively used for diverse sets of metabolomics data. Misra (2020) provided a recent review that summarized over 95 metabolomics tools, software, and database.

In addition, the fine-scale dynamics between the PSMs-microbiome are of special importance to improve our understanding on plant–microbe interactions. Indeed, there is a growing interest in tracing and narrowing PSMs analyses down to single-cell level, which will be helpful to observe in-situ metabolism and trace metabolites in plant–microbiome interaction. Sensitive detection technologies and innovative cell-sampling techniques are needed to profile and trace metabolites in single cells. However, the field is still in its infancy for plant research. As PSMs are accumulated due to the activities of both host plant and its microbiome, strategies that allow metabolite traceability should be used to elucidate the origin of metabolites and to interpret their actions. The studies of the intestinal microbiome from humans and animals provide good references from which to design similar studies on plant–microbiome interactions (Koh et al., 2018; Duncan et al., 2019; Nemet et al., 2020).

One approach called Exometabolomics could provide novel insights into root microbiome. This approach investigates the root-derived compounds as carbon sources consumed by individual microbial strains and identifies substrate preferences of individual microbes from a mixture of exuded metabolites (Jacoby et al., 2018). Unfortunately, tracking the dynamics of root–microbiome interactions at high spatial resolution is still time consuming and requires significant expertise. Other methods include a microfluidics-based approach termed tracking root interactions system (TRIS) (Massalha et al., 2017) and a root-microbe interaction chip (RMI-Chip) (Noirot-Gros et al., 2020). These biosensors (Pini et al., 2017) or microfluidics (Millet et al., 2019) based methods enable direct imaging of root–microbiome interactions in real time, and provide spatiotemporal non-destructive analysis of samples *in situ* (Lenzewski et al., 2018). On the other hand, methods for whole-sample metabolic profiling of non-sterile rhizosphere soil have also been explored (Petriacq et al., 2017). These novel approaches thus allow researchers to investigate microbiome substrate preference for a number of metabolites at the same time, mimicking the real PSMs–microbiome interactions.

Bioinformatics of Plant Metabolome–Microbiome Interactions

Aside from the development in hardware for data acquisition, software that integrates information from both the metabolome

and the microbiome is also needed. For example, statistical methods for metabolome-microbiome data integration have been developed to identify the potential molecular markers driving their interactions (Lamichhane et al., 2018). Unfortunately, though improvements have been made, complete annotation of plant metabolomes is not yet possible (Lucaciu et al., 2019). Furthermore, there is a lack of in-depth understanding on how to integrate multi-omics data such as those from the proteome and transcriptome. The development of more reliable bioinformatics tools such as neural networks method is also urgent.

Despite these potential problems, recent studies suggested that untargeted metabolomics datasets showed a closer correlation with the microbiome data than those of targeted approaches, especially when they are compared with specific microbial metabolites (Melnik et al., 2017; Lamichhane et al., 2018). At present, several metabolite analysis methods are available and their use depends mainly on study objectives. These metabolic profiling methods include nontargeted metabolomics, widely targeted metabolites, metabolite target analysis (targeted), and metabolite fingerprinting (Tahir et al., 2019). A new integrated method named ESI-triple quadrupole-linear ion trap (Q TRAP)-MS (Luo et al., 2016) or ESI-QqTOF-MS (Chen et al., 2013) for large-scale detection, identification, and quantification of common metabolites has also been developed (Duncan et al., 2019; Kozuka et al., 2020). However, there is no specialized database for plant microbiome–metabolome information that is linked to environmental conditions (Lucaciu et al., 2019). Priority needs to be given to the development of such databases for functional interpretation of the increasingly common large-scale multi-omics plant microbiome data.

Confirming Causal Relationship Between PSMs–Microbiome Interactions Using Synthetic Communities

Both the plant microbiome and PSMs play important roles in plant health, impacting agriculture and food security (Haney et al., 2015). Though progresses have been made in our understanding of their interactions, many questions remain. For example, which special microbiome was attracted by PSMs and how to maintain the activities and abundance of PSMs? How do PSMs discriminate beneficial microbiome from pathogenic ones? Future research efforts should be devoted to understanding the modes with which microbiome affects PSMs in various plant tissues, evaluating the direction and magnitude of changes in microbiome as mediated by PSMs. Similarly, understanding how changes in PSMs are affected by the plant microbiomes is also important. A promising approach to understand reciprocal effects of plants and their microbiota is through SynCom, using sequenced and cultivated bacteria to provide simple and reproducible systems to study PSMs–microbiome interactions (Durán et al., 2018; Liu et al., 2019). Such a system allows precise variations in stress levels, exposure to infectious agents, phytohormone concentrations and metabolism, nutrition supply, etc. (Koprivova et al., 2019; Liu H. et al., 2020). Another study developed a machine learning computational approach to design SynCom, making it possible to infer causal relationships between microbiome membership and host plant phenotypes, potentially allowing the design of novel communities (Herrera Paredes et al., 2018). In addition, SynComs can be further combined with PSMs detection technologies. In summary, SynCom systems can provide crucial insights into the two-way interactions between PSMs and plant microbiome.

Connecting PSMs–Microbiome Relationships With Plant Breeding

Plant scientists are beginning to consider the plant microbiome as plants' "secondary genome" that can provide host plants with microbe-derived metabolites and traits. During traditional crop breeding, breeders select traits for yield or nutrition but not for PSMs-microbiome relationships. However, as shown above, microbiomes can respond rapidly to changes in PSMs such as those in root exudates and in the phyllosphere. Consequently, the plant microbiome could be treated as a selectable trait during breeding that could be manipulated chemically through PSMs. A pre-requisite for success in such a breeding program is a broad understanding of the relationships and changes of PSMs and microbiome during crop domestication. As described above, domestication can modify PSMs – microbiome relationship. Furthermore, previous studies have shown that different corps attract different microbiomes and that the microbiomes can have different effects on different host plants. A recent review proposed using desirable microbiomes as selective markers to identify potential beneficial microbiome for specific crop varieties (Pascale et al., 2020). In this proposal, PSMs play a key role in the selection process, by attracting various beneficial microbes and/or repelling detrimental one.

At present, in-depth and systematic investigations on the effects of the PSMs and functional microbiome on economic crops are relatively limited. Indeed, the function and dynamics of PSMs-functional microbiome interactions remain unexplored in most economic crops. Some economic crops, such as garlic and ginger, can be widely used as models to analyze such interactions and to enhance the production of the desirable metabolites such as allicin and curcumin for commercial purposes. Indeed, understanding the relationship between economic crop PSMs and functional microbiome can lead to improved agricultural practices that enhance plant fitness and increase the yield of functional secondary metabolites.

AUTHOR CONTRIBUTIONS

YC and JX conceived, structured, and finalized the manuscript. ZP coordinated the literature research and drafted the initial version of the manuscript. All authors contributed to the literature search and reviewing and finalizing the manuscript.

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REFERENCES

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., et al. (2011). Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiol.* 157, 317–327. doi: 10.1104/pp.111.180224

Amicucci, M. J., Galermo, A. G., Guerrero, A., Treves, G., Nandita, E., Kailemia, M. J., et al. (2019). Strategy for structural elucidation of polysaccharides: elucidation of a maize mucilage that harbors diazotrophic bacteria. *Anal. Chem.* 91, 7254–7265. doi: 10.1021/acs.analchem.9b00789

Bakker, M. G., Manter, D. K., Sheflin, A. M., Weir, T. L., and Vivanco, J. M. (2012). Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil.* 360, 1–13. doi: 10.1007/s11104-012-1361-x

Bakker, O. B., Aguirre-Gamboa, R., Sanna, S., Oosting, M., Smeekens, S. P., Jaeger, M., et al. (2018). Integration of multi-omics data and deep phenotyping enables prediction of cytokine responses. *Nat. Immunol.* 19, 776–786. doi: 10.1038/s41590-018-0121-3

Bakker, P. A. H. M., Pieterse, C. M. J., de Jonge, R., and Berendsen, R. L. (2018). The soil-borne legacy. *Cell* 172, 1178–1180. doi: 10.1016/j.cell.2018.02.024

Battin, T. J., Besemer, K., Bengtsson, M. M., Romani, A. M., and Packmann, A. I. (2016). The ecology and biogeochemistry of stream biofilms. *Nat. Rev. Microbiol.* 14, 251–263. doi: 10.1038/nrmicro.2016.15

Bennett, A. B., Pankievicz, V. C. S., and Ane, J. M. (2020). A model for nitrogen fixation in cereal crops. *Trends Plant Sci.* 25, 226–235. doi: 10.1016/j.tplants.2019.12.004

Bhattacharjee, A., Velickovic, D., Wietsma, T. W., Bell, S. L., Jansson, J. K., Hofmockel, K. S., et al. (2020). Visualizing microbial community dynamics via a controllable soil environment. *Msystems* 5:e645-19. doi: 10.1128/mSystems.00645-19

Boachon, B., Burdloff, Y., Ruan, J.-X., Rojo, R., Junker, R. R., Vincent, B., et al. (2019a). A promiscuous cyp706a3 reduces terpene volatile emission from *Arabidopsis* flowers, affecting florivores and the floral microbiome. *Plant Cell* 31, 2947–2972. doi: 10.1105/tpc.19.00320

Boachon, B., Lynch, J. H., Ray, S., Yuan, J., Caldo, K. M. P., Junker, R. R., et al. (2019b). Natural fumigation as a mechanism for volatile transport between flower organs. *Nat. Chem. Biol.* 15, 583–588. doi: 10.1038/s41589-019-0287-5

Bodenhausen, N., Bortfeld-Miller, M., Ackermann, M., and Vorholt, J. A. (2014). A synthetic community approach reveals plant genotypes affecting the phyllosphere microbiota. *PLoS Genet.* 10:e1004283. doi: 10.1371/journal.pgen.1004283

Bont, Z., Züst, T., Arce, C. C. M., Huber, M., and Erb, M. (2020). Heritable variation in root secondary metabolites is associated with recent climate. *J. Ecol.* 108, 2611–2624. doi: 10.1111/1365-2745.13441

Boughton, B. A., and Thinagaran, D. (2018). "Mass spectrometry imaging (msi) for plant metabolomics," in *Plant Metabolomics: Methods and Protocols*, ed. C. António (New York, NY: Springer).

Brader, G., Compant, S., Mitter, B., Trognitz, F., and Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. *Curr. Opin. Biotechnol.* 27, 30–37. doi: 10.1016/j.copbio.2013.09.012

Broekgaarden, C., Caarls, L., Vos, I. A., Pieterse, C. M., and Van Wees, S. C. (2015). Ethylene: traffic controller on hormonal crossroads to defense. *Plant Physiol.* 169, 2371–2379. doi: 10.1104/pp.15.01020

Bulgarelli, D., Garrido-Oter, R., Münch, Philipp, C., Weiman, A., Dröge, J., et al. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe.* 17, 392–403. doi: 10.1016/j.chom.2015.01.011

Burdon, R. C. F., Junker, R. R., Scofield, D. G., and Parachnowitsch, A. L. (2018). Bacteria colonising penstemon digitalis show volatile and tissue-specific responses to a natural concentration range of the floral volatile linalool. *Chemoecology* 28, 11–19. doi: 10.1007/s00049-018-0252-x

Buza, T. M., Tonui, T., Stomeo, F., Tiambo, C., Katani, R., Schilling, M., et al. (2019). Imap: an integrated bioinformatics and visualization pipeline for microbiome data analysis. *BMC Bioinformatics* 20:374. doi: 10.1186/s12859-019-2965-4

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carrion, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., de Hollander, M., Ruiz-Buck, D., et al. (2019). Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science* 366, 606–612. doi: 10.1126/science.aaw9285

Carvalhais, L. C., Dennis, P. G., Badri, D. V., Kidd, B. N., Vivanco, J. M., and Schenk, P. M. (2015). Linking jasmonic acid signaling, root exudates, and rhizosphere microbiomes. *Mol. Plant Microbe Interact.* 28, 1049–1058. doi: 10.1094/MPMI-01-15-0016-R

Carvalhais, L. C., Dennis, P. G., Badri, D. V., Tyson, G. W., Vivanco, J. M., and Schenk, P. M. (2013). Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. *PLoS One* 8:e56457. doi: 10.1371/journal.pone.0056457

Carvalhais, L. C., Dennis, P. G., and Schenk, P. M. (2014). Plant defence inducers rapidly influence the diversity of bacterial communities in a potting mix. *Appl. Soil Ecol.* 84, 1–5. doi: 10.1016/j.apsoil.2014.06.011

Carvalhais, L. C., Schenk, P. M., and Dennis, P. G. (2017). Jasmonic acid signalling and the plant holobiont. *Curr. Opin. Microbiol.* 37, 42–47. doi: 10.1016/j.mib.2017.03.009

Castrillo, G., Teixeira, P. J., Paredes, S. H., Law, T. F., de Lorenzo, L., Feltcher, M. E., et al. (2017). Root microbiota drive direct integration of phosphate stress and immunity. *Nature* 543, 513–518. doi: 10.1038/nature21417

Chen, H., Wu, H., Yan, B., Zhao, H., Liu, F., Zhang, H., et al. (2018). Core microbiome of medicinal plant salvia miltiorrhiza seed: a rich reservoir of beneficial microbes for secondary metabolism? *Int. J. Mol. Sci.* 19:672. doi: 10.3390/ijms19030672

Chen, J., Li, Z. M., Cheng, Y., Gao, C. S., Guo, L. T., Wang, T. H., et al. (2020). Sphinganine-analog mycotoxins (SAMs): chemical structures, bioactivities, and genetic controls. *J. Fungi.* 6:312. doi: 10.3390/jof6040312

Chen, L., Fang, K., Zhou, J., Yang, Z. P., Dong, X. F., Dai, G. H., et al. (2019). Enrichment of soil rare bacteria in root by an invasive plant ageratina adenophora. *Sci. Total Environ.* 683, 202–209. doi: 10.1016/j.scitotenv.2019.05.220

Chen, M. X., Wang, S. Y., Kuo, C. H., and Tsai, I. L. (2019). Metabolome analysis for investigating host-gut microbiota interactions. *J. Formos. Med. Assoc.* 118(Suppl. 1), S10–S22. doi: 10.1016/j.jfma.2018.09.007

Chen, Q., Jiang, T., Liu, Y. X., Liu, H., Zhao, T., Liu, Z., et al. (2019). Recently duplicated sesterterpene (c25) gene clusters in *Arabidopsis thaliana* modulate root microbiota. *Sci. China Life Sci.* 62, 947–958. doi: 10.1007/s11427-019-9521-2

Chen, Q.-L., Hu, H.-W., Zhu, D., Ding, J., Yan, Z.-Z., He, J.-Z., et al. (2020). Host identity determines plant associated resistomes. *Environ. Pollut.* 258:113709. doi: 10.1016/j.envpol.2019.113709

Chen, W., Gong, L., Guo, Z., Wang, W., Zhang, H., Liu, X., et al. (2013). A novel integrated method for large-scale detection, identification, and quantification of widely targeted metabolites: application in the study of rice metabolomics. *Mol. Plant.* 6, 1769–1780. doi: 10.1093/mp/sst080

Chen, X., Marszałkowska, M., and Reinhold-Hurek, B. (2020). Jasmonic acid, not salicyclic acid restricts endophytic root colonization of rice. *Front. Plant Sci.* 10:1758. doi: 10.3389/fpls.2019.01758

Chen, Y., Bonkowski, M., Shen, Y., Griffiths, B. S., Jiang, Y., Wang, X., et al. (2020). Root ethylene mediates rhizosphere microbial community reconstruction when chemically detecting cyanide produced by neighbouring plants. *Microbiome* 8:4. doi: 10.1186/s40168-019-0775-6

Cheng, Y. T., Zhang, L., and He, S. Y. (2019). Plant-microbe interactions facing environmental challenge. *Cell Host Microbe.* 26, 183–192. doi: 10.1016/j.chom.2019.07.009

Comeau, A. M., Douglas, G. M., and Langille, M. G. I. (2017). Microbiome helper: a custom and streamlined workflow for microbiome research. *mSystems* 2:e127-16. doi: 10.1128/mSystems.00127-16

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004

Contreras-Cornejo, H. A., Macìias-Rodrìguez, L. I., Cortés-Penagos, C., and Loìpez-Bucio, J. (2009). Trichoderma virens, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. *Plant Physiol.* 149, 1579–1592. doi: 10.1104/pp.108.130369

Cotton, T. E. A., Petriacq, P., Cameron, D. D., Meselmani, M. A., Schwarzenbacher, R., Rolfe, S. A., et al. (2019). Metabolic regulation of the maize rhizobiome by benzoxazinoids. *ISME J.* 13, 1647–1658. doi: 10.1038/s41396-019-0375-2

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6:226. doi: 10.1186/s40168-018-0605-2

de Vries, F. T., Griffiths, R. I., Knight, C. G., Nicolitch, O., and Williams, A. (2020). Harnessing rhizosphere microbiomes for drought-resilient crop production. *Science* 368:270. doi: 10.1126/science.aaz5192

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., and Xia, J. (2017). Microbiomeanalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* 45, W180–W188. doi: 10.1093/nar/gkx295

Doornbos, R. F., Geraats, B. P., Kuramae, E. E., Van Loon, L. C., and Bakker, P. A. (2011). Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 24, 395–407. doi: 10.1094/mpmi-05-10-0115

Duan, L.-X., and Qi, X. (2015). "Metabolite qualitative methods and the introduction of metabolomics database," in *Plant metabolomics: Methods and applications*, eds X. Qi, X. Chen, and Y. Wang (Dordrecht: Springer).

Dubey, O., Dubey, S., Schnee, S., Glauser, G., Nawrath, C., Gindro, K., et al. (2020). Plant surface metabolites as potent antifungal agents. *Plant Physiol. Biochem.* 150, 39–48. doi: 10.1016/j.plaphy.2020.02.026

Dudenhöffer, J.-H., Scheu, S., and Jousset, A. (2016). Systemic enrichment of antifungal traits in the rhizosphere microbiome after pathogen attack. *J. Ecol.* 104, 1566–1575. doi: 10.1111/1365-2745.12626

Duncan, K. D., Fyrestam, J., and Lanekoff, I. (2019). Advances in mass spectrometry based single-cell metabolomics. *Analyst* 144, 782–793. doi: 10.1039/c8an01581c

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis* survival. *Cell* 175, 973.e14–983.e14. doi: 10.1016/j.cell.2018.10.020

Edgar, R. C. (2013). Uparse: highly accurate otu sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., and Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* 31, 3476–3482. doi: 10.1093/bioinformatics/btv401

Edwards, J. A., Santos-Medellin, C. M., Liechty, Z. S., Nguyen, B., Lurie, E., Eason, S., et al. (2018). Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. *PLoS Biol.* 16:e2003862. doi: 10.1371/journal.pbio.2003862

Escudero-Martinez, C., and Bulgarelli, D. (2019). Tracing the evolutionary routes of plant–microbiota interactions. *Curr. Opin. Microbiol.* 49, 34–40. doi: 10.1016/j.mib.2019.09.013

Etalo, D. W., Díez-Simón, C., de Vos, R. C. H. and Hall, R. D. (2018a). "Laser ablation electrospray ionization-mass spectrometry imaging (laesi-ms) for spatially resolved plant metabolomics," in *Plant Metabolomics: Methods and Protocols*, ed. C. António (New York, NY: Springer).

Etalo, D. W., Jeon, J. S., and Raaijmakers, J. M. (2018b). Modulation of plant chemistry by beneficial root microbiota. *Nat. Prod. Rep.* 35, 398–409. doi: 10.1039/c7np00057j

Fabiańska, I., Gerlach, N., Almario, J., and Bucher, M. (2019). Plant-mediated effects of soil phosphorus on the root-associated fungal microbiota in *Arabidopsis thaliana*. *New Phytol.* 221, 2123–2137. doi: 10.1111/nph.15538

Fakhri, S., Moradi, S. Z., Farzaei, M. H., and Bishayee, A. (2020). Modulation of dysregulated cancer metabolism by plant secondary metabolites: a mechanistic review. *Semin. Cancer Biol.* doi: 10.1016/j.semcancer.2020.02.007 Online ahead of print

Fang, C., Fernie, A. R., and Luo, J. (2019). Exploring the diversity of plant metabolism. *Trends Plant Sci.* 24, 83–98. doi: 10.1016/j.tplants.2018.09.006

Fang, H., Huang, C., Zhao, H., and Deng, M. (2015). Cclasso: correlation inference for compositional data through lasso. *Bioinformatics* 31, 3172–3180. doi: 10.1093/bioinformatics/btv349

Farré-Armengol, G., Filella, I., Llusia, J., and Peñuelas, J. (2016). Bidirectional interaction between phyllospheric microbiotas and plant volatile emissions. *Trends Plant Sci.* 21, 854–860. doi: 10.1016/j.tplants.2016.06.005

Faust, K., Sathirapongsasuti, J. F., Izard, J., Segata, N., Gevers, D., Raes, J., et al. (2012). Microbial co-occurrence relationships in the human microbiome. *PLoS Comput. Biol.* 8:e1002606. doi: 10.1371/journal.pcbi.1002606

Finkel, O. M., Salas-González, I., Castrillo, G., Conway, J. M., Law, T. F., Teixeira, P. J. P. L., et al. (2020). A single bacterial genus maintains root growth in a complex microbiome. *Nature* 587, 103–108. doi: 10.1038/s41586-020-2778-7

Finkel, O. M., Salas-González, I., Castrillo, G., Spaepen, S., Law, T. F., Teixeira, P. J. P. L., et al. (2019). The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response. *PLoS Biol.* 17:e3000534. doi: 10.1371/journal.pbio.3000534

Friedman, J., and Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLoS Comput. Biol.* 8:e1002687. doi: 10.1371/journal.pcbi.1002687

Furtado, B. U., Golebiewski, M., Skorupa, M., Hulisz, P., and Hrynkiewicz, K. (2019). Bacterial and fungal endophytic microbiomes of salicornia europaea. *Appl. Environ. Microbiol.* 85:e305-19. doi: 10.1128/AEM.00305-19

Garcia, K., Delaux, P. M., Cope, K. R., and Ane, J. M. (2015). Molecular signals required for the establishment and maintenance of ectomycorrhizal symbioses. *New Phytol.* 208, 79–87. doi: 10.1111/nph.13423

Garcia-Lemos, A. M., Großkinsky, D. K., Saleem, A. S., Nicolaisen, M. H., Roitsch, T., Nybroe, O., et al. (2020). Identification of root-associated bacteria that influence plant physiology, increase seed germination, or promote growth of the christmas tree species *Abies nordmanniana*. *Front. Microbiol.* 11:566613. doi: 10.3389/fmicb.2020.566613)

Gargallo-Garriga, A., Preece, C., Sardans, J., Oravec, M., Urban, O., and Peñuelas, J. (2018). Root exudate metabolomes change under drought and show limited capacity for recovery. *Sci. Rep.* 8:12696. doi: 10.1038/s41598-018-30150-0

Gargallo-Garriga, A., Sardans, J., Pérez-Trujillo, M., Guenther, A., Llusià, J., Rico, L., et al. (2016). Shifts in plant foliar and floral metabolomes in response to the suppression of the associated microbiota. *BMC Plant Biol.* 16:78. doi: 10.1186/s12870-016-0767-7

Geier, B., Sogin, E. M., Michellod, D., Janda, M., Kompauer, M., Spengler, B., et al. (2020). Spatial metabolomics of in situ host–microbe interactions at the micrometre scale. *Nat. Microbiol.* 5, 498–510. doi: 10.1038/s41564-019-0664-6

Gevers, D., Kugathasan, S., Denson, L. A., Vazquez-Baeza, Y., Van Treuren, W., Ren, B., et al. (2014). The treatment-naive microbiome in new-onset crohn's disease. *Cell Host Microbe.* 15, 382–392. doi: 10.1016/j.chom.2014.02.005

Górniak, I., Bartoszewski, R., and Króliczewski, J. (2019). Comprehensive review of antimicrobial activities of plant flavonoids. *Phytochem. Rev.* 18, 241–272. doi: 10.1007/s11101-018-9591-z

Greenblum, S., Turnbaugh, P. J., and Borenstein, E. (2012). Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. *Proc. Natl. Acad. Sci. U.S.A.* 109, 594–599. doi: 10.1073/pnas.1116053109

Guerrieri, A., Dong, L., and Bouwmeester, H. J. (2019). Role and exploitation of underground chemical signaling in plants. *Pest Manag. Sci.* 75, 2455–2463. doi: 10.1002/ps.5507

Gweon, H. S., Oliver, A., Taylor, J., Booth, T., Gibbs, M., Read, D. S., et al. (2015). Pipits: an automated pipeline for analyses of fungal internal transcribed spacer sequences from the illumina sequencing platform. *Methods Ecol. Evol.* 6, 973–980. doi: 10.1111/2041-210x.12399

Haney, C. H., Samuel, B. S., Bush, J., and Ausubel, F. M. (2015). Associations with rhizosphere bacteria can confer an adaptive advantage to plants. *Nat. Plants* 1:15051. doi: 10.1038/nplants.2015.51

Hansen, A. K., and Moran, N. A. (2014). The impact of microbial symbionts on host plant utilization by herbivorous insects. *Mol. Ecol.* 23, 1473–1496. doi: 10.1111/mec.12421

Hartman, K., van der Heijden, M. G., Roussely-Provent, V., Walser, J. C., and Schlaeppi, K. (2017). Deciphering composition and function of the root microbiome of a legume plant. *Microbiome* 5:2. doi: 10.1186/s40168-016-0220-z

Helletsgruber, C., Dötterl, S., Ruprecht, U., and Junker, R. R. (2017). Epiphytic bacteria alter floral scent emissions. *J. Chem. Ecol.* 43, 1073–1077. doi: 10.1007/s10886-017-0898-9

Herrera Paredes, S., Gao, T., Law, T. F., Finkel, O. M., Mucyn, T., Teixeira, P., et al. (2018). Design of synthetic bacterial communities for predictable plant phenotypes. *PLoS Biol.* 16:e2003962. doi: 10.1371/journal.pbio.2003962

Hill, C. J., Lynch, D. B., Murphy, K., Ulaszewska, M., Jeffery, I. B., O'Shea, C. A., et al. (2017). Evolution of gut microbiota composition from birth to 24 weeks in the infantmet cohort. *Microbiome* 5:4. doi: 10.1186/s40168-016-0213-y

Holmer, R., Rutten, L., Kohlen, W., van Velzen, R., and Geurts, R. (2017). "Commonalities in symbiotic plant-microbe signalling," in *Advances in Botanical Research*, ed. G. Becard (Cambridge, MA: Academic Press).

Hu, L., Robert, C. A. M., Cadot, S., Zhang, X., Ye, M., Li, B., et al. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nat. Commun.* 9:2738. doi: 10.1038/s41467-018-05122-7

Huang, A. C., Jiang, T., Liu, Y. X., Bai, Y. C., Reed, J., Qu, B., et al. (2019). A specialized metabolic network selectively modulates *Arabidopsis* root microbiota. *Science* 364:eaau6389. doi: 10.1126/science.aau6389

Huang, W., Long, C., and Lam, E. (2018). Roles of plant-associated microbiota in traditional herbal medicine. *Trends Plant Sci.* 23, 559–562. doi: 10.1016/j.tplants.2018.05.003

Huang, Y. M., Zou, Y. N., and Wu, Q. S. (2017). Alleviation of drought stress by mycorrhizas is related to increased root h2o2 efflux in trifoliate orange. *Sci Rep.* 7:42335. doi: 10.1038/srep42335

Iannucci, A., Fragasso, M., Beleggia, R., Nigro, F., and Papa, R. (2017). Evolution of the crop rhizosphere: impact of domestication on root exudates in tetraploid wheat (*Triticum* turgidum l.). *Front. Plant Sci.* 8:2124. doi: 10.3389/fpls.2017.02124

Ilhan, Z. E., DiBaise, J. K., Dautel, S. E., Isern, N. G., Kim, Y.-M., Hoyt, D. W., et al. (2020). Temporospatial shifts in the human gut microbiome and metabolome after gastric bypass surgery. *NPJ Biofilms Microbiomes.* 6:12. doi: 10.1038/s41522-020-0122-5

Isah, T. (2019). Stress and defense responses in plant secondary metabolites production. *Biol. Res.* 52:39. doi: 10.1186/s40659-019-0246-3

Jacoby, R., Chen, L., Schwier, M., Koprivova, A., and Kopriva, S. (2020). Recent advances in the role of plant metabolites in shaping the root microbiome [version 1; peer review: 3 approved]. *F1000Res* 9:F1000FacultyRev-151. doi: 10.12688/f1000research.21796.1

Jacoby, R. P., Martyn, A., and Kopriva, S. (2018). Exometabolomic profiling of bacterial strains as cultivated using *Arabidopsis* root extract as the sole carbon source. *Mol. Plant Microbe Interact.* 31, 803–813. doi: 10.1094/mpmi-10-17-0253-r

Jha, P., Panwar, J., and Jha, P. N. (2018). Mechanistic insights on plant root colonization by bacterial endophytes: a symbiotic relationship for sustainable agriculture. *Environ. Sustain.* 1, 25–38. doi: 10.1007/s42398-018-0011-5

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., and Piechulla, B. (2009). Bacterial volatiles and their action potential. *Appl. Microbiol. Biotechnol.* 81, 1001–1012. doi: 10.1007/s00253-008-1760-3

Kamutando, C. N., Vikram, S., Kamgan-Nkuekam, G., Makhalanyane, T. P., Greve, M., Le Roux, J. J., et al. (2019). The functional potential of the rhizospheric microbiome of an invasive tree species, acacia dealbata. *Microb. Ecol.* 77, 191–200. doi: 10.1007/s00248-018-1214-0

Kang, S. M., Khan, A. L., Waqas, M., Asaf, S., Lee, K. E., Park, Y. G., et al. (2019). Integrated phytohormone production by the plant growth-promoting rhizobacterium *Bacillus* tequilensis SSB07 induced thermotolerance in soybean. *J. Plant Interact.* 14, 416–423. doi: 10.1080/17429145.2019.1640294

Kessler, A., and Kalske, A. (2018). Plant secondary metabolite diversity and species interactions. *Annu. Rev. Ecol. Evol. Syst.* 49, 115–138. doi: 10.1146/annurev-ecolsys-110617-062406

Kniskern, J. M., Traw, M. B., and Bergelson, J. (2007). Salicylic acid and jasmonic acid signaling defense pathways reduce natural bacterial diversity on *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 20, 1512–1522. doi: 10.1094/mpmi-20-12-1512

Köberl, M., Schmidt, R., Ramadan, E. M., Bauer, R., and Berg, G. (2013). The microbiome of medicinal plants: diversity and importance for plant growth, quality and health. *Front. Microbiol.* 4:400. doi: 10.3389/fmicb.2013.00400

Koh, A., Molinaro, A., Stahlman, M., Khan, M. T., Schmidt, C., Manneras-Holm, L., et al. (2018). Microbially produced imidazole propionate impairs insulin signaling through mtorc1. *Cell* 175, 947.e17–961.e17. doi: 10.1016/j.cell.2018.09.055

Kong, C. H., Zhang, S. Z., Li, Y. H., Xia, Z. C., Yang, X. F., Meiners, S. J., et al. (2018). Plant neighbor detection and allelochemical response are driven by root-secreted signaling chemicals. *Nat. Commun.* 9:3867. doi: 10.1038/s41467-018-06429-1

Kong, H. G., Song, G. C., Sim, H.-J., and Ryu, C.-M. (2020). Achieving similar root microbiota composition in neighbouring plants through airborne signalling. *ISME J.* 15, 397–408. doi: 10.1038/s41396-020-00759-z

Koprivova, A., Schuck, S., Jacoby, R. P., Klinkhammer, I., Welter, B., Leson, L., et al. (2019). Root-specific camalexin biosynthesis controls the plant growth-promoting effects of multiple bacterial strains. *Proc. Natl. Acad. Sci. U.S.A.* 116, 15735–15744. doi: 10.1073/pnas.1818604116

Korenblum, E., and Aharoni, A. (2019). Phytobiome metabolism: beneficial soil microbes steer crop plants' secondary metabolism. *Pest. Manag. Sci.* 75, 2378–2384. doi: 10.1002/ps.5440

Korenblum, E., Dong, Y., Szymanski, J., Panda, S., Jozwiak, A., Massalha, H., et al. (2020). Rhizosphere microbiome mediates systemic root metabolite exudation by root-to-root signaling. *Proc. Natl. Acad. Sci. U.S.A.* 117, 3874–3883. doi: 10.1073/pnas.1912130117

Kosmacz, M., Sokołowska, E. M., Bouzaa, S., and Skirycz, A. (2020). Towards a functional understanding of the plant metabolome. *Curr. Opin. Plant Biol.* 55, 47–51. doi: 10.1016/j.pbi.2020.02.005

Kostic, A. D., Gevers, D., Siljander, H., Vatanen, T., Hyotylainen, T., Hamalainen, A. M., et al. (2015). The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. *Cell Host Microbe.* 17, 260–273. doi: 10.1016/j.chom.2015.01.001

Kozuka, T., Sawada, Y., Imai, H., Kanai, M., Hirai, M. Y., Mano, S., et al. (2020). Regulation of sugar and storage oil metabolism by phytochrome during de-etiolation. *Plant Physiol.* 182, 1114–1129. doi: 10.1104/pp.19.00535

Kudjordjie, E. N., Sapkota, R., Steffensen, S. K., Fomsgaard, I. S., and Nicolaisen, M. (2019). Maize synthesized benzoxazinoids affect the host associated microbiome. *Microbiome* 7:59. doi: 10.1186/s40168-019-0677-7

Kurtz, Z. D., Muller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., and Bonneau, R. A. (2015). Sparse and compositionally robust inference of microbial ecological networks. *PLoS Comput. Biol.* 11:e1004226. doi: 10.1371/journal.pcbi.1004226

Lamichhane, S., Sen, P., Dickens, A. M., Orešič, M., and Bertram, H. C. (2018). Gut metabolome meets microbiome: a methodological perspective to understand the relationship between host and microbe. *Methods* 149, 3–12. doi: 10.1016/j.ymeth.2018.04.029

Larsen, P. E., Collart, F. R., Field, D., Meyer, F., Keegan, K. P., Henry, C. S., et al. (2011). Predicted relative metabolomic turnover (prmt): determining metabolic turnover from a coastal marine metagenomic dataset. *Microb. Inform. Exp.* 1:4. doi: 10.1186/2042-5783-1-4

Lavelle, A., and Sokol, H. (2020). Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat. Rev. Gastroenterol. Hepatol.* 17, 223–237. doi: 10.1038/s41575-019-0258-z

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., McDonald, M., et al. (2015). Plant microbiome. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* 349, 860–864. doi: 10.1126/science.aaa8764

Lenzewski, N., Mueller, P., Meier, R. J., Liebsch, G., Jensen, K., and Koop-Jakobsen, K. (2018). Dynamics of oxygen and carbon dioxide in rhizospheres of lobelia dortmanna – a planar optode study of belowground gas exchange between plants and sediment. *New Phytol.* 218, 131–141. doi: 10.1111/nph.14973

Li, B., Li, Y.-Y., Wu, H.-M., Zhang, F.-F., Li, C.-J., Li, X.-X., et al. (2016). Root exudates drive interspecific facilitation by enhancing nodulation and n2 fixation. *Proc. Natl. Acad. Sci. U.S.A.* 113, 6496–6501. doi: 10.1073/pnas.1523580113

Liang, D., Li, M., Wei, R., Wang, J., Li, Y., Jia, W., et al. (2019). Strategy for intercorrelation identification between metabolome and microbiome. *Anal. Chem.* 91, 14424–14432. doi: 10.1021/acs.analchem.9b02948

Liu, H., and Brettell, L. E. (2019). Plant defense by voc-induced microbial priming. *Trends Plant Sci.* 24, 187–189. doi: 10.1016/j.tplants.2019.01.008

Liu, H., Brettell, L. E., Qiu, Z., and Singh, B. K. (2020). Microbiome-mediated stress resistance in plants. *Trends Plant Sci.* 25, 733–743. doi: 10.1016/j.tplants.2020.03.014

Liu, H., Carvalhais, L. C., Schenk, P. M., and Dennis, P. G. (2017). Effects of jasmonic acid signalling on the wheat microbiome differ between body sites. *Sci. Rep.* 7:41766. doi: 10.1038/srep41766

Liu, Y. X., Qin, Y., and Bai, Y. (2019). Reductionist synthetic community approaches in root microbiome research. *Curr. Opin. Microbiol.* 49, 97–102. doi: 10.1016/j.mib.2019.10.010

Liu, Y. X., Qin, Y., Chen, T., Lu, M., Qian, X., Guo, X., et al. (2020). A practical guide to amplicon and metagenomic analysis of microbiome data. *Protein Cell* doi: 10.1007/s13238-020-00724-8 Online ahead of print

Lock, E. F., Hoadley, K. A., Marron, J. S., and Nobel, A. B. (2013). Joint and individual variation explained (jive) for integrated analysis of multiple data types. *Ann. Appl. Stat.* 7, 523–542. doi: 10.1214/12-AOAS597

Lovell, D., Pawlowsky-Glahn, V., Egozcue, J. J., Marguerat, S., and Bahler, J. (2015). Proportionality: a valid alternative to correlation for relative data. *PLoS Comput. Biol.* 11:e1004075. doi: 10.1371/journal.pcbi.1004075

Lu, W., Su, X., Klein, M. S., Lewis, I. A., Fiehn, O., and Rabinowitz, J. D. (2017). Metabolite measurement: pitfalls to avoid and practices to follow. *Annu. Rev. Biochem.* 86, 277–304. doi: 10.1146/annurev-biochem-061516-044952

Lucaciu, R., Pelikan, C., Gerner, S. M., Zioutis, C., Köstlbacher, S., Marx, H., et al. (2019). A bioinformatics guide to plant microbiome analysis. *Front. Plant Sci.* 10:1313. doi: 10.3389/fpls.2019.01313

Lundberg, D. S., and Teixeira, P. (2018). Root-exuded coumarin shapes the root microbiome. *Proc. Natl. Acad. Sci. U.S.A.* 115, 5629–5631. doi: 10.1073/pnas.1805944115

Luo, P., Yin, P., Zhang, W., Zhou, L., Lu, X., Lin, X., et al. (2016). Optimization of large-scale pseudotargeted metabolomics method based on liquid chromatography–mass spectrometry. *J. Chromatogr. A* 1437, 127–136. doi: 10.1016/j.chroma.2016.01.078

Maggini, V., Bandeira Reidel, R. V., De Leo, M., Mengoni, A., Gallo, E. R., Miceli, E., et al. (2019a). Volatile profile of echinacea purpurea plants after in vitro endophyte infection. *Nat. Prod. Res.* 34, 2232–2237. doi: 10.1080/14786419.2019.1579810

Maggini, V., De Leo, M., Granchi, C., Tuccinardi, T., Mengoni, A., Gallo, E. R., et al. (2019b). The influence of echinacea purpurea leaf microbiota on chicoric acid level. *Sci. Rep.* 9:10897. doi: 10.1038/s41598-019-47329-8

Maggini, V., De Leo, M., Mengoni, A., Gallo, E. R., Miceli, E., Reidel, R. V. B., et al. (2017). Plant-endophytes interaction influences the secondary metabolism in echinacea purpurea (l.) moench: an in vitro model. *Sci. Rep.* 7:16924. doi: 10.1038/s41598-017-17110-w

Maggini, V., Mengoni, A., Bogani, P., Firenzuoli, F., and Fani, R. (2020). Promoting model systems of microbiota-medicinal plant interactions. *Trends Plant Sci.* 25, 223–225. doi: 10.1016/j.tplants.2019.12.013

Maggini, V., Miceli, E., Fagorzi, C., Maida, I., Fondi, M., Perrin, E., et al. (2018). Antagonism and antibiotic resistance drive a species-specific plant microbiota differentiation in echinacea spp. *FEMS Microbiol. Ecol.* 94, 1–18. doi: 10.1093/femsec/fiy118

Magnúsdóttir, S., Heinken, A., Kutt, L., Ravcheev, D. A., Bauer, E., Noronha, A., et al. (2017). Generation of genome-scale metabolic reconstructions for 773 members of the human gut microbiota. *Nat. Biotechnol.* 35, 81–89. doi: 10.1038/nbt.3703

Mallick, H., Franzosa, E. A., McLver, L. J., Banerjee, S., Sirota-Madi, A., Kostic, A. D., et al. (2019). Predictive metabolomic profiling of microbial communities using amplicon or metagenomic sequences. *Nat. Commun.* 10:3136. doi: 10.1038/s41467-019-10927-1

Mao, S.-Y., Huo, W.-J., and Zhu, W.-Y. (2016). Microbiome–metabolome analysis reveals unhealthy alterations in the composition and metabolism of ruminal microbiota with increasing dietary grain in a goat model. *Environ. Microbiol.* 18, 525–541. doi: 10.1111/1462-2920.12724

Mariano, D. C., Pereira, F. L., Aguiar, E. L., Oliveira, L. C., Benevides, L., Guimaraes, L. C., et al. (2016). Simba: a web tool for managing bacterial genome assembly generated by ion pgm sequencing technology. *BMC Bioinformatics* 17:456. doi: 10.1186/s12859-016-1344-7

Massalha, H., Korenblum, E., Malitsky, S., Shapiro, O. H., and Aharoni, A. (2017). Live imaging of root-bacteria interactions in a microfluidics setup. *Proc. Natl. Acad. Sci. U.S.A.* 114, 4549–4554. doi: 10.1073/pnas.1618584114

Mastan, A., Bharadwaj, R. K. B., Kushwaha, R. K., and Vivek Babu, C. S. (2019). Functional fungal endophytes in coleus forskohlii regulate labdane diterpene biosynthesis for elevated forskolin accumulation in roots. *Microb. Ecol.* 78, 914–926. doi: 10.1007/s00248-019-01376-w

Masteling, R., Lombard, L., de Boer, W., Raaijmakers, J. M., and Dini-Andreote, F. (2019). Harnessing the microbiome to control plant parasitic weeds. *Curr. Opin. Microbiol.* 49, 26–33. doi: 10.1016/j.mib.2019.09.006

Masuda, K., Abouleila, Y., Ali, A., and Yanagida, T. and Masujima, T. (2018). "Live single-cell mass spectrometry (lsc-ms) for plant metabolomics," in *Plant Metabolomics: Methods and Protocols*, eds C. António (New York, NY: Springer New York).

McGregor, K., Labbe, A., and Greenwood, C. M. T. (2020). Mdine: a model to estimate differential co-occurrence networks in microbiome studies. *Bioinformatics* 36, 1840–1847. doi: 10.1093/bioinformatics/btz824

McHardy, I. H., Goudarzi, M., Tong, M., Ruegger, P. M., Schwager, E., Weger, J. R., et al. (2013). Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. *Microbiome* 1:17. doi: 10.1186/2049-2618-1-17

McIver, L. J., Abu-Ali, G., Franzosa, E. A., Schwager, R., Morgan, X. C., Waldron, L., et al. (2018). Biobakery: a meta'omic analysis environment. *Bioinformatics* 34, 1235–1237. doi: 10.1093/bioinformatics/btx754

Mehmood, A., Hussain, A., Irshad, M., Hamayun, M., Iqbal, A., Tawab, A., et al. (2020). Yucasin and cinnamic acid inhibit iaa and flavonoids biosynthesis minimizing interaction between maize and endophyteaspergillus nomius. *Symbiosis* 12, 149–160. doi: 10.1007/s13199-020-00690-z

Melnik, A. V., da Silva, R. R., Hyde, E. R., Aksenov, A. A., Vargas, F., Bouslimani, A., et al. (2017). Coupling targeted and untargeted mass spectrometry for metabolome-microbiome-wide association studies of human fecal samples. *Anal. Chem.* 89, 7549–7559. doi: 10.1021/acs.analchem.7b01381

Mendes-Soares, H., Mundy, M., Soares, L. M., and Chia, N. (2016). Mminte: an application for predicting metabolic interactions among the microbial species in a community. *BMC Bioinformatics* 17:343. doi: 10.1186/s12859-016-1230-3

Millet, L. J., Aufrecht, J., Labbe, J., Uehling, J., Vilgalys, R., Estes, M. L., et al. (2019). Increasing access to microfluidics for studying fungi and other branched biological structures. *Fungal Biol. Biotechnol.* 6:1. doi: 10.1186/s40694-019-0071-z

Misra, B. B. (2020). The connection and disconnection between microbiome and metabolome: a critical appraisal in clinical research. *Biol. Res. Nurs.* 22, 561–576. doi: 10.1177/1099800420903083

Morton, J. T., Aksenov, A. A., Nothias, L. F., Foulds, J. R., Quinn, R. A., Badri, M. H., et al. (2019). Learning representations of microbe-metabolite interactions. *Nat. Methods* 16, 1306–1314. doi: 10.1038/s41592-019-0616-3

Nascimento, F. X., Rossi, M. J., and Glick, B. R. (2018). Ethylene and 1-aminocyclopropane-1-carboxylate (acc) in plant–bacterial interactions. *Front. Plant Sci.* 9:114. doi: 10.3389/fpls.2018.00114

Nasir, F., Li, W., Tran, L.-S. P., and Tian, C. (2020). Does karrikin signaling shape the rhizomicrobiome via the strigolactone biosynthetic pathway? *Trends Plant Sci.* 25, 1184–1187. doi: 10.1016/j.tplants.2020.08.005

Nasir, F., Shi, S., Tian, L., Chang, C., Ma, L., Li, X., et al. (2019). Strigolactones shape the rhizomicrobiome in rice (*Oryza sativa*). *Plant Sci.* 286, 118–133. doi: 10.1016/j.plantsci.2019.05.016

Naylor, D., and Coleman-Derr, D. (2017). Drought stress and root-associated bacterial communities. *Front. Plant Sci.* 8:2223. doi: 10.3389/fpls.2017.02223

Nemet, I., Saha, P. P., Gupta, N., Zhu, W., Romano, K. A., Skye, S. M., et al. (2020). A cardiovascular disease-linked gut microbial metabolite acts

via adrenergic receptors. *Cell* 180, 862.e22–877.e22. doi: 10.1016/j.cell.2020.02.016

Ni, Y., Yu, G., Chen, H., Deng, Y., Wells, P. M., Steves, C. J., et al. (2020). M2ia: a web server for microbiome and metabolome integrative analysis. *Bioinformatics*. 36, 3493–3498. doi: 10.1093/bioinformatics/btaa188

Nilsson, R. H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., and Tedersoo, L. (2019). Mycobiome diversity: high-throughput sequencing and identification of fungi. *Nat. Rev. Microbiol.* 17, 95–109. doi: 10.1038/s41579-018-0116-y

Noecker, C., Chiu, H.-C., McNally, C. P., and Borenstein, E. (2019). Defining and evaluating microbial contributions to metabolite variation in microbiome-metabolome association studies. *mSystems* 4:e579-19. doi: 10.1128/mSystems.00579-19

Noecker, C., Eng, A., Srinivasan, S., Theriot, C. M., Young, V. B., Jansson, J. K., et al. (2016). Metabolic model-based integration of microbiome taxonomic and metabolomic profiles elucidates mechanistic links between ecological and metabolic variation. *mSystems* 1:e00013-15. doi: 10.1128/mSystems.00013-15

Noirot-Gros, M. F., Shinde, S. V., Akins, C., Johnson, J. L., Zerbs, S., Wilton, R., et al. (2020). Functional imaging of microbial interactions with tree roots using a microfluidics setup. *Front. Plant Sci.* 11:408. doi: 10.3389/fpls.2020.00408

Okutani, F., Hamamoto, S., Aoki, Y., Nakayasu, M., Nihei, N., Nishimura, T., et al. (2020). Rhizosphere modelling reveals spatiotemporal distribution of daidzein shaping soybean rhizosphere bacterial community. *Plant Cell Environ.* 43, 1036–1046. doi: 10.1111/pce.13708

Ortíz-Castro, R., Contreras-Cornejo, H. A., Macías-Rodríguez, L., and López-Bucio, J. (2009). The role of microbial signals in plant growth and development. *Plant Signal. Behav.* 4, 701–712. doi: 10.4161/psb.4.8.9047

Pagnani, G., Pellegrini, M., Galieni, A., D'Egidio, S., Matteucci, F., Ricci, A., et al. (2018). Plant growth-promoting rhizobacteria (pgpr) in cannabis sativa 'finola' cultivation: an alternative fertilization strategy to improve plant growth and quality characteristics. *Indust. Crops Prod.* 123, 75–83. doi: 10.1016/j.indcrop.2018.06.033

Pangesti, N., Reichelt, M., van de Mortel, J. E., Kapsomenou, E., Gershenzon, J., van Loon, J. J., et al. (2016). Jasmonic acid and ethylene signaling pathways regulate glucosinolate levels in plants during rhizobacteria-induced systemic resistance against a leaf-chewing herbivore. *J. Chem. Ecol.* 42, 1212–1225. doi: 10.1007/s10886-016-0787-7

Pascale, A., Proietti, S., Pantelides, I. S., and Stringlis, I. A. (2020). Modulation of the root microbiome by plant molecules: the basis for targeted disease suppression and plant growth promotion. *Front. Plant Sci.* 10:1741. doi: 10.3389/fpls.2019.01741

Pei, Y. C., Siemann, E., Tian, B. L., and Ding, J. Q. (2020). Root flavonoids are related to enhanced amf colonization of an invasive tree. *AoB Plants* 12:laa002. doi: 10.1093/aobpla/plaa002

Peñuelas, J., Farré-Armengol, G., Llusia, J., Gargallo-Garriga, A., Rico, L., Sardans, J., et al. (2014). Removal of floral microbiota reduces floral terpene emissions. *Sci. Rep.* 4:6727. doi: 10.1038/srep06727

Perez-Jaramillo, J. E., de Hollander, M., Ramirez, C. A., Mendes, R., Raaijmakers, J. M., and Carrion, V. J. (2019). Deciphering rhizosphere microbiome assembly of wild and modern common bean (*Phaseolus vulgaris*) in native and agricultural soils from colombia. *Microbiome* 7:114. doi: 10.1186/s40168-019-0727-1

Peter, A., Pola, S. P., Sandeep, B., and Rao, B. (2019). *Antimicrobial and Anti-Quorum Sensing Activities of Medicinal Plants*. Singapore: Springer.

Petriacq, P., Williams, A., Cotton, A., McFarlane, A. E., Rolfe, S. A., and Ton, J. (2017). Metabolite profiling of non-sterile rhizosphere soil. *Plant J.* 92, 147–162. doi: 10.1111/tpj.13639

Piasecka, A., Jedrzejczak-Rey, N., and Bednarek, P. (2015). Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. *New Phytol.* 206, 948–964. doi: 10.1111/nph.13325

Pini, F., East, A. K., Appia-Ayme, C., Tomek, J., Karunakaran, R., Mendoza-Suarez, M., et al. (2017). Bacterial biosensors for in vivo spatiotemporal mapping of root secretion. *Plant Physiol.* 174, 1289–1306. doi: 10.1104/pp.16.01302

Rajniak, J., Giehl, R. F. H., Chang, E., Murgia, I., von Wiren, N., and Sattely, E. S. (2018). Biosynthesis of redox-active metabolites in response to iron deficiency in plants. *Nat. Chem. Biol.* 14, 442–450. doi: 10.1038/s41589-018-0019-2

Ray, S., Singh, J., Rajput, R. S., Yadav, S., Singh, S., and Singh, H. B. (2019). "A thorough comprehension of host endophytic interaction entailing the biospherical benefits: a metabolomic perspective," in *Endophytes and Secondary Metabolites*, ed. S. Jha (Cham: Springer International Publishing).

Ray, T., Pandey, S. S., Pandey, A., Srivastava, M., Shanker, K., and Kalra, A. (2019). Endophytic consortium with diverse gene-regulating capabilities of benzylisoquinoline alkaloids biosynthetic pathway can enhance endogenous morphine biosynthesis in papaver somniferum. *Front. Microbiol.* 10:925. doi: 10.3389/fmicb.2019.00925

Raza, W., Wang, J., Jousset, A., Friman, V.-P., Mei, X., Wang, S., et al. (2020). Bacterial community richness shifts the balance between volatile organic compound-mediated microbe–pathogen and microbe–plant interactions. *Proc. R. Soc. B Biol. Sci.* 287:20200403. doi: 10.1098/rspb.2020.0403

Reshef, D. N., Reshef, Y. A., Finucane, H. K., Grossman, S. R., McVean, G., Turnbaugh, P. J., et al. (2011). Detecting novel associations in large data sets. *Science* 334, 1518–1524. doi: 10.1126/science.1205438

Rodriguez, P. A., Rothballer, M., Chowdhury, S. P., Nussbaumer, T., Gutjahr, C., and Falter-Braun, P. (2019). Systems biology of plant-microbiome interactions. *Mol. Plant* 12, 804–821. doi: 10.1016/j.molp.2019.05.006

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahe, F. (2016). Vsearch: a versatile open source tool for metagenomics. *PeerJ.* 4:e2584. doi: 10.7717/peerj.2584

Sasse, J., Martinoia, E., and Northen, T. (2018). Feed your friends: do plant exudates shape the root microbiome? *Trends Plant Sci.* 23, 25–41. doi: 10.1016/j.tplants.2017.09.003

Schandry, N., and Becker, C. (2020). Allelopathic plants: models for studying plant–interkingdom interactions. *Trends Plant Sci.* 25, 176–185. doi: 10.1016/j.tplants.2019.11.004

Schenkel, D., Deveau, A., Niimi, J., Mariotte, P., Vitra, A., Meisser, M., et al. (2019). Linking soil's volatilome to microbes and plant roots highlights the importance of microbes as emitters of belowground volatile signals. *Environ. Microbiol.* doi: 10.1111/1462-2920.14599 Online ahead of print

Schlechter, R. O., Miebach, M., and Remus-Emsermann, M. N. P. (2019). Driving factors of epiphytic bacterial communities: a review. *J. Adv. Res.* 19, 57–65. doi: 10.1016/j.jare.2019.03.003

Schulz-Bohm, K., Gerards, S., Hundscheid, M., Melenhorst, J., de Boer, W., and Garbeva, P. (2018). Calling from distance: attraction of soil bacteria by plant root volatiles. *ISME J.* 12, 1252–1262. doi: 10.1038/s41396-017-0035-3

Schütz, V., Bigler, L., Girel, S., Laschke, L., Sicker, D., and Schulz, M. (2019). Conversions of benzoxazinoids and downstream metabolites by soil microorganisms. *Front. Ecol. Evol.* 7:238. doi: 10.3389/fevo.2019.00238

Shtark, O. Y., Shishova, M. F., Povydysh, M. N., Avdeeva, G. S., Zhukov, V. A., and Tikhonovich, I. A. (2018). Strigolactones as regulators of symbiotrophy of plants and microorganisms. *Russ. J. Plant Physiol.* 65, 151–167. doi: 10.1134/S1021443718020073

Smilde, A. K., Måge, I., Næs, T., Hankemeier, T., Lips, M. A., Kiers, H. A. L., et al. (2017). Common and distinct components in data fusion. *J. Chemom.* 31:e2900. doi: 10.1002/cem.2900

Sokol, N. W., Kuebbing, S. E., Karlsen-Ayala, E., and Bradford, M. A. (2019). Evidence for the primacy of living root inputs, not root or shoot litter, in forming soil organic carbon. *New Phytol.* 221, 233–246. doi: 10.1111/nph.15361

Stassen, M. J. J., Hsu, S.-H., Pieterse, C. M. J., and Stringlis, I. A. (2020). Coumarin communication along the microbiome–root–shoot axis. *Trends Plant Sci.* 26, 169–183. doi: 10.1016/j.tplants.2020.09.008

Stefanowicz, A. M., Stanek, M., Nobis, M., and Zubek, S. (2017). Few effects of invasive plants reynoutria japonica, rudbeckia laciniata and solidago gigantea on soil physical and chemical properties. *Sci. Total Environ.* 574, 938–946. doi: 10.1016/j.scitotenv.2016.09.120

Stringlis, I. A., de Jonge, R., and Pieterse, C. M. J. (2019). The age of coumarins in plant-microbe interactions. *Plant Cell Physiol.* 60, 1405–1419. doi: 10.1093/pcp/pcz076

Stringlis, I. A., Yu, K., Feussner, K., de Jonge, R., Van Bentum, S., Van Verk, M. C., et al. (2018). Myb72-dependent coumarin exudation shapes root microbiome assembly to promote plant health. *Proc. Natl. Acad. Sci. U.S.A.* 115, E5213–E5222. doi: 10.1073/pnas.1722335115

Sugiyama, A. (2019). The soybean rhizosphere: metabolites, microbes, and beyond-a review. *J. Adv. Res.* 19, 67–73. doi: 10.1016/j.jare.2019.03.005

Sugiyama, A., and Yazaki, K. (2014). Flavonoids in plant rhizospheres: secretion, fate and their effects on biological communication. *Plant Biotechnol.* 31, 431–443. doi: 10.5511/plantbiotechnology.14.0917a

Szoboszlay, M., White-Monsant, A., and Moe, L. A. (2016). The effect of root exudate 7,4'-dihydroxyflavone and naringenin on soil bacterial community structure. *PLoS One* 11:e0146555. doi: 10.1371/journal.pone.0146555

Taghinasab, M., and Jabaji, S. (2020). Cannabis microbiome and the role of endophytes in modulating the production of secondary metabolites: an overview. *Microorganisms* 8:355. doi: 10.3390/microorganisms8030355

Tahir, A. T., Fatmi, Q., Nosheen, A., Imtiaz, M., and Khan, S (2019). "Metabolomic approaches in plant research," in *Essentials of Bioinformatics, Volume III: In Silico Life Sciences: Agriculture*, eds K. R. Hakeem, N. A. Shaik, and B. Banaganapalli (Cham: Springer International Publishing).

Tian, T., Reverdy, A., She, Q., Sun, B., and Chai, Y. (2020). The role of rhizodeposits in shaping rhizomicrobiome. *Environ. Microbiol. Rep.* 12, 160–172. doi: 10.1111/1758-2229.12816

Tian, Y., Amand, S., Buisson, D., Kunz, C., Hachette, F., Dupont, J., et al. (2014). The fungal leaf endophyte paraconiothyrium variabile specifically metabolizes the host-plant metabolome for its own benefit. *Phytochemistry* 108, 95–101. doi: 10.1016/j.phytochem.2014.09.021

Tidke, S. A., Kiran, S., Giridhar, P. and Gokare, R. A. (2019). "Current understanding and future perspectives of endophytic microbes vis-a-vis production of secondary metabolites," in *Endophytes and Secondary Metabolites*, ed. S. Jha (Cham: Springer International Publishing).

Trda, L., Janda, M., Mackova, D., Pospichalova, R., Dobrev, P. I., Burketova, L., et al. (2019). Dual mode of the saponin aescin in plant protection: antifungal agent and plant defense elicitor. *Front. Plant Sci.* 10:1448. doi: 10.3389/fpls.2019.01448

Ullrich, C. I., Aloni, R., Saeed, M. E. M., Ullrich, W., and Efferth, T. (2019). Comparison between tumors in plants and human beings: mechanisms of tumor development and therapy with secondary plant metabolites. *Phytomedicine* 64:153081. doi: 10.1016/j.phymed.2019.153081

van der Kloet, F. M., Sebastian-Leon, P., Conesa, A., Smilde, A. K., and Westerhuis, J. A. (2016). Separating common from distinctive variation. *BMC Bioinformatics* 17(Suppl. 5):195. doi: 10.1186/s12859-016-1037-2

Van Deynze, A., Zamora, P., Delaux, P. M., Heitmann, C., Jayaraman, D., Rajasekar, S., et al. (2018). Nitrogen fixation in a landrace of maize is supported by a mucilage-associated diazotrophic microbiota. *PLoS Biol.* 16:e2006352. doi: 10.1371/journal.pbio.2006352

Varoquaux, N., Cole, B., Gao, C., Pierroz, G., Baker, C. R., Patel, D., et al. (2019). Transcriptomic analysis of field-droughted sorghum from seedling to maturity reveals biotic and metabolic responses. *Proc Natl Acad Sci U S A*. doi: 10.1073/pnas.1907500116 Online ahead of print

Venturi, V., and Keel, C. (2016). Signaling in the rhizosphere. *Trends Plant Sci.* 21, 187–198. doi: 10.1016/j.tplants.2016.01.005

Verbeek, J. D., and Kotanen, P. M. (2019). Soil-mediated impacts of an invasive thistle inhibit the recruitment of certain native plants. *Oecologia* 190, 619–628. doi: 10.1007/s00442-019-04435-8

Vives-Peris, V., de Ollas, C., Gomez-Cadenas, A., and Perez-Clemente, R. M. (2020). Root exudates: from plant to rhizosphere and beyond. *Plant Cell Rep.* 39, 3–17. doi: 10.1007/s00299-019-02447-5

Voges, M., Bai, Y., Schulze-Lefert, P., and Sattely, E. S. (2019). Plant-derived coumarins shape the composition of an *Arabidopsis* synthetic root microbiome. *Proc. Natl. Acad. Sci. U.S.A.* 116, 12558–12565. doi: 10.1073/pnas.1820691116

Wang, D., Xu, Z., Zhang, G., Xia, L., Dong, X., Li, Q., et al. (2019). A genomic island in a plant beneficial rhizobacterium encodes novel antimicrobial fatty acids and a self-protection shield to enhance its competition. *Environ. Microbiol.* doi: 10.1111/1462-2920.14683 Online ahead of print

Wang, P., and Niu, B. (2019). Plant specialized metabolites modulate root microbiomes. *Sci. China Life Sci.* 62, 1111–1113. doi: 10.1007/s11427-019-9579-6

Wang, W., Yang, J., Zhang, J., Liu, Y. X., Tian, C., Qu, B., et al. (2020). An *Arabidopsis* secondary metabolite directly targets expression of the bacterial type iii secretion system to inhibit bacterial virulence. *Cell Host Microbe* 27, 601.e7–613.e7. doi: 10.1016/j.chom.2020.03.004

Wasternack, C., and Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in annals of botany. *Ann. Bot.* 111, 1021–1058. doi: 10.1093/aob/mct067

Weiss, S., Van Treuren, W., Lozupone, C., Faust, K., Friedman, J., Deng, Y., et al. (2016). Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. *ISME J.* 10, 1669–1681. doi: 10.1038/ismej.2015.235

White, L. J., Ge, X., Brözel, V. S., and Subramanian, S. (2017). Root isoflavonoids and hairy root transformation influence key bacterial taxa in the soybean rhizosphere. *Environ. Microbiol.* 19, 1391–1406. doi: 10.1111/1462-2920.13602

Williams, A., and de Vries, F. T. (2020). Plant root exudation under drought: implications for ecosystem functioning. *New Phytol.* 225, 1899–1905. doi: 10.1111/nph.16223

Xu, L., Naylor, D., Dong, Z., Simmon, T., Pierroz, G., Hixson, K. K., et al. (2018). Drought delays development of the sorghum root microbiome and enriches for monoderm bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 115:E4952. doi: 10.1073/pnas.1807275115

Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F., and Wang, Q. (2018). Response of plant secondary metabolites to environmental factors. *Molecules* 23:762. doi: 10.3390/molecules23040762

Yang, W., Zhang, D., Cai, X., Xia, L., Luo, Y., Cheng, X., et al. (2019). Significant alterations in soil fungal communities along a chronosequence of spartina alterniflora invasion in a chinese yellow sea coastal wetland. *Sci. Total Environ.* 693:133548. doi: 10.1016/j.scitotenv.2019.07.354

You, M., Fang, S. M., MacDonald, J., Xu, J., and Yuan, Z. C. (2020). Isolation and characterization of Burkholderia cenocepacia CR318, a phosphate solubilizing bacterium promoting corn growth. *Microbiol. Res.* 233:126395. doi: 10.1016/j.micres.2019.126395

Youens-Clark, K., Bomhoff, M., Ponsero, A. J., Wood-Charlson, E. M., Lynch, J., Choi, I., et al. (2019). Imicrobe: tools and data-dreaiven discovery platform for the microbiome sciences. *Gigascience* 8:giz083. doi: 10.1093/gigascience/giz083

Yuan, J., Zhao, J., Wen, T., Zhao, M., Li, R., Goossens, P., et al. (2018). Root exudates drive the soil-borne legacy of aboveground pathogen infection. *Microbiome* 6:156. doi: 10.1186/s40168-018-0537-x

Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., da Rocha, U. N., Shi, S., et al. (2018). Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nat. Microbiol.* 3, 470–480. doi: 10.1038/s41564-018-0129-3

Zhan, Z. J., Tian, T., Xu, Y. L., Yu, H. F., Zhang, C. X., Zhang, Z. D., et al. (2019). Biotransformation of huperzine b by a fungal endophyte of huperzia serrata. *Chem. Biodivers.* 16:e1900299. doi: 10.1002/cbdv.201900299

Zhang, B., Zhang, J., Liu, Y., Shi, P., and Wei, G. (2018). Co-occurrence patterns of soybean rhizosphere microbiome at a continental scale. *Soil Biol. Biochem.* 118, 178–186. doi: 10.1016/j.soilbio.2017.12.011

Zhang, H., Kim, M. S., Krishnamachari, V., Payton, P., Sun, Y., Crimson, M., et al. (2007). Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. *Planta* 226, 839–851. doi: 10.1007/s00425-007-0530-2

Zhang, J., Liu, Y. X., Zhang, N., Hu, B., Jin, T., Xu, H., et al. (2019). Nrt1.1b is associated with root microbiota composition and nitrogen use in field-grown rice. *Nat. Biotechnol.* 37, 676–684. doi: 10.1038/s41587-019-0104-4

Zhang, Y., Li, S. S., Li, H. X., Wang, R. R., Zhang, K. Q., and Xu, J. (2020). Fungal-nematode interactions: diversity, ecology and biocontrol prospects in agriculture. *J. Fungi.* 6:206. doi: 10.3390/jof6040206

Zhang, Y., Xu, J., Riera, N., Jin, T., Li, J., and Wang, N. (2017). Huanglongbing impairs the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome. *Microbiome* 5:97. doi: 10.1186/s40168-017-0304-4

Zheng, Q., Bartow-McKenney, C., Meisel, J. S., and Grice, E. A. (2018). Hmmufotu: an hmm and phylogenetic placement based ultra-fast taxonomic assignment and otu picking tool for microbiome amplicon sequencing studies. *Genome Biol.* 19:82. doi: 10.1186/s13059-018-1450-0

Zhou, F., Emonet, A., Dénervaud Tendon, V., Marhavy, P., Wu, D., Lahaye, T., et al. (2020). Co-incidence of damage and microbial patterns controls localized immune responses in roots. *Cell* 180, 440.e18–453.e18. doi: 10.1016/j.cell.2020.01.013

Zhou, F., and Pichersky, E. (2020). More is better: the diversity of terpene metabolism in plants. *Curr. Opin. Plant Biol.* 55, 1–10. doi: 10.1016/j.pbi.2020.01.005

Zhou, J.-L., Xu, J., Jiao, A.-G., Yang, L., Chen, J., Callac, P., et al. (2019). Patterns of PCR amplification artifacts of the fungal barcode marker in a hybrid mushroom. *Front. Microbiol.* 10:2686. doi: 10.3389/fmicb.2019.02686

Zhou, J. Y., Sun, K., Chen, F., Yuan, J., Li, X., and Dai, C. C. (2018). Endophytic *Pseudomonas* induces metabolic flux changes that enhance medicinal sesquiterpenoid accumulation in atractylodes lancea. *Plant Physiol. Biochem.* 130, 473–481. doi: 10.1016/j.plaphy.2018.07.016

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Exploring Biocontrol Agents From Microbial Keystone Taxa Associated to Suppressive Soil: A New Attempt for a Biocontrol Strategy

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Recent studies have observed differing microbiomes between disease-suppressive and disease-conducive soils. However, it remains unclear whether the microbial keystone taxa in suppressive soil are critical for the suppression of diseases. Bacterial wilt is a common soil-borne disease caused by *Ralstonia solanacearum* that affects tobacco plants. In this study, two contrasting tobacco fields with bacterial wilt disease incidences of 0% (disease suppressive) and 100% (disease conducive) were observed. Through amplicon sequencing, as expected, a high abundance of *Ralstonia* was found in the disease-conducive soil, while large amounts of potential beneficial bacteria were found in the disease-suppressive soil. In the fungal community, an abundance of the *Fusarium* genus, which contains species that cause *Fusarium* wilt, showed a positive correlation ($p < 0.001$) with the abundance of *Ralstonia*. Network analysis revealed that the healthy plants had more complex bacterial networks than the diseased plants. A total of 9 and 13 bacterial keystone taxa were identified from the disease-suppressive soil and healthy root, respectively. Accumulated abundance of these bacterial keystones showed a negative correlation ($p < 0.001$) with the abundance of *Ralstonia*. To complement network analysis, culturable strains were isolated, and three species belonging to *Pseudomonas* showed high 16S rRNA gene similarity (98.4–100%) with keystone taxa. These strains displayed strong inhibition on pathogens and reduced the incidence of bacterial wilt disease in greenhouse condition. This study highlighted the importance of keystone species in the protection of crops against pathogen infection and proposed an approach to obtain beneficial bacteria through identifying keystone species, avoiding large-scale bacterial isolation and cultivation.

Keywords: bacterial wilt disease, microbiome, Biocontrol Agents, network, biocontrol, suppressive soil

INTRODUCTION

Bacterial wilt, a common soil-borne disease caused by *Ralstonia solanacearum*, occurs annually in most tobacco cultivation regions and causes huge economic losses (Jiang et al., 2017a). It has been reported that plants can adjust their microbiome and specifically recruit disease-resistant and growth-promoting microbes or microbial consortia when they encounter

a disease (Berendsen et al., 2018). The soil with low disease incidence even in the coexistence of susceptible plants and pathogens was defined as disease-suppressive soil. The mechanisms behind soil-mediated suppression of diseases, such as bacterial wilt disease in tobacco (Qi et al., 2019; Zhang et al., 2020), *Fusarium* wilt in vanilla (Xiong et al., 2017), *Rhizoctonia solani* in sugar beet (Mendes et al., 2011), and black root rot in tobacco (Kyselkova et al., 2009), have been extensively investigated. The soil microbiome has been deciphered in detail, and it has been found that the soil compositions between suppressive and conducive soils are distinct, although they share similar geographical and climatic conditions, as well as the same agronomic management.

To prevent and control bacterial wilt disease, hundreds of beneficial microbes that exhibit suppressive activity against *R. solanacearum* have been isolated (Jiang et al., 2017a), e.g., *Pseudomonas* spp. (Raaijmakers and Weller, 1998; Mendes et al., 2011; Hu et al., 2016), *Bacillus* spp. (Wei et al., 2011; Guo et al., 2020), *Burkholderia* spp. (Nion and Toyota, 2008), and *Streptomyces* spp. (Boukaew et al., 2011). They control bacterial wilt disease by producing antibacterial substance and inducing host systemic resistance. Moreover, it has been indicated that multiple beneficial microbial consortia or synthetic community (SynCom) has been shown to be more efficient than the single isolates in controlling bacterial wilt (Hu et al., 2016; Jiang et al., 2017a), suggesting that interactions and synergistic effects among microbes are critical.

Network analysis is a particularly useful tool to understand microbe-microbe associations and to visualize co-occurrence patterns among communities (Barberan et al., 2012; de Vries et al., 2018; Banerjee et al., 2019). Previous studies have shown distinct bacterial networks between healthy and diseased plants (Xiong et al., 2017; Wei et al., 2019). The bacterial network of healthy soil was more complex and stable than bacterial wilt-susceptible soil (Qi et al., 2019). Network scores can be used to identify keystone taxa, i.e., species highly associated with a microbial community (Berry and Widder, 2014; Banerjee et al., 2018), which have a significant influence on the community (Agler et al., 2016; Banerjee et al., 2016; Hu et al., 2020). Extensive keystone taxa have been identified in various terrestrial ecosystems, such as forest (Ding et al., 2015), agricultural (Jiang et al., 2017b; Wang et al., 2017a), and grassland soils (Lupatini et al., 2014). These keystone taxa can be indicators of plant or soil microbiome shifts linked to abiotic factors (Zhou et al., 2011; Liang et al., 2016) and host genotypes (Jiang et al., 2017b). Moreover, Agler et al. have suggested that microbial keystone taxa are promising targets in control of plant-pathogen relationships and in construction of beneficial host-associated microbial communities (Agler et al., 2016). A previous study showed that keystone taxa contributed to the divergences in the microbiome between healthy and diseased tomato status and demonstrated that the *Massilia*, *Dyadobacter*, *Terrabacter*, *Arachidicoccus*, and *Dyella* genera were potential keystone taxa in healthy plants (Wei et al., 2019). Qi et al. found more key microorganisms (e.g., *Bacillus* and *Actinobacteria*) in healthy soil than in bacterial wilt-susceptible soil through network analysis. However, it remains unclear if these keystone microbial species have the ability to suppress bacterial wilt. Additionally, previous studies mainly focused on bacterial community and network structure, but the impact on fungal composition and network made by bacterial wilt disease are little known.

In this study, we observed two contrasting fields. Bacterial wilt disease incidence was 0% in suppressive soil but 100% in conducive soil, although they were geographically proximate and shared the same management. Here, we aimed to answer the following: (i) How does the bacterial wilt disease change the microbe-microbe interaction and network structure? (ii) What is the keystone taxa in suppressive soil? (iii) What is the relationship between keystone taxa and bacterial wilt disease pathogen? (iv) Can these keystone taxa reduce the incidence of bacterial wilt disease? To achieve this, we sampled tobacco plants from these contrasting fields. Bacterial and fungal communities were determined by 16S ribosomal RNA (rRNA) gene and internal transcribed spacer (ITS) amplicon sequencing, respectively. Network analyses were used to uncover microbe-microbe interactions and identify keystone taxa from suppressive fields. Culturable keystone species were isolated, and their role in protecting against disease was evaluated using a greenhouse experiment.

MATERIALS AND METHODS

Sampling

We collected 40 samples from four model experiment stations in Zunyi city (Guizhou province, China), all of which harbored two contrasting soils, bacterial wilt disease-suppressive and disease-conducive soils. Bacterial wilt disease rarely occurred in disease-suppressive soil, although tobacco had been continuously planted for ~20 years. In disease-conducive soil, high disease incidences were observed every year. Rhizosphere soil and root samples were collected from each field on August 10, 2018 (70 days after transplanting). When we collected samples, none of the plants showed wilt symptoms in the suppressive soil, while all plants suffered from different levels of wilt disease in the conducive soil. Thus, healthy plants were collected from the suppressive soil, and plants with slight disease symptoms were collected from the conducive soil. From each field, two or three random tobacco plants were chosen. The roots were shaken to remove loosely adhering soil, put into sterile plastic bags, and transported to the laboratory on ice. Finally, the four groups in the study were rhizosphere soil (SS, n = 10) and healthy tobacco roots (HR, n = 10) in suppressive fields and rhizosphere soil (CS, n = 10) and infected tobacco roots (IR, n = 10) in conducive fields.

Sample Processing and DNA Extraction

Soil attached firmly to the root was collected with a brush and considered to be the rhizosphere soil. The root was shaken vigorously with phosphate-buffered saline (PBS) solution

in order to clean all the soil from the root surface. They were then soaked in alcohol (75%) for 2 min and sodium hypochlorite (5%) for 5 min. The final rinse with sterile water was performed three times for 1 min. We refer to the community on surface-sterilized roots as the root microbiome. All samples were stored at −80°C until DNA extraction. Rhizosphere soil (0.5 g) and root samples were used to perform DNA extractions with the DNeasy® PowerSoil Kit (Qiagen) according to the manufacturer's instructions. DNA concentration and purity were monitored using a nanodrop and 1% agarose gels, respectively.

PCR Amplification and 16S rRNA Gene Amplicon Sequencing

For the community analysis, the V5–V7 regions of the 16S rRNA gene and the ITS region 1 of the nuclear ribosomal coding cistron were amplified using primers 799F and 1193R and ITS1F and ITS2R (**Supplementary Table S1**). The PCR reactions were performed in 30 μl reactions with 15 μl of the Phusion® High-Fidelity PCR Master Mix (2×, New England Biolabs), 0.2 μM of primers, and 10 ng of the template DNA. Thermal cycling for the 16S rRNA gene consisted of an initial denaturation at 98°C for 1 min, followed by 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 5 min. Thermal cycling for ITS region 1 consisted of 95°C for 5 min, followed by 34 cycles of 94°C for 1 min, 57°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 10 min. PCR products were detected using 2% agarose gel electrophoresis. Then, the PCR product mixture in equi-density ratios was purified with the GeneJETTM Gel Extraction Kit (Thermo Scientific). Libraries were constructed using the Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific). The qualified libraries were sequenced on an Ion S5TM platform (Thermo Fisher Scientific Inc., Waltham, MA, United States) at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

Quantification of Bacteria and *R. solanacearum*

Bacterial abundance was determined using the Eub338F/Eub518R primers (**Supplementary Table S1**). The specific primer Rsol_*fliC* (**Supplementary Table S1**) that targets the *fliC* gene encoding the flagellum subunit was used to quantify *R. solanacearum* densities. The quantitative PCR (qPCR) analyses were performed using an Applied Biosystems 7,500 Real-Time PCR System (Applied Biosystems, United States). Standard curves were generated using 10-fold serial dilutions of a plasmid containing the *16S rRNA* gene from *Arthrobacter pokkalii* and a fragment copy of *R. solanacearum fliC*. qPCR amplifications for standard and DNA samples included a 20-μl mixture containing 2 μl of templates, 10 μl of the SYBR Green premix Ex Taq (2×), 0.4 μl of ROX Reference Dye II, 0.4 μl of each primer, and distilled water. The PCR thermal cycling conditions for bacteria and *R. solanacearum* were conducted according to previous procedures (Muyzer et al., 1993; Schonfeld et al., 2003; Fierer et al., 2005) with three technical replicates.

Amplicon Sequence Processing and Statistical Analysis

Raw reads were quality filtered using Cutadapt v1.9.1 (Martin, 2011) to remove low-quality reads and obtain high-quality clean reads. Chimera sequences were detected using the UCHIME algorithm (Edgar et al., 2011) and then removed to obtain the final clean reads. Operational taxonomic unit (OTU) assignment was performed by Uparse (v7.0.1001; Edgar, 2013) at a similarity of 97%. For each OTU representative sequence, the Silva (release 132, https://www.arb-silva.de) and Unite database (version 7.2, https://unite.ut.ee) were used to annotate bacterial and fungal taxonomic information, respectively. To equalize sequencing depth, each sample was rarefied to the lowest sequence number across all samples (41,326 reads for bacteria and 63,022 reads for fungi) for downstream analyses. Alpha and beta diversities were calculated with Qiime (version 1.7.0; Caporaso et al., 2010) and visualized using R software.

Differences between the microbial communities of the suppressive and conducive field samples (including soil and root) were calculated using analysis of similarities (ANOSIM) in PRIMER 6 (Clarke and Gorley, 2006). The microbiome composition in the different samples was ordinated by principal coordinates analysis (PCoA) by unweighted UniFrac distance with WGCNA package, stat packages, and the ggplot2 package in R software. In this study, the top 100 abundant bacterial genera were used for co-occurrence network construction. The Spearman correlation matrix was calculated [absolute correlation coefficient values >0.6; the false discovery rate (FDR) adjusted $p < 0.05$] using the psych package in the R environment (Revelle, 2017). The network was analyzed and visualized using Gephi (version 0.9.2; Bastian et al., 2009). The taxa harboring the highest degree and closeness centrality and the lowest betweenness centrality values were considered as the keystone taxa (Berry and Widder, 2014). Based on this, genera with a degree >40, a closeness centrality >0.61, and a betweenness centrality <0.18 were considered as the keystone taxa for the soil and root in suppressive fields. We further used the online NetShift tool,[1] which could identify potential driver taxa that maintain the healthy microbiome against diseased samples. Statistical analyses were performed using *t* tests in GraphPad Prism 7. Linear regression analysis was performed in Microsoft Excel to determine the association between the absolute abundance (qPCR) and relative abundance (amplicon sequencing) of *R. solanacearum* as well as the abundances of *Ralstonia* and *Fusarium*.

Bacterial Isolation and Identification

Surface sterile root samples from the suppressive soil were ground. The homogenate was serially diluted to 10^{-2}, and 100 μl of each dilution was spread on nutrient agar (NA) for bacterial isolation. Media were prepared according to methods described by Dhingra and Sinclair (1985). All plates were incubated at 28°C for 2–4 days. Morphologically different bacterial isolates were picked from plates containing approximately 30–300

[1]https://web.rniapps.net/netshift/

colonies. All the isolates were purified three times and stored at −80°C with 15% (v/v) glycerol. The bacterial genomic DNA was extracted using an EasyPure Bacteria Genomic DNA Kit according to the manufacturer's instructions. Bacterial universal primers 27F/1492R (**Supplementary Table S1**) were used to amplify the full-length 16S rRNA genes. PCR products were tested by 1.0% agarose gel electrophoresis and sequenced by TsingKe Biological Technology Co., Ltd. (Beijing, China). Identification and sequence similarity were achieved using the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). Sequences of these isolates and OTUs from high-throughput sequencing were aligned using the CLUSTAL_X program (Thompson et al., 1997), and a neighbor-joining phylogenetic tree was constructed using MEGA 7.0 with a bootstrap value of 1,000 replicates (Kumar et al., 2016).

Inhibition and Greenhouse Experiment

Three isolates (*Pseudomonas lurida* FGD5-2, *Pseudomonas koreensis* HCH2-3, and *Pseudomonas rhodesiae* MTD4-1) that showed high 16S rRNA gene similarity with keystone taxa were selected. Their inhibitory effect on *R. solanacearum* were determined by the Oxford cup method. Briefly, 100 μl of *R. solanacearum* culture was spread on NA plates. Then, sterilized Oxford cups were placed on the plates, and 200 μl culture of each isolate was put into these cups in triplicate. All plates were incubated at 28°C for 24 h. The diameter of the inhibition zone was recorded. For the greenhouse experiment, tobacco seeds were sown into a seedling tray. At the three-leaf stage, tobacco plants were transplanted to a plastic pot containing 300 g of soil collected from a farmland in Qingdao city, Shandong province, China. Bacteria were cultivated using nutrient broth medium for 24 h and centrifuged at 4°C, 4000 rpm to concentrate bacterial cells and then resuspended them in the sterile water. After 5 days of transplantation, bacterial cells were inoculated by the use of root drenching methods with a final density of ~10^7 cells per gram of soil three times at intervals of 5 days (Wei et al., 2013). Microbial consortia, a mixture of all isolates with a 1:1 ratio, were also inoculated. The control group was treated with equal amounts of sterile water. After 5 days postinoculation of beneficial stains, the pathogen of *R. solanacearum* Rs10 (prepared as the same methods of keystone species) was inoculated, resulting in ~10^7 cells/g soil. Each treatment was replicated three times, and each replicate contained 12 plants. Plants were arranged in a climate chamber at 30°C with a relative humidity of 70% and watered regularly with sterile water. The disease index was assessed 15 days after pathogen inoculation.

RESULTS

The Abundances of Bacteria and *R. solanacearum*

Based on the quantitative PCR results, the bacterial 16S rRNA gene abundance ranged from 6.15×10^8 to 1.51×10^9 copies/g. No significant differences in bacterial abundance were observed among the different samples (**Supplementary Figure S1**). As expected, the densities of *R. solanacearum* increased significantly ($p < 0.01$) with 5.12×10^6 and 9.13×10^7 copies of *fliC* gene per gram in conducive soil and infected roots, respectively. These values in suppressive soil and healthy roots were 2.39×10^4 and 6.86×10^4 copies/g, respectively (**Supplementary Figure S1**). Additionally, the fold changes in *R. solanacearum* densities between the suppressive and conducive fields were larger in the roots (~1,330-fold) than in the soil (~214-fold), suggesting that *R. solanacearum* infection might exert more of an impact on the root compartment.

Diversity of Microbial Communities

A total of 3,798,025 and 3,846,278 clean reads were produced from 16S rRNA gene and ITS amplicon sequencing, respectively. Good's coverage of all samples was >99.4%, and most rarefaction curves tended to reach the saturation plateau (**Supplementary Figure S2**), which suggested that the sequencing depth was sufficient to represent the microbial community in these environments. These sequences were grouped into 2,469 bacterial and 1,606 fungal OTUs. The Venn diagram showed that 591 bacterial OTUs were shared among the four groups, while 248, 286, 63, and 9 OTUs were unique to suppressive soil, conducive soil, healthy root, and infected root, respectively. The four groups shared 499 fungal OTUs, while suppressive soil, conducive soil, healthy root, and infected root harbored 106, 198, 93, and 104 specific OTUs, respectively (**Supplementary Figure S3**). According to the diversity indices, we observed that both bacterial and fungal species were significantly lower ($p < 0.05$) in the suppressive soil than in the conducive soil (**Figures 1A**,**C**). In contrast, for the root samples, the observed bacterial species in the healthy plants were significantly higher ($p < 0.001$) than those in the infected plants, whereas there was no significant difference in fungal species. These diversity patterns were confirmed by the Chao1 indices (**Figures 1B**,**D**).

For the beta diversity, principal coordinate analysis (PCoA) showed that both the bacterial and fungal communities from the suppressive soil and healthy root were clearly separated from the conducive soil and infected root (**Figure 2**). This result was confirmed by the analysis of similarity (ANOSIM) of Bray-Curtis measures, which indicated that significant differences were observed between the suppressive and conducive soils ($R = 0.530$, $p < 0.01$ for bacteria; $R = 0.339$, $p < 0.01$ for fungi) and the healthy and infected roots ($R = 0.656$, $p < 0.01$ for bacteria; $R = 0.553$, $p < 0.01$ for fungi). Additionally, there were no significant differences between the soil and root of healthy plants ($R = 0.0408$, $p = 0.203$) as well as the soil and root of infected plants ($R = 0.0644$, $p = 0.147$) regarding the fungal community, suggesting that a healthy status rather than the sample type was the key factor affecting the fungal profile in this study.

Specific Differences in Microbiome Between Suppressive and Conducive Fields

At the phylum level, all samples shared three dominant bacterial phyla, Proteobacteria, Actinobacteria, and Firmicutes, accounting

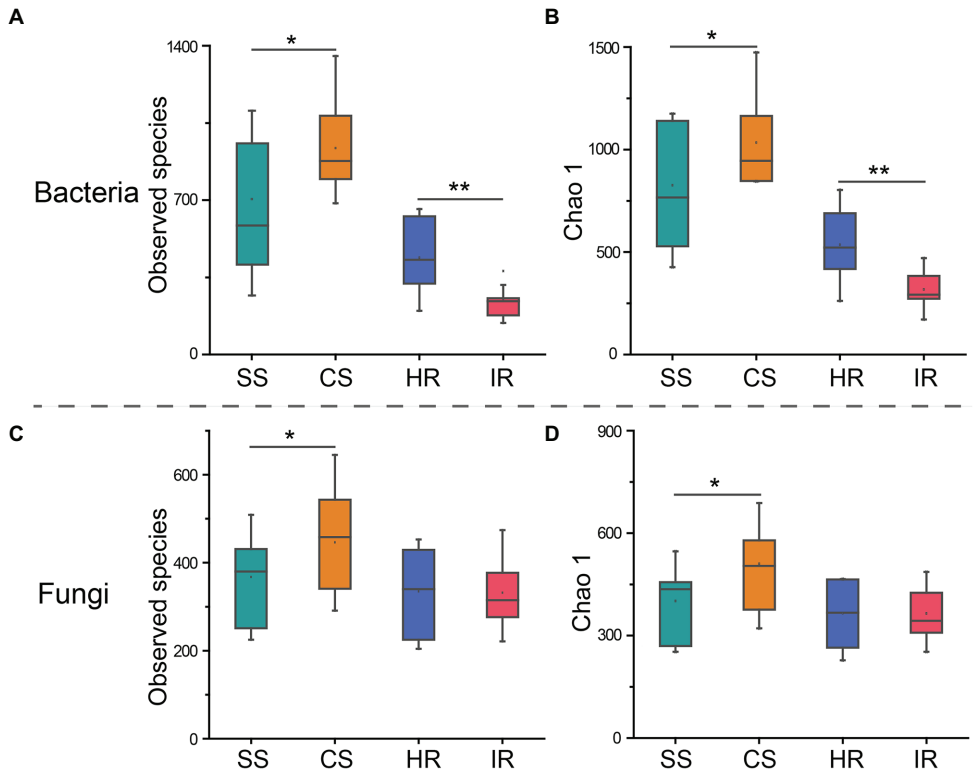

FIGURE 1 | Alpha diversity indices of bacteria and fungi in different samples. **(A)** Bacterial operational taxonomic unit (OTU) numbers, **(B)** bacterial Chao 1, **(C)** fungal OTU numbers, and **(D)** fungal Chao 1. SS, suppressive rhizosphere soil; CS, conducive rhizosphere soil; HR, root of healthy tobacco; IR. root of infected tobacco. The asterisks (*) indicate significant differences determined by the Student's *t* test. $^{*}p < 0.05$; $^{**}p < 0.01$.

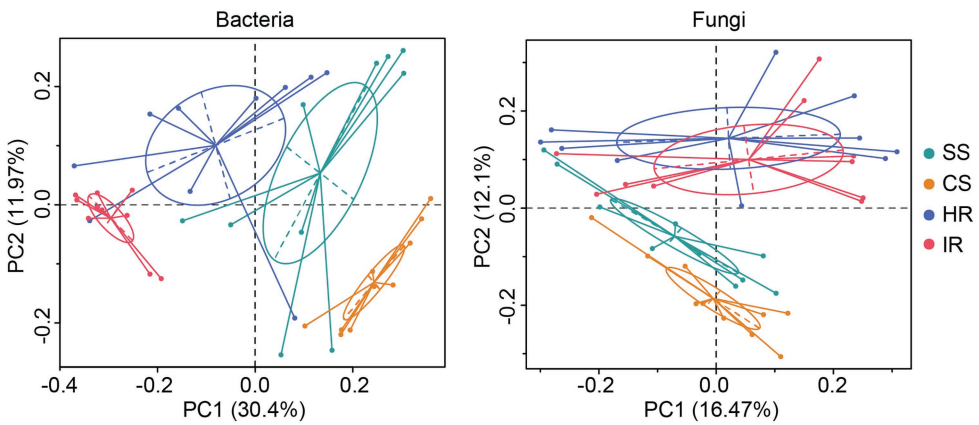

FIGURE 2 | Principal coordinate analysis of the bacterial and fungal communities in rhizosphere soil and root based on unweighted UniFrac distance. SS, suppressive rhizosphere soil; CS, conducive rhizosphere soil; HR, root of healthy tobacco; IR, root of infected tobacco.

for 61.5–89.3%, 6.4–19.0%, and 1.9–13.7% of the total bacterial communities, respectively. However, these three phyla varied in their relative abundance in the different samples. More Firmicutes and less Proteobacteria and Bacteroidetes were observed in the suppressive soil than in the conducive soil, and a similar trend was found in the root samples (**Figure 3A**). The most dominant phyla in the fungal community were Ascomycota (52.1–80.8%), Basidiomycota (1.8–25.9%), and Mortierellomycota (0.3–5.0%). Ascomycota were more abundant in the suppressive soil and healthy roots than in the conducive soil and infected roots, whereas Basidiomycota showed the opposite trend (**Figure 3B**).

To further investigate the taxa in order to discriminate between the microbiomes of the different samples, MetaStat analysis was performed based on the relative abundance of the microbial community at the genus level (**Supplementary Figure S4**). The relative abundance of the *Ralstonia* genus, which contains the pathogenic species of bacterial wilt disease, was much higher in the conducive soil (12.8%) and infected root (39.0%) than in the suppressive soil (0.3%) and healthy root (0.6%; **Figure 3C**). Although we could not attest that all OTUs assigned to *Ralstonia* in this study were pathogenic species, the relative abundance of *Ralstonia* from 16S rRNA gene amplicon sequencing correlated positively (R^2 = 0.843, p < 0.001) with the density of *R. solanacearum* determined by qPCR (**Supplementary Figure S5**). This indicates that the abundance of the *Ralstonia* genus can represent the density of the pathogen *R. solanacearum*. In addition to *Ralstonia*, the abundances of *Ochrobactrum* and *Kluyvera* were also higher in the conducive soil than in the suppressive soil (**Supplementary Figure S4A**). In the fungal community, the *Fusarium* genus, which contains the fungal wilt pathogens in plants, was the most dominant group in the conducive soil and infected root (**Figure 3D**; **Supplementary Figure S4B**), accounting for 15.0 and 21.6% of the total fungal sequences, respectively (only 0.6 and 1.7% in the suppressive soil and healthy root, respectively). Interestingly, the relative abundance of *Fusarium* positively correlated with *Ralstonia* (**Supplementary Figure S6**), suggesting that bacterial wilt disease is probably accompanied by *Fusarium* wilt disease.

Network Analysis of Bacterial and Fungal Communities

To explore the microbial co-occurrence and potential keystone taxa in the suppressive soil and healthy root samples, network analyses were carried out based on the top 100 abundant genera. The networks showed remarkable differences in their structure and topological properties (**Figure 4**; **Supplementary Table S2**). For the bacterial community, the suppressive and conducive soils consisted of 97 and 100 nodes with a modularity of 0.444 and 0.731, with average path lengths of 2.343 and 3.125, respectively (**Supplementary Table S2**). Moreover, the suppressive soil harbored higher values of edges

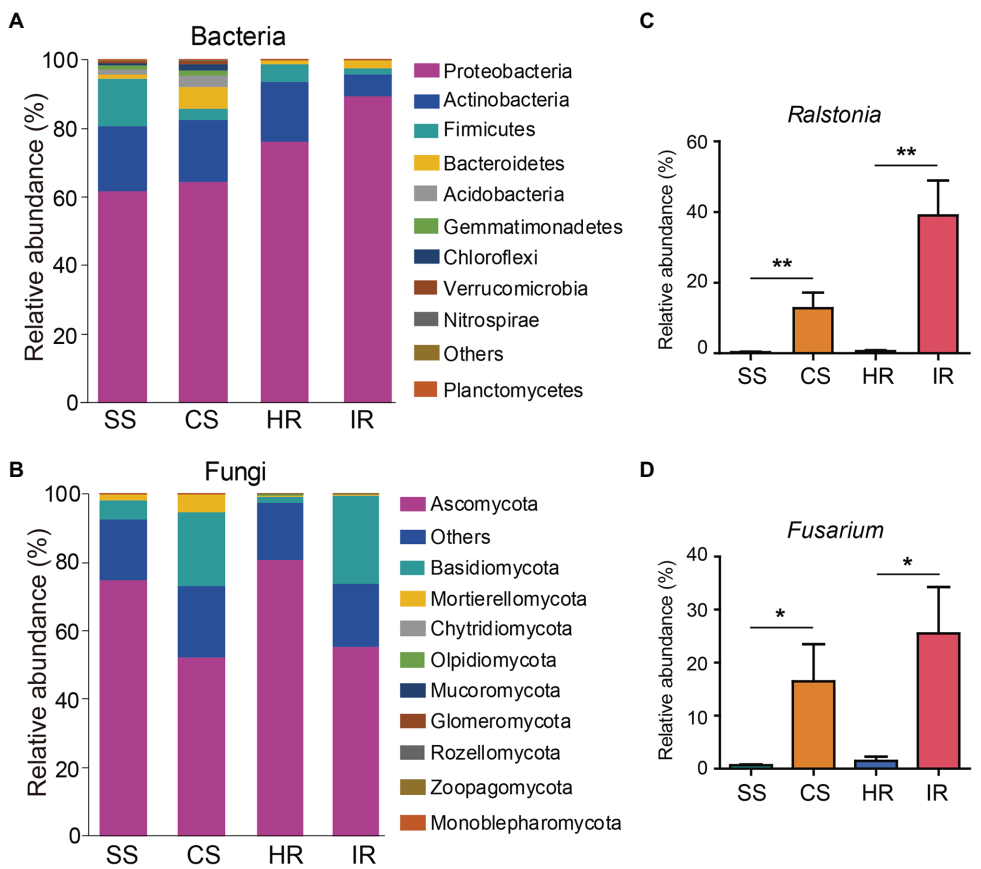

FIGURE 3 | Changes in the rhizosphere soil and root microbiome composition in different samples. **(A)** Bacterial community at the phylum level. **(B)** Fungal community at the phylum level. The relative abundances of Ralstonia and Fusarium in different samples were shown in Panels (**C** and **D**), respectively. Bars represent the standard error of the mean, and asterisks (*) indicate significant differences determined by the Student's *t* test. ns, no significant; *p < 0.05; **p < 0.01. SS, suppressive rhizosphere soil; CS, conducive rhizosphere soil; HR, root of healthy tobacco; IR, root of infected tobacco.

(total of 1,649: positive, 1,449; negative, 200), clustering coefficient (0.474), and average degree (17.0) than the conducive soil, which presented values of 900 (positive, 701; negative, 199), 0.277, and 9.0, respectively (**Figures 4A**,**B**; **Supplementary Table S2**). Similarly, the healthy roots also had a higher number of edges, clustering coefficient, average degree, lower modularity, and shorter average path length compared to the infected roots (**Figures 4D**,**E**; **Supplementary Table S2**). The above results revealed that the bacteria in the suppressive soil and healthy roots formed a highly interactive and complex network. Moreover, the infected root samples harbored a much simpler network than the conducive soil, suggesting that bacterial wilt disease may have a greater influence on the endophytic microbiome. Similar to the general community analysis, the phyla Proteobacteria, Actinobacteria, and Firmicutes seem to play a key role in the establishment of the microbiota sociability (**Figure 4**).

With respect to fungi, networks of the top 100 abundant genera were also constructed (**Supplementary Figure S7**; **Supplementary Table S3**). In contrast to the bacterial network, the conducive soil had higher edges (425), average degree (4.5), and clustering coefficient (0.203) than those (352, 3.8, and 0.189, respectively) in the suppressive soil. The healthy root samples had higher edges (418), average degree (5.1), and clustering coefficient (0.218) than those (331, 3.9, and 0.162, respectively) in the infected root samples. This indicated that the healthy roots possessed a more complex network compared to the infected roots, which was consistent with the bacterial network.

Keystone Taxa Associated With Bacterial and Fungal Communities

To investigate the driving taxa behind the disease-suppressive soil and healthy root samples, the taxa with the high degree, high closeness centrality, and low betweenness centrality were calculated. The results showed that no bacterial keystone taxon was found in the conducive soils and infected roots, while 9 and 13 bacterial keystone taxa were obtained in the suppressive soil and healthy roots, respectively (**Supplementary Table S4**). Three and 10 fungal keystone taxa were found in the suppressive soil and healthy roots, respectively (**Supplementary Table S5**).

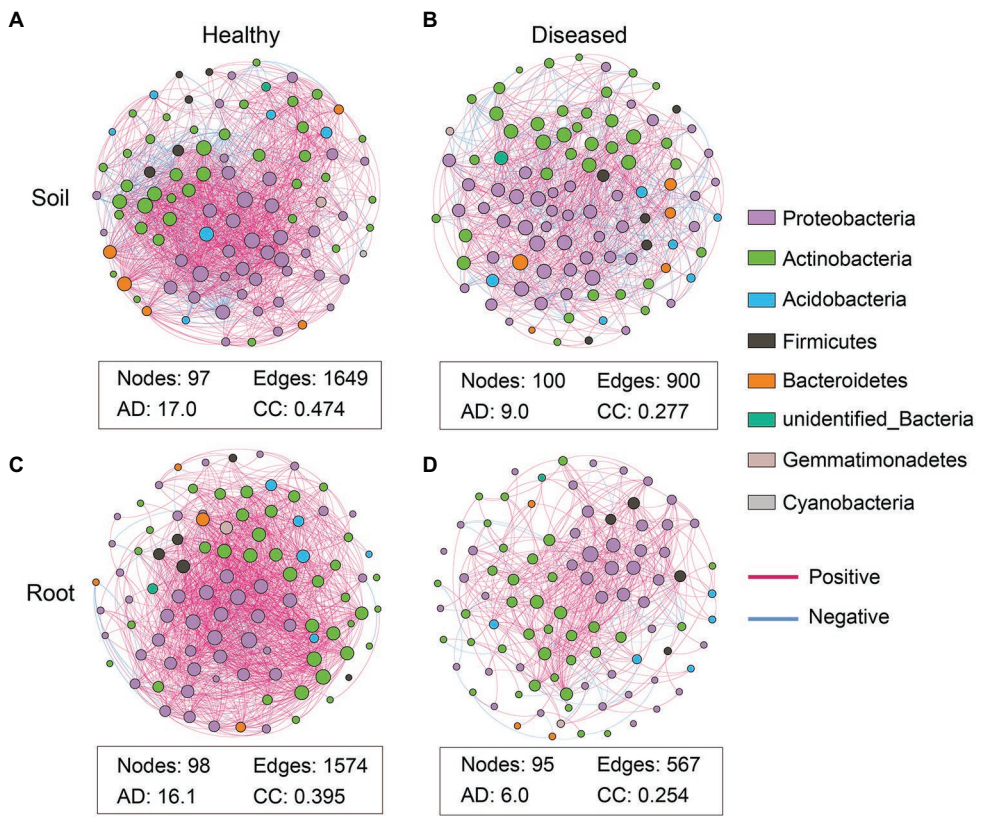

FIGURE 4 | Co-occurrence network analyses of the rhizosphere soil and root based on top 100 abundant bacterial genera. Soil networks are shown in Panels (**A**; suppressive soil) and (**B**; conducive soil). Root network are shown in Panels (**C**; healthy root) and (**D**; infected root). The nodes in these networks represent genera and the edges represent correlations between the nodes. AD, average degree; the average number of direct correlations to a node in the network; CC, clustering coefficient. The probability that the adjacent nodes of a node are connected. A connection indicates a statistically significant [false discovery rate (FDR) adjusted $p < 0.05$] correlation with magnitude >0.6 (positive correlation: blue edges) or <–0.6 (negative correlation: red edges). The size of each node is proportional to the connection numbers of node (i.e., degree). Each node color represents a bacterial genus at phylum level.

Most of the bacterial keystone taxa were from Proteobacteria and Actinobacteria. Among all bacterial keystone taxa, *Pseudomonas* was the most abundant genus in both the suppressive soil and healthy roots, followed by *Streptomyces* and *Gaiella* (**Supplementary Table S4**). More importantly, the accumulated abundance of all bacterial keystone taxa negatively correlated with *Ralstonia* in the soil (R^2 = 0.115, p < 0.05; **Figure 5A**) and root (R^2 = 0.414, p < 0.001; **Figure 5B**), implying their potential beneficial effects on disease control. No correlation between fungal keystone taxa and *Ralstonia* was observed. NetShift analysis was carried out to identify drivers in the conducive soil and infected root samples (**Supplementary Figure S8**). A total of 24 and 25 bacterial genera whose betweenness (importance) increased from the suppressive soil to the conducive soil and from the healthy root to the infected root samples, respectively, were identified as potential driver taxa. Among these taxa, 13 taxa were shared by the conducive soil and infected roots. *Ralstonia*, *Kluyvera*, *Terrabacter*, *Ochrobactrum*, and *Massilia* were considered to play significant roles in maintaining the network structure of the conducive soil and infected roots.

Inhibitory Effects of Isolates on *R. solanacearum* and Greenhouse Experiments

To complement the network analysis and further investigate the important role keystone taxa might play in suppressing disease, we performed an isolation campaign and isolated 83 bacterial strains from a healthy root sample. These root-derived isolates represented four bacterial phyla and nine bacterial orders (**Figure 6A**). Unfortunately, only *Pseudomonas* sp. of the keystone taxa was obtained. Three *Pseudomonas* isolates, *P. lurida* FGD5-2, *P. koreensis* HCH2-3, and *P. rhodesiae* MTD4-1 showed 98.4–100% of 16S rRNA gene similarity to *Pseudomonas* OTU sequences derived from high-throughput amplicon sequencing and clustered together (**Figure 6B**), indicating that these strains represent indigenous key species of healthy plants. Thus, they were selected for further experiment. We found that three *Pseudomonas* isolates displayed strong inhibitory effects on *R. solanacearum*, with an inhibition zone ranging from 2.9 to 4.2 cm (**Supplementary Table S6**). Moreover, greenhouse experiments were performed to determine whether they could protect plants against *R. solanacearum* infection. The results showed that all *Pseudomonas* addition groups could significantly reduce disease indices (Tukey's test, p < 0.05) when compared to the control group (**Figure 6C**). The consortium consisted of three *Pseudomonas* isolates, thereby showing similar protection against disease with strain MTD4-1. Overall, this indicated that *Pseudomonas* species (one of the keystone taxa) might play a significant role in maintaining tobacco health while decreasing bacterial wilt incidence.

DISCUSSION

In this study, we observed that in two adjacent tobacco experimental fields, plants in conducive soil suffered severe wilt disease, but in suppressive soil, they grew well. Our objective was to find microbial keystone taxa behind this phenomenon using network analysis and explore their relationship with bacterial wilt pathogen. We found that both bacterial and fungal network complexity between the suppressive and conducive soils were different. Several keystone taxa were identified from the suppressive soil. Importantly, we further found that isolates presumably related to keystone taxa have inhibitory effects on the pathogen and reduction of bacterial wilt disease incidence, highlighting the importance of keystone taxa in suppressing disease.

Large numbers of previous studies have analyzed the soil microbial community, abundance, and diversity when bacterial wilt disease outbreak (Wang et al., 2017b; Qi et al., 2019; Wei et al., 2019; Lee et al., 2020). As they reported, we also found that the relative abundance of Firmicutes was higher in the suppressive soil compared to that in the conducive soil

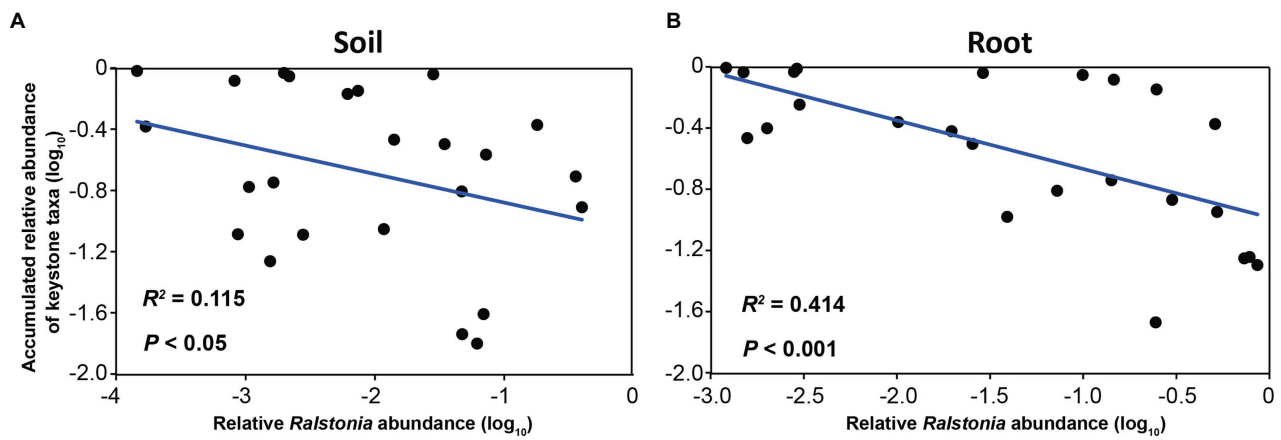

FIGURE 5 | Relationship between the relative abundance of *Ralstonia* and the accumulated abundances of keystone taxa in **(A)** soil and **(B)** root.

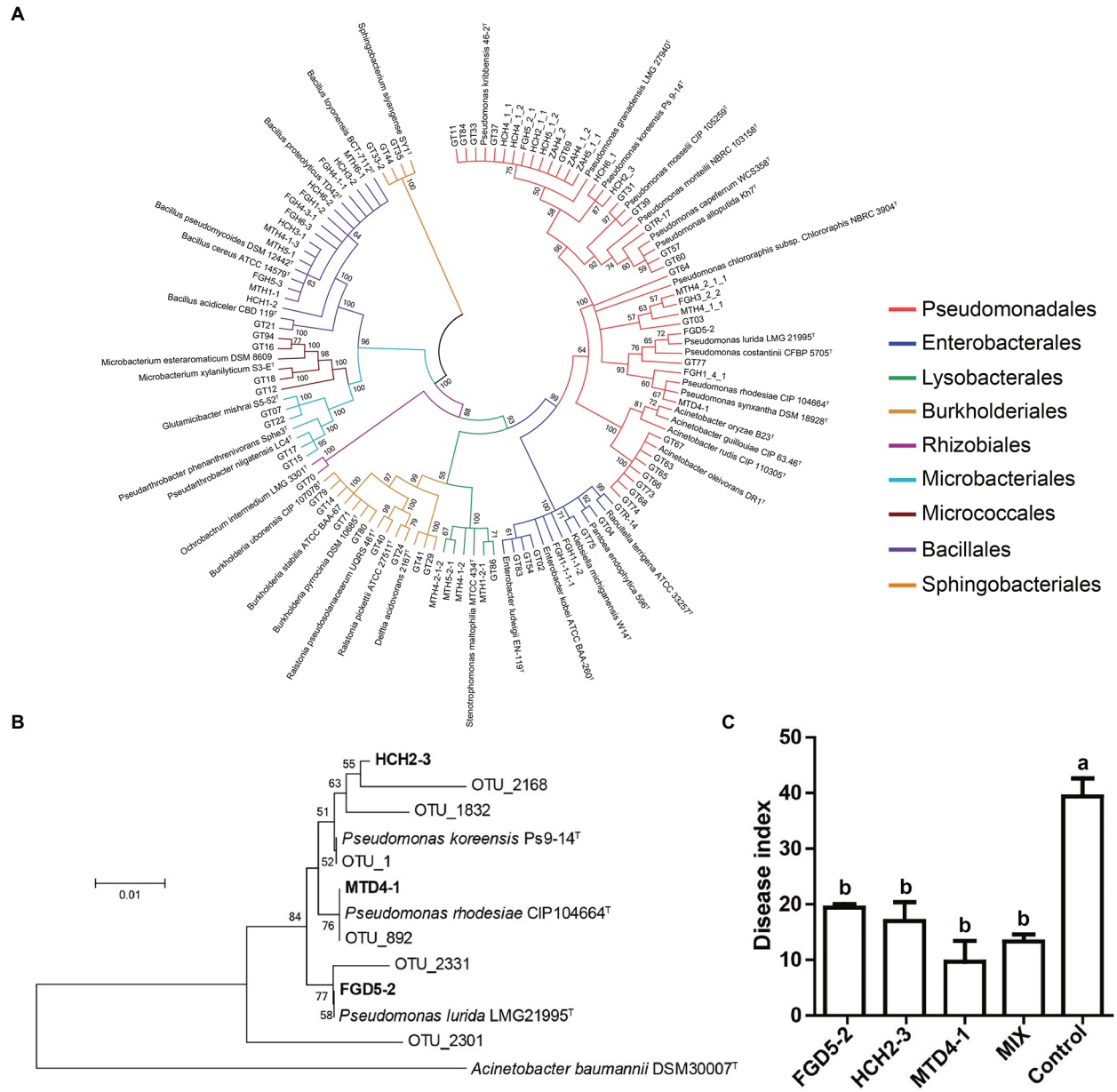

FIGURE 6 | The phylogenetic and disease suppression analyses of potential keystone bacteria. **(A)** The neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of 83 culturable isolates and their type strain. **(B)** The neighbor-joining phylogenetic tree constructed with the 16S rRNA gene sequences obtained from high-throughput amplicon sequencing derived operational taxonomic units (OTUs) and the isolated strains. **(C)** The disease index of tobacco seedlings after *R. solanacearum* inoculation for 15 days. Bars (the standard error of the mean) represent the average of three biological replicates (n = 12) per treatment. Different letters above the bars in Panel **(C)** indicate a significant difference ($p < 0.05$) according to the Tukey's test.

(**Figure 3A**). The phylum Firmicutes is well known to include species that produce various secondary metabolites beneficial to plant growth (Kim et al., 2011), and disruption of its abundance in tomato rhizosphere causes the incidence of bacterial wilt disease (Lee et al., 2020). In the fungal community, Ascomycota and Basidiomycota were the most abundant phyla in all the samples (**Figure 3B**), broadly corresponding to extensive surveys of the soil microbiome (Hartmann et al., 2015; Fu et al., 2017; Xiong et al., 2017). In this study, we found that bacterial wilt disease causes the reduction in Ascomycota and enrichment of Basidiomycota.

Consistent with field observations of the plants, OTUs assigned to the potential pathogenic *Ralstonia* showed high abundances in the conducive soil and infected roots but were rarely found in the suppressive soil and healthy roots (**Figure 3C**). In the fungal community, the relative abundances of *Fusarium*

in the conducive soil and infected roots were 25- and 13-fold higher than those in the suppressive soil and healthy roots, respectively (**Figure 3D**), indicating that the occurrence of bacterial wilt disease might be accompanied by a high abundance of *Fusarium*. This is supported by the positive correlation between the abundance of *Fusarium* and *Ralstonia*.

It has been suggested that the function of a plant microbiome is not the sum of its individual members, since microbial taxa can interact with each other and shape a complex network (van der Heijden and Hartmann, 2016). Our study revealed that disease altered the network features of the soil and root microbiomes. In particular, the co-occurrence networks of the suppressive soil and healthy roots were more complex, with a higher number of edges, higher average degree, and clustering coefficient compared with the networks of the conducive soil and infected roots (**Figure 4**); this is consistent with previous studies (Qi et al., 2019; Wei et al., 2019; Zhang et al., 2020). It was indicated that complex networks are more robust to external biotic and abiotic stresses than simple networks (Santolini and Barabási, 2018). Thus, the high-complexity network of the suppressive soil and healthy root in this study might be critical for the suppression of disease. The key members of microbial communities are defined as keystone species, which frequently interact with other microbial taxa in co-occurrence networks. In this study, no keystone taxon was observed in the conducive soil and infected roots, while 9 and 13 keystone taxa, most of them belonging to Proteobacteria and Actinobacteria, were identified in the suppressive soil and healthy roots, respectively (**Supplementary Table S4**). The high abundance and diversity of these keystone taxa can increase the complexity of healthy plant networks, leading to a highly resilient microbiome. It has been found that keystone taxa play a determinant role within the microbiome, and their removal causes significant changes in network complexity, microbial composition, and function (Banerjee et al., 2018, 2019). Furthermore, the accumulated abundance of all keystone taxa exhibited a negative correlation with the abundance of *Ralstonia* (**Figure 5**), indicating the importance of their roles in suppressing bacterial wilt disease.

Keystones identified by network-based scores must be complemented with experimental evidence to uncover their true importance (Banerjee et al., 2018). Therefore, we performed an isolation campaign to obtain culturable keystone taxa. Unfortunately, only species of the *Pseudomonas* genus within the keystone taxa were obtained in our study. By constructing a phylogenetic tree with sequences of OTUs and isolates, three *Pseudomonas* species were considered as indigenous strains, and all of them showed strong inhibitory effects on *R. solanacearum* plates. Furthermore, a greenhouse experiment suggested that these *Pseudomonas* species could significantly reduce the disease indices of tobacco plants (**Figure 6C**). Consistently, Hu et al. (2016) found that diverse *Pseudomonas* consortia enhanced rhizosphere microbiome function and plant disease suppression. We know that numerous *Pseudomonas* species have been found from suppressive soil and are used as protectants against soil-borne diseases (Schroth and Hancock, 1982; Keel et al., 1992; Haas and Keel, 2003). However, to our

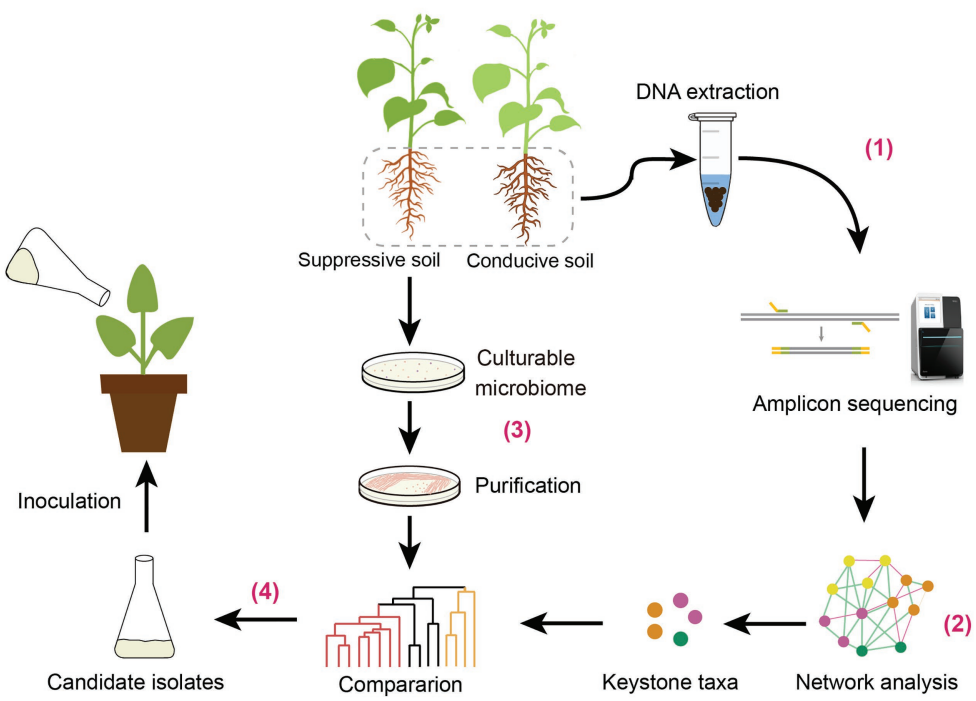

FIGURE 7 | The strategy we proposed for biocontrol agent exploring based on rhizosphere keystone taxa.

knowledge, this is the first study to reveal their indispensable role as keystone taxa in sustaining the stability of healthy plant microbiomes. Many other keystone taxa, such as *Streptomyces* (Tan et al., 2011), *Ensifer* (Kumar et al., 2011), *Bradyrhizobium* (Omar and Abd-Alla, 1998), and *Microbacterium* (Freitas et al., 2019) have been reported to be beneficial for plant growth and/or antagonistic to *R. solanacearum*.

It has been indicated that most exogenous microorganisms cannot colonize in plant rhizosphere under field conditions (Gómez Expósito et al., 2017). Recently, synthetic community (SynCom) comprising multiple microbes has been constructed in sterile soil to imitate disease-suppressive community (Bai et al., 2015; Liu et al., 2019). Identification of keystone taxa from disease-suppressive soil may provide insights to design an artificial SynCom against soil-borne diseases. In this study, we identified keystone taxa through network analysis at the genus level and confirmed their preventive effect on disease suppression. Based on the results of previous and current studies, we propose a new strategy for biocontrol agent exploring based on rhizosphere core microbiome. The potential strategy may include the following steps (**Figure 7**): (1) rhizosphere microbiome analysis through high-throughput sequencing techniques; (2) screening microbial keystone taxa associated to plant health by network analysis; (3) the cultivable biocontrol agents screening; and (4) select candidate agent by comparison to the microbial keystone taxa. It should be noted that the biocontrol effect of these strains selected should be further verified by field experiments.

CONCLUSION

The suppressive and conducive soils showed substantial shifts in microbial abundance and community composition. High abundance of *Fusarium* in the conducive soil and infected roots suggests that *R. solanacearum* infection probably boosts the growth of *Fusarium* within the fungal community. The network complexity and abundance of keystone taxa are higher in the suppressive soil than in the conducive soil. A negative association was observed between the accumulated abundance of keystone taxa and the abundance of *Ralstonia*. The genus *Pseudomonas* was the most abundant keystone taxon, and greenhouse experiments showed that several species in this genus could reduce the disease index. Thus, we propose that the keystone species in suppressive soil and/or healthy plants play an important role in the suppression of soil-borne pathogens.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA632641.

AUTHOR CONTRIBUTIONS

C-SZ and ML designed the experiments, analyzed the data, and wrote the manuscript. YZ conducted qPCR, analyzed the sequence data, wrote the manuscript, and prepared all figures and tables. XH collected samples and analyzed data. DZ and KW extracted community DNA and isolated strains. KW performed greenhouse experiment. YY and YL discussed the results and provide critical idea in greenhouse experiment. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.655673/full#supplementary-material

REFERENCES

Agler, M. T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S. -T., Weigel, D., et al. (2016). Microbial hub taxa link host and abiotic factors to plant microbiome variation. *PLoS Biol.* 14:e1002352. doi: 10.1371/journal.pbio.1002352

Bai, Y., Muller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., et al. (2015). Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature* 528, 364–369. doi: 10.1038/nature16192

Banerjee, S., Kirkby, C. A., Schmutter, D., Bissett, A., Kirkegaard, J. A., and Richardson, A. E. (2016). Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil. *Soil Biol. Biochem.* 97, 188–198. doi: 10.1016/j.soilbio.2016.03.017

Banerjee, S., Schlaeppi, K., and van der Heijden, M. G. A. (2018). Keystone taxa as drivers of microbiome structure and functioning. *Nat. Rev. Microbiol.* 16, 567–576. doi: 10.1038/s41579-018-0024-1

Banerjee, S., Walder, F., Büchi, L., Meyer, M., Held, A. Y., Gattinger, A., et al. (2019). Agricultural intensification reduces microbial network complexity and the abundance of keystone taxa in roots. *ISME J.* 13, 1722–1736. doi: 10.1038/s41396-019-0383-2

Barberan, A., Bates, S. T., Casamayor, E. O., and Fierer, N. (2012). Using network analysis to explore co-occurrence patterns in soil microbial communities. *ISME J.* 6, 343–351. doi: 10.1038/ismej.2011.119

Bastian, M., Heymann, S., and Jacomy, M. (2009). "Gephi: an open source software for exploring and manipulating networks" *in Third international AAAI conference on weblogs and social media*; San Jose, CA, 361–362.

Berendsen, R. L., Vismans, G., Yu, K., Song, Y., de Jonge, R., Burgman, W. P., et al. (2018). Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME J.* 12, 1496–1507. doi: 10.1038/s41396-018-0093-1

Berry, D., and Widder, S. (2014). Deciphering microbial interactions and detecting keystone species with co-occurrence networks. *Front. Microbiol.* 5:219. doi: 10.3389/fmicb.2014.00219

Boukaew, S., Chuenchit, S., and Petcharat, V. (2011). Evaluation of *Streptomyces* spp. for biological control of Sclerotium root and stem rot and *Ralstonia* wilt of chili pepper. *BioControl* 56, 365–374. doi: 10.1007/s10526-010-9336-4

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7:335. doi: 10.1038/nmeth.f.303

Clarke, K., and Gorley, R. (2006). Primer v6: User Manual/Tutorial (PRIMER-E, 2006).

de Vries, F. T., Griffiths, R. I., Bailey, M., Craig, H., Girlanda, M., Gweon, H. S., et al. (2018). Soil bacterial networks are less stable under drought than fungal networks. *Nat. Commun.* 9:3033. doi: 10.1038/s41467-018-05516-7

Ding, J., Zhang, Y., Wang, M., Sun, X., Cong, J., Deng, Y., et al. (2015). Soil organic matter quantity and quality shape microbial community compositions of subtropical broadleaved forests. *Mol. Ecol.* 24, 5175–5185. doi: 10.1111/mec.13384

Dhingra, O. D., and Sinclair, J. B. (1995). *Basic plant pathology methods. 2nd Edn.* Boca Raton, Fl, USA: CRC Press, Inc.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10:996. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Fierer, N., Jackson, J. A., Vilgalys, R., and Jackson, R. B. (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* 71, 4117–4120. doi: 10.1128/AEM.71.7.4117-4120.2005

Freitas, M. A., Medeiros, F. H., Melo, I. S., Pereira, P. F., Peñaflor, M. F. G., Bento, J. M., et al. (2019). Stem inoculation with bacterial strains *Bacillus amyloliquefaciens* (GB03) and *Microbacterium imperiale* (MAIIF2a) mitigates Fusarium root rot in cassava. *Phytoparasitica* 47, 135–142. doi: 10.1007/s12600-018-0706-2

Fu, L., Penton, C. R., Ruan, Y., Shen, Z., Xue, C., Li, R., et al. (2017). Inducing the rhizosphere microbiome by biofertilizer application to suppress banana Fusarium wilt disease. *Soil Biol. Biochem.* 104, 39–48. doi: 10.1016/j.soilbio.2016.10.008

Gómez Expósito, R., De Bruijn, I., Postma, J., and Raaijmakers, J. M. (2017). Current insights into the role of rhizosphere bacteria in disease suppressive soils. *Front. Microbiol.* 8:2529. doi: 10.3389/fmicb.2017.02529

Guo, Q., Shi, M., Chen, L., Zhou, J., Zhang, L., Li, Y., et al. (2020). The biocontrol agent *Streptomyces pactum* increases *Pseudomonas koreensis* populations in the rhizosphere by enhancing chemotaxis and biofilm formation. *Soil Biol. Biochem.* 144:107755. doi: 10.1016/j.soilbio.2020.107755

Haas, D., and Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annu. Rev. Phytopathol.* 41, 117–153. doi: 10.1146/annurev.phyto.41.052002.095656

Hartmann, M., Frey, B., Mayer, J., Mader, P., and Widmer, F. (2015). Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J.* 9, 1177–1194. doi: 10.1038/ismej.2014.210

Hu, Q., Tan, L., Gu, S., Xiao, Y., Xiong, X., Zeng, W. A., et al. (2020). Network analysis infers the wilt pathogen invasion associated with non-detrimental bacteria. *NPJ Biofilms Microbi.* 6:8. doi: 10.1038/s41522-020-0117-2

Hu, J., Wei, Z., Friman, V. P., Gu, S. H., Wang, X. F., Eisenhauer, N., et al. (2016). Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression. *MBio* 7, e01790–e01716. doi: 10.1128/mBio.01790-16

Jiang, Y., Li, S., Li, R., Zhang, J., Liu, Y., Lv, L., et al. (2017b). Plant cultivars imprint the rhizosphere bacterial community composition and association networks. *Soil Biol. Biochem.* 109, 145–155. doi: 10.1016/j.soilbio.2017.02.010

Jiang, G., Wei, Z., Xu, J., Chen, H., Zhang, Y., She, X., et al. (2017a). Bacterial wilt in China: history, current status, and future perspectives. *Front. Plant Sci.* 8:1549. doi: 10.3389/fpls.2017.01549

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., et al. (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2, 4-diacetylphloroglucinol. *Mol. Plant Microbe Interact.* 5, 4–13. doi: 10.1094/MPMI-5-004

Kim, Y. C., Leveau, J., Gardener, B. B. M., Pierson, E. A., Pierson, L. S., and Ryu, C. -M. (2011). The multifactorial basis for plant health promotion by plant-associated bacteria. *Appl. Environ. Microbiol.* 77, 1548–1555. doi: 10.1128/AEM.01867-10

Kumar, H., Dubey, R., and Maheshwari, D. (2011). Effect of plant growth promoting rhizobia on seed germination, growth promotion and suppression of Fusarium wilt of fenugreek (*Trigonella foenum-graecum* L.). *Crop Prot.* 30, 1396–1403. doi: 10.1016/j.cropro.2011.05.001

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Kyselkova, M., Kopecky, J., Frapolli, M., Defago, G., Sagova-Mareckova, M., Grundmann, G. L., et al. (2009). Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. *ISME J.* 3, 1127–1138. doi: 10.1038/ismej.2009.61

Lee, S. M., Kong, H. G., Song, G. C., and Ryu, C. M. (2020). Disruption of Firmicutes and Actinobacteria abundance in tomato rhizosphere causes the incidence of bacterial wilt disease. *ISME J.* 15, 330–347. doi: 10.1038/s41396-020-00785-x

Liang, Y., Zhao, H., Deng, Y., Zhou, J., Li, G., and Sun, B. (2016). Long-term oil contamination alters the molecular ecological networks of soil microbial functional genes. *Front. Microbiol.* 7:60. doi: 10.3389/fmicb.2016.00060

Liu, Y. -X., Qin, Y., and Bai, Y. (2019). Reductionist synthetic community approaches in root microbiome research. *Curr. Opin. Microbiol.* 49, 97–102. doi: 10.1016/j.mib.2019.10.010

Lupatini, M., Suleiman, A. K., Jacques, R. J., Antoniolli, Z. I., de Siqueira Ferreira, A., Kuramae, E. E., et al. (2014). Network topology reveals high connectance levels and few key microbial genera within soils. *Front. Environ. Sci.* 2:10. doi: 10.3389/fenvs.2014.00010

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700. doi: 10.1128/AEM.59.3.695-700.1993

Nion, Y. A., and Toyota, K. (2008). Suppression of bacterial wilt and Fusarium wilt by a *Burkholderia nodosa* strain isolated from Kalimantan soils, Indonesia. *Microbes Environ.* 23, 134–141. doi: 10.1264/jsme2.23.134

Omar, S., and Abd-Alla, M. (1998). Biocontrol of fungal root rot diseases of crop plants by the use of rhizobia and bradyrhizobia. *Folia Microbiol.* 43, 431–437. doi: 10.1007/BF02818587

Qi, G., Ma, G., Chen, S., Lin, C., and Zhao, X. (2019). Microbial network and soil properties are changed in bacterial wilt-susceptible soil. *Appl. Environ. Microbiol.* 85, e00162–e00119. doi: 10.1128/AEM.00162-19

Raaijmakers, J. M., and Weller, D. M. (1998). Natural plant protection by 2, 4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. *Mol. Plant Microbe Interact.* 11, 144–152. doi: 10.1094/MPMI.1998.11.2.144

Revelle, W. R. (2017). psych: Procedures for personality and psychological research.

Santolini, M., and Barabási, A. -L. (2018). Predicting perturbation patterns from the topology of biological networks. *Proc. Natl. Acad. Sci. U.S.A.* 115, E6375–E6383. doi: 10.1073/pnas.1720589115

Schonfeld, J., Heuer, H., Van Elsas, J. D., and Smalla, K. (2003). Specific and sensitive detection of *Ralstonia solanacearum* in soil on the basis of PCR amplification of *fliC* fragments. *Appl. Environ. Microbiol.* 69, 7248–7256. doi: 10.1128/AEM.69.12.7248-7256.2003

Schroth, M. N., and Hancock, J. G. (1982). Disease-suppressive soil and root-colonizing bacteria. *Science* 216, 1376–1381. doi: 10.1126/science.216.4553.1376

Tan, H., Zhou, S., Deng, Z., He, M., and Cao, L. (2011). Ribosomal-sequence-directed selection for endophytic streptomycete strains antagonistic to *Ralstonia solanacearum* to control tomato bacterial wilt. *Biol. Control* 59, 245–254. doi: 10.1016/j.biocontrol.2011.07.018

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876–4882. doi: 10.1093/nar/25.24.4876

van der Heijden, M. G., and Hartmann, M. (2016). Networking in the plant microbiome. *PLoS Biol.* 14:e1002378. doi: 10.1371/journal.pbio.1002378

Wang, H., Wei, Z., Mei, L., Gu, J., Yin, S., Faust, K., et al. (2017a). Combined use of network inference tools identifies ecologically meaningful bacterial associations in a paddy soil. *Soil Biol. Biochem*. 105, 227–235. doi: 10.1016/j.soilbio.2016.11.029

Wang, R., Zhang, H., Sun, L., Qi, G., Chen, S., and Zhao, X. (2017b). Microbial community composition is related to soil biological and chemical properties and bacterial wilt outbreak. *Sci. Rep.* 7:343. doi: 10.1038/s41598-017-00472-6

Wei, Z., Gu, Y., Friman, V. -P., Kowalchuk, G. A., Xu, Y., Shen, Q., et al. (2019). Initial soil microbiome composition and functioning predetermine future plant health. *Sci. Adv.* 5:eaaw0759. doi: 10.1126/sciadv.aaw0759

Wei, Z., Huang, J., Tan, S., Mei, X., Shen, Q., and Xu, Y. (2013). The congeneric strain *Ralstonia pickettii* QL-A6 of *Ralstonia solanacearum* as an effective biocontrol agent for bacterial wilt of tomato. *Biol. Control* 65, 278–285. doi: 10.1016/j.biocontrol.2012.12.010

Wei, Z., Yang, X., Yin, S., Shen, Q., Ran, W., and Xu, Y. (2011). Efficacy of *Bacillus*-fortified organic fertiliser in controlling bacterial wilt of tomato in the field. *Appl. Soil Ecol.* 48, 152–159. doi: 10.1016/j.apsoil.2011.03.013

Xiong, W., Li, R., Ren, Y., Liu, C., Zhao, Q., Wu, H., et al. (2017). Distinct roles for soil fungal and bacterial communities associated with the suppression of vanilla Fusarium wilt disease. *Soil Biol. Biochem.* 107, 198–207. doi: 10.1016/j.soilbio.2017.01.010

Zhang, Y., Hu, A., Zhou, J., Zhang, W., and Li, P. (2020). Comparison of bacterial communities in soil samples with and without tomato bacterial wilt caused by *Ralstonia solanacearum* species complex. *BMC Microbiol.* 20:89. doi: 10.1186/s12866-020-01774-y

Zhou, J., Deng, Y., Luo, F., He, Z., and Yang, Y. (2011). Phylogenetic molecular ecological network of soil microbial communities in response to elevated CO_2. *MBio* 2, e00122–e00111. doi: 10.1128/mBio.00122-11

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Composition of Rhizosphere Microbial Communities Associated With Healthy and *Verticillium* Wilt Diseased Cotton Plants

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Rhizosphere microbial communities are known to be related to plant health; using such an association for crop management requires a better understanding of this relationship. We investigated rhizosphere microbiomes associated with *Verticillium* wilt symptoms in two cotton cultivars. Microbial communities were profiled by amplicon sequencing, with the total bacterial and fungal DNA quantified by quantitative polymerase chain reaction based on the respective 16S and internal transcribed spacer primers. Although the level of *V. dahliae* inoculum was higher in the rhizosphere of diseased plants than in the healthy plants, such a difference explained only a small proportion of variation in wilt severities. Compared to healthy plants, the diseased plants had much higher total fungal/bacterial biomass ratio, as represented by quantified total fungal or bacterial DNA. The variability in the fungal/bacterial biomass ratio was much smaller than variability in either fungal or bacterial total biomass among samples within diseased or healthy plants. Diseased plants generally had lower bacterial alpha diversity in their rhizosphere, but such differences in the fungal alpha diversity depended on cultivars. There were large differences in both fungal and bacterial communities between diseased and healthy plants. Many rhizosphere microbial groups differed in their abundance between healthy and diseased plants. There was a decrease in arbuscular mycorrhizal fungi and an increase in several plant pathogen and saprophyte guilds in diseased plants. These findings suggested that *V. dahliae* infection of roots led to considerable changes in rhizosphere microbial communities, with large increases in saprophytic fungi and reduction in bacterial community.

Keywords: cotton, *Verticillium dahliae*, rhizosphere soil, amplicon-sequencing, microbial community composition

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is an important fiber crop. Cotton *Verticillium* wilt, caused by *Verticillium dahliae*, is one of the most devastating plant diseases worldwide (Klosterman et al., 2009). The pathogen can survive in the soil as resting microsclerotia without a host plant for more than 14 years. In China, its hosts include a number of economically important crops

such as potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Miller), strawberry (*Fragaria* × *ananassa*), sunflower (*Helianthus annuus*), eggplant (*Solanum melongena* L.), and pepper (*Capsicum annuum* L.). Incidence of cotton wilt increases with increasing densities of *V. dahliae* microsclerotia in soil (Wei et al., 2015). Controlling *Verticillium* wilt is difficult because of the inaccessibility of the pathogen during infection, long-term survival of microsclerotia in soil, and its broad host range (Klosterman et al., 2009). There has been limited success in planting resistant cultivars of upland cotton against wilt in heavy infested fields (Zhang et al., 2012b). In Xinjiang, the main cotton production region in China, crop rotation with non-hosts of *V. dahliae* has not been adopted because of the difficulties in changing cropping systems and saline–alkali soils. Soil fumigation with methyl bromide was very effective against *V. dahliae* but has already been banned under the Montreal Protocol (Martin, 2003). Although several remaining fumigants, such as chloropicrin and dazomet, can be used to manage wilt, farmers in China are reluctant to use them in cotton production because of their limited economic benefits.

Plants harbor diverse microbiota both inside and outside their tissues (Vandenkoornhuyse et al., 2015). Rhizosphere microbiota, which closely interact with plant roots, are important for plant health (Berendsen et al., 2012) and crop yield potential (Xu et al., 2015) and influenced by many factors such as plant species and developmental stage, soil properties, nutrient status, land use, and climatic conditions. Selective recruitment of specific microbes by plant roots has been observed (Peiffer et al., 2013; Bai et al., 2015; Zarraonaindia et al., 2015). Suppression of soilborne disease has long been considered as one of the key benefits associated with beneficial microbes in soil (Mendes et al., 2011; Bai et al., 2015; Finkel et al., 2017; Xiong et al., 2017). Microbial diversity and composition are related to plant disease resistance (Wei et al., 2019); high microbial diversity provides greater protection against soilborne pathogens (van Elsas et al., 2002; Mallon et al., 2015). Understanding the association of plant health with rhizosphere microbiota may provide a basis for manipulating soil microbiomes directly (e.g., amending soil with specific microbes) and/or indirectly (e.g., altering management practice) to promote plant health.

Biocontrol of soilborne pathogens has long been a goal of sustainable agriculture, but because of the complexity of the soil environment and resident microbial communities, there are limited numbers of commercial biocontrol products against soilborne diseases in commercial agriculture (Mazzola and Freilich, 2017). To ensure that introduced microorganisms remain effective against pathogens over time in the soil environment, a clear understanding of how the introduced microbes interact with soilborne pathogens and other soil microorganisms is necessary. This knowledge may assist in development of cultural measures to increase the suppressiveness of soil microbiomes against soilborne pathogens and to improve survival (and hence enhance efficacy) of introduced biocontrol microbes.

Multinutrient interactions among resident microbes may be disturbed by plant pathogens, which could cause community reorganization and lead to large-scale collapse and serious degradation of soil ecosystems (van der Putten et al., 2007). There is, however, limited knowledge on the changes in rhizosphere microbiota due to infection of plant roots by pathogens. Recently, several studies have shown that soilborne pathogens can significantly affect soil bacterial composition under field conditions (Shanmugam et al., 2011; Zhang et al., 2011; Wu et al., 2015) or in greenhouse (Mendes et al., 2011; Li et al., 2014). Specific fungal groups, e.g., *Mortierella* spp., may play an important role in the development of soil suppressiveness against *Fusarium* wilt disease in vanilla (*Vanilla planifolia*) (Xiong et al., 2017). However, bacterial and fungal communities are rarely investigated together to understand the nature of disease suppressive soil.

The present study focuses on the changes in rhizosphere microbiome associated with the occurrence of *Verticillium* wilt on two cotton cultivars. Specifically, we quantified *V. dahliae* inoculum in rhizosphere soil and assessed the wilt severity for a number of pairs of plants (healthy and diseased plants) in two cultivars. Then we used amplicon metabarcoding to profile rhizosphere microbiome of these paired healthy–wilted plants and quantified the total biomass of fungi and bacteria DNA using quantitative polymerase chain reaction (qPCR) with generic internal transcribed spacer (ITS) and 16S primers. Finally, we established the differences in microbial communities in the rhizosphere between healthy and diseased plants.

MATERIALS AND METHODS

Site Description and Sample Collection

Two commercial monoculture cotton fields (∼300 m apart) with the incidence of plants with *Verticillium* wilt >50% were used for sampling in August 2018 in Anyang, Henan Province, China. The soil at the two sites is classified as cambisol type soil (FAO, 1998). At one site (36°03′44″ N, 114°28′52″ E) cv. "Zhongzhimian2" [ZHM2] was grown and cv. "Lumianyan28" [LM28] at the other site (36°03′36″ N, 114°29′04″ E). Both fields were cultivated by farmers using standard cultural practices. Thirty random pairs of neighboring healthy and diseased plants were selected (120 samples in total, 60 plants [30 pairs] per cultivar) for sampling rhizosphere soil on September 3, 2018 (at the boll-forming stage of cotton plants). Each diseased plant was assessed for wilt on an ordinal scale of 0 to 4 as described previously (Wei et al., 2019). For cv. ZHM2, there were 30, 6, 16, and 8 sampled plants with severity score of 0, 1, 2, and 3, respectively; for cv. LM28, there were 30, 8, 16, and 6 sampled plants with wilt severity score of 0, 1, 2, and 3, respectively. Rhizosphere soil samples were collected and stored as described previously (Wei et al., 2019). We first removed the top soil and then dug out of the roots along the base of plants with a soil sampler to maintain the root system integrity as much as possible. Roots were first shaken to remove loosely adhering soil particles; then, each root sample was placed in a sterile plastic bag, kept on ice, and transported to the laboratory within 4 h of sampling the soil. Fine roots were cut into pieces of ∼2-cm length with a pair of sterile scissors. Rhizosphere samples were harvested in aliquots of 20-g roots in 1:50 TE buffer by shaking, filtering, and centrifuging. At the same time, soil samples (∼100 g) were collected from the position of each sampled plant

to estimate the density of *V. dahliae* inoculum based on a wet sieving and plating method (Wei et al., 2016).

DNA Extraction and qPCR

DNA extraction of rhizosphere samples (250 mg) was performed using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following published procedures (Wei et al., 2019). Rhizosphere samples (250 mg) were resuspended in 500 μL MoBio PowerSoil bead solution, and DNA was extracted according to the manufacturer's instructions. The extracts were checked on a 1% agarose gel, and the DNA concentration was estimated with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was stored at −80°C until further analysis.

qPCR of the 16S rRNA and ITS rRNA genes was performed for each sample in triplicate to estimate the total bacterial and fungal abundances (to represent biomass) with a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). Primer sets F515/R806 (Caporaso et al., 2011) and ITS1f/5.8s (Fierer et al., 2005) were used to quantify bacteria and fungi, respectively. The reactions were conducted in a 20-μL mixture containing 10 μL of SYBR® Premix Ex Taq™ (Tli RNaseH Plus; Takara, China), 0.2 μL of each primer (10 μmol L^{-1}), 1 μL of DNA, and 8.6 μL of ultrapure water. Plasmids containing either the 16S rRNA gene fragment or ITS gene fragment were constructed to prepare the respective standard curves, and the plasmid copy numbers were automatically calculated using an online calculator (http://cels.uri.edu/gsc/cndna.html). PCR conditions were as follows: (i) for the bacterial 16S rRNA gene: 95°C for 30 s; 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 40 total cycles; and (ii) for the fungal ITS rRNA gene: 95°C for 30 s; 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s for 40 total cycles. No-template controls as well as positive controls with known cycle threshold (Ct) values were included in every qPCR reaction.

Amplicon Sequencing of Rhizosphere Samples

For bacteria, the V3–V4 hypervariable region of the 16S rRNA gene was amplified in triplicate for each sample using the 341F/805R primers (Herlemann et al., 2011). For fungi, primers ITS5/ITS2 (White et al., 1990) were used to amplify the ITS1 region in triplicates for each sample. PCR reactions and subsequent extraction and purification of amplicons were performed according to the method we used previously (Wei et al., 2019). Sequencing libraries were generated with the Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, USA) following the manufacturer's recommendations. The quality of each library was assessed on a Qubit 2.0 Fluorometer (Life Technologies, USA). Finally, the libraries were sequenced on an Ion S5™ XL platform (Thermo Fisher Scientific, Waltham, MA) to generate single-end reads. In total, 240 libraries were sequenced: 120 samples (2 cultivars × 30 pairs of healthy and diseased plants) each for 16S rRNA gene and ITS rRNA gene.

Processing and Analysis of the Sequencing Data

Sequences were processed and filtered separately for 16S and ITS data to retain high-quality sequences following the general pipeline we used previously (Wei et al., 2019) to generate operational taxonomic units (OTUs). These high-quality sequences were first dereplicated, and only those unique reads with at least 2 copies were used in cluster analysis to generate OTUs at 97% sequence identity together with a representative sequence for each OTU. Clustered reads were checked for chimeras using the UPARSE pipeline. An OTU count table was then generated as described previously (Wei et al., 2019). All OTU processing was carried out with the UPARSE pipeline (version 10.0) (Edgar, 2013) unless specified otherwise.

The UTAX algorithm (https://www.drive5.com/usearch/manual9/utax_algo.html) was used to assign each ITS OTU and 16S rRNA OTU representative sequence to taxonomic ranks by alignment with the gene sequences against Unite V7 fungal database (Koljalg et al., 2013) and RDP training set 15 bacterial database (Cole et al., 2014), respectively.

Statistical Analysis of Sequence Data

The differences in the level of inoculum (microsclerotia) between different levels of disease severities, cultivars, and within-field locations were assessed via analysis of variance (ANOVA) in which inoculum density was logarithmically transformed. Ordinal regression was used to establish whether wilt severity scores are related to cultivars, locations (represented by the pairs of healthy and diseased plants), the level of inoculum, and microbial (bacterial and fungal) biomass (represented by qPCR data). The ordinal package for R version 3.5.4 was used to carry out the ordinal regression analysis.

Before statistical analysis of sequence data, both bacterial and fungal OTU tables were transformed to abundance data by multiplying the relative abundance (sequence reads) of each OTU for each sample with the number of total bacterial 16S rRNA or ITS rRNA copies of the respective sample as determined in the qPCR. All subsequent data analyses were based on this transformed (normalized) OTU data set.

General statistical methodology was similar to previous publications (Tilston et al., 2018; Wei et al., 2019). It should be noted that differences between the two cultivars are confounded with the differences between the two fields; hence, cautions should be excised when interpreting cultivar differences. However, the present study focuses on the differences between healthy and wilted plants.

Alpha-diversity indices, including the observed OTUs, Shannon, and Simpson indices, were calculated with the R vegan 2.3-1 package (Dixon, 2003). The rank of alpha-diversity indices was subjected to ANOVA to assess the differences between healthy and diseased plants with statistical significance derived from a permutation test. Beta-diversity (Bray–Curtis metric) indices were calculated and subjected to non-dimensional scaling analysis as implemented in the vegan package. The effects of cultivar, location within a field (namely, among pairs of plants), and disease status on the beta-diversity indices were

assessed with permutational multivariate ANOVA using distance matrices (via the "Adonis" function as implement in the R vegan package version 2.5-7). Similarly, the effects of these experimental factors on the first three principle components were determined via ANOVA.

Further analysis was carried out to identify specific microbial OTUs that differed significantly in their abundances between healthy and diseased plants for each cultivar separately, as well as together through DESeq2 (McMurdie and Holmes, 2013). DESeq2 also implements an algorithm for automatic filtering of OTUs before differential abundance analysis using several criteria, including variance in abundance across samples and overall abundance level. The Benjamini–Hochberg adjustment was used with DESeq2 (Benjamin and Aikman, 1995) to correct for the false discovery rate associated with multiple testing. For tree view graphs, OTU abundances were aggregated at each taxonomic rank (at the SINTAX confidence of 0.7), and these aggregated values were tested for differential abundance between diseased and healthy plants with DESeq2 as above. FunGuild (Nguyen et al., 2016) was used to classify those fungal OTUs with differential abundance between diseased and healthy plants into several broad groups and tested for enrichment using a Fisher exact test in which significance was taken at 0.05 and not corrected for multiple testing.

RESULTS

Relationship of Wilt Severity With Bacterial and Fungal Abundances

The overall bacterial and fungal abundances in the rhizosphere soil were estimated with the qPCR technique. The coefficient of determination of the standard curve was 0.993 and 0.988 for bacteria and fungi, respectively; the corresponding efficiencies were 100 and 97%. The number of quantified 16S copies ranged from 1.40×10^8 to 7.06×10^9 per sample (0.25-g soil), with the respective mean and median of 1.29×10^9 and 1.08×10^9. The number of quantified ITS copies ranged from 3.18×10^7 to 1.02×10^{10} per sample (0.25-g soil), with the respective mean and median of 1.45×10^9 and 5.85×10^8 copies.

The density of *V. dahliae* microsclerotia in soil quantified with the wet-sieving method increased with wilt severity ($P < 0.05$) independent of cultivars, but this relationship accounted for only limited variation in the wilt severity (**Supplementary Figure 1**). None of sampled plants reached a wilt score of 4; no visible root decay was found in all samples. Average colony-forming units (CFU) value was 3.70 per gram of dry soil for healthy plants; average CFU values were 3.26, 4.21, and 5.71 per gram of dry soil for diseased plants with wilt scores of 1, 2, and 3, respectively (**Supplementary Figure 1**). The ratio between the overall fungal and bacterial abundance also increased ($P < 0.001$) with the increasing wilt score, particularly from healthy to wilt symptoms irrespective of wilt severities (**Figure 1**). Such a relationship of the wilt severity with the fungal/bacterial biomass ratio was independent of cultivars (**Figure 1**). This increase in the fungi and bacteria ratio is primarily due to the pronounced increase in the fungal ITS copy number associated with diseased plants (**Figure 1**). Average ratios of fungal/bacterial abundance were 0.23, 2.31, 2.66, and 2.25 for plants with wilt scores of 0 (healthy) 1, 2, and 3, respectively. There was also less variation in the ratio among healthy samples than among diseased samples (**Figure 1**) despite the large sample-to-sample variability in both the total bacterial and fungal biomass in the healthy plant samples.

Overview of the Sequencing Results

There were 4,406 bacterial OTUs, with the top 166 OTUs accounting for 90% of the total reads. The number of sequence reads clustered into OTUs ranged from 27,987 to 72,236 per sample, with the respective mean and median of 49,441 and 47,161. There were 3,856 fungal OTUs, with the top 159 OTUs accounting for 90% of the total reads. Three samples failed the ITS sequencing. Of the remaining 117 samples, the number of sequence reads clustered into OTUs ranged from 31,937 to 71,478 per sample, with the respective mean and median of 64,388 and 68,426. For both ITS and 16S, rarefication curves indicated that sequencing depth is sufficient for all samples (**Supplementary Figures 2**, **3**), except those three sampled that failed ITS sequencing.

Most of the bacterial OTUs could not be assigned to a taxonomic rank below the class with high confidence. At a confidence of 0.8, 85.2, 65.5, 31.9, 21.7, and 15.6% of bacterial OTUs were assigned to the taxonomic rank of phylum, class, order, family, and genus, respectively. The corresponding values for assigning taxonomy to fungal OTUs were 68.8, 37.7, 27.8, 18.9, and 10.8%.

Alpha Diversity

For all three bacterial alpha diversity measures, rhizosphere soil samples from healthy plants had much larger ($P < 0.001$) values than from diseased plants (**Figure 2A**). Moreover, this difference was even greater on cv. LM28 than cv. ZHM2, as indicated by the interactions ($P < 0.001$) between cultivars and plant disease status. However, fungal alpha-diversity indices showed a different pattern (**Figure 2B**). LM28 had the same pattern as for bacteria; namely, alpha-diversity indices were greater for healthy plants than for diseased plants. The opposite was true for ZHM2. Such a difference in the response between the two cultivars, i.e., the interaction between cultivar and plant disease status, was highly significant ($P < 0.001$). For both fungi and bacteria, there were no significant differences in the alpha-diversity indices among 30 pairs (i.e., locations within a field) of plant samples.

Beta Diversity Indices

In contrast to the alpha-diversity indices, beta diversity (Bray–Curtis metric) indices showed consistent differences between diseased and healthy plant samples of both cultivars (**Figure 3**). In addition, this consistent difference in both fungal and bacterial communities was further supported by the results from principal component analysis (**Table 1**). Two general patterns can be observed. First, variability among samples from cv. LM28 was less than from cv. ZHM2. Second, variability among diseased samples was far less than among healthy plant samples, particularly for the bacterial community in the rhizosphere of

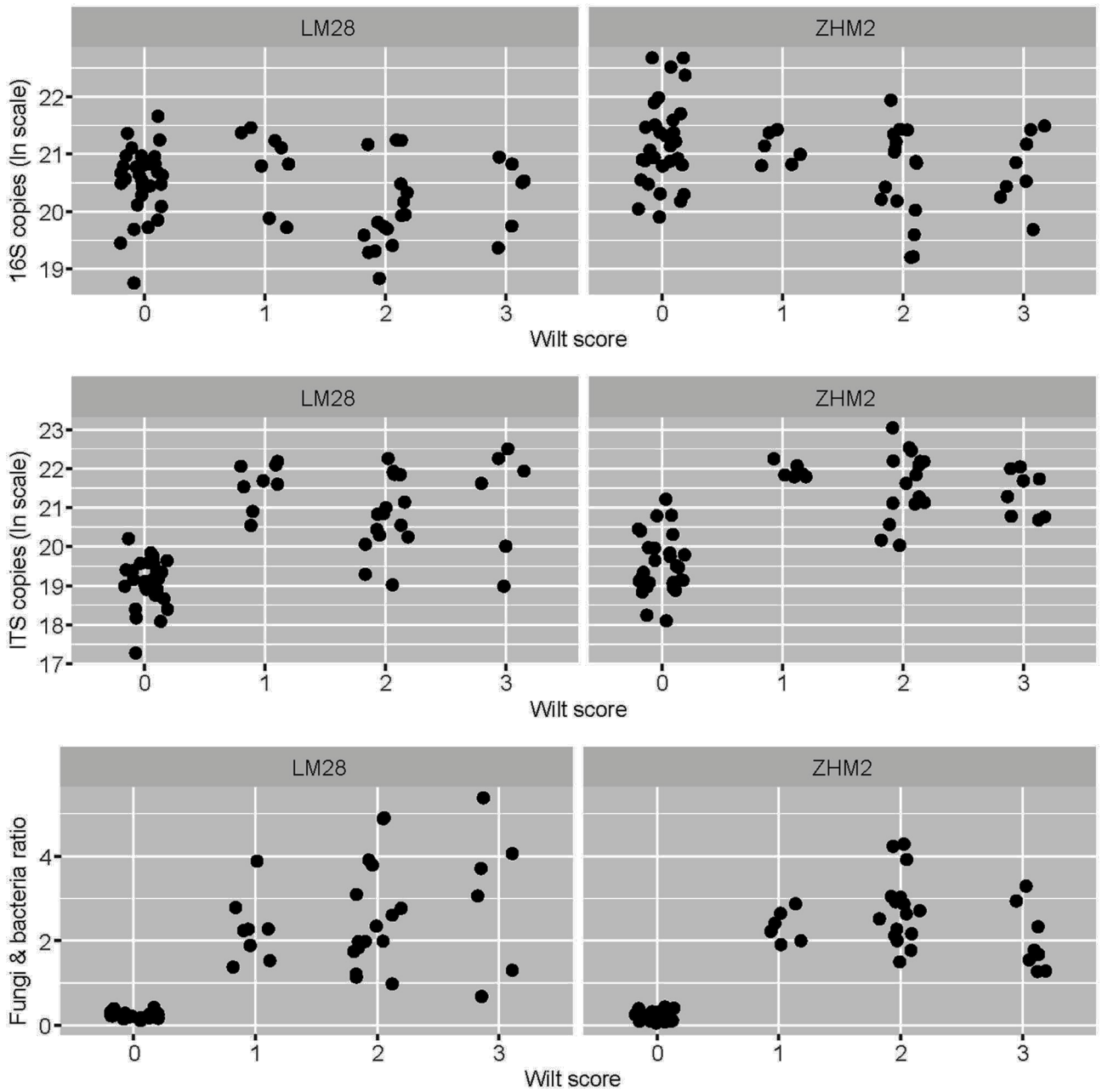

FIGURE 1 | Bacterial 16S and fungal ITS copy number as estimated by qPCR for each rhizosphere soil sample in relation to plant healthy status—healthy plants with the wilt score of zero. Rhizosphere soil samples were collected from neighboring pairs of healthy and diseased (showing *Verticillium* wilt symptoms) cotton plants of two cultivars in two separated fields ~300 m apart.

cv. LM28 plants (**Figure 3**). There were significant differences among locations, namely, between 30 pairs of plants within each cultivar, in the beta-diversity indices as shown by the Adonis analysis.

For both bacterial and fungal communities, the main effects of cultivar and plant wilt status, as well as their interaction on beta-diversity indices, were highly significant (Adonis). For bacteria, cultivar, plant wilt status, and their interactions accounted for 9.1, 16.7, and 6.9% of the total variability, respectively; the corresponding values for the fungal community were 10.3, 18.4, and 4.9%. The interaction was primarily because the difference between the healthy and diseased plant samples was greater for cv. LM28 than cv. ZHM2, especially for the bacterial community.

The first PC accounted for 44.3% of the total variability in the bacterial community, whereas the second and third PCs accounted for only 9.2 and 2.3%, respectively. For the first

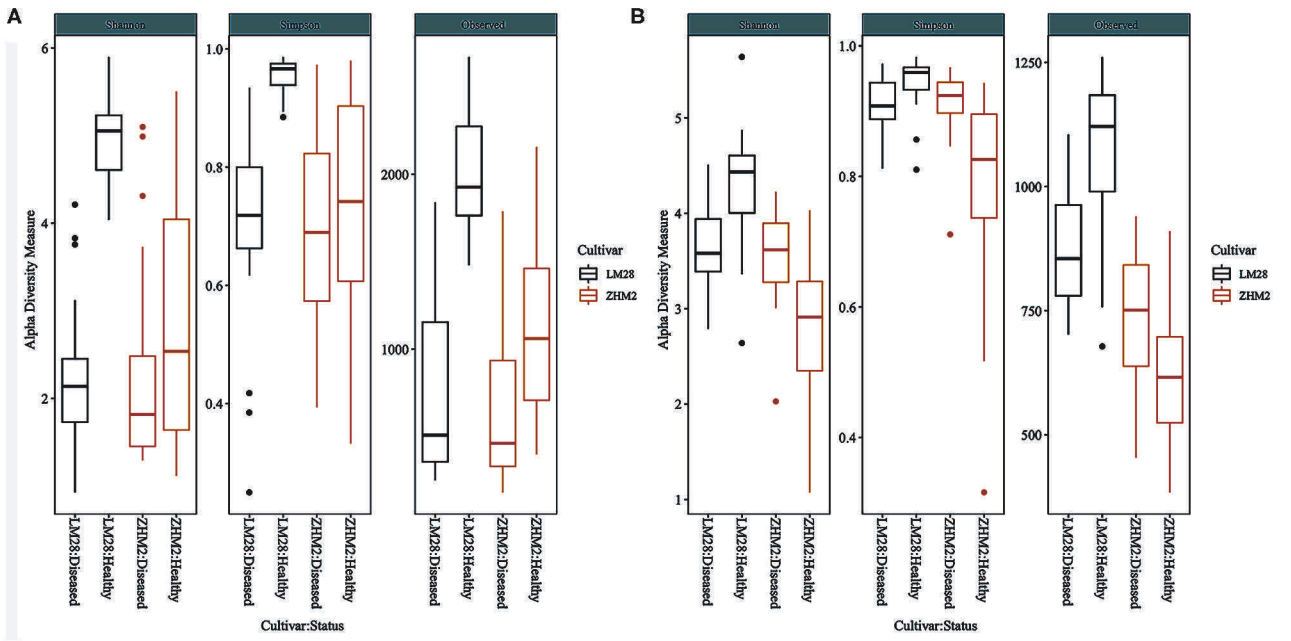

FIGURE 2 | Bacterial **(A)** and fungal **(B)** alpha-diversity indices of rhizosphere soil samples collected from neighboring pairs of healthy and diseased (showing wilt symptoms) cotton plants of two cultivars in two separated fields ∼300 m apart. The indices were calculated from 16S and ITS amplicon sequences.

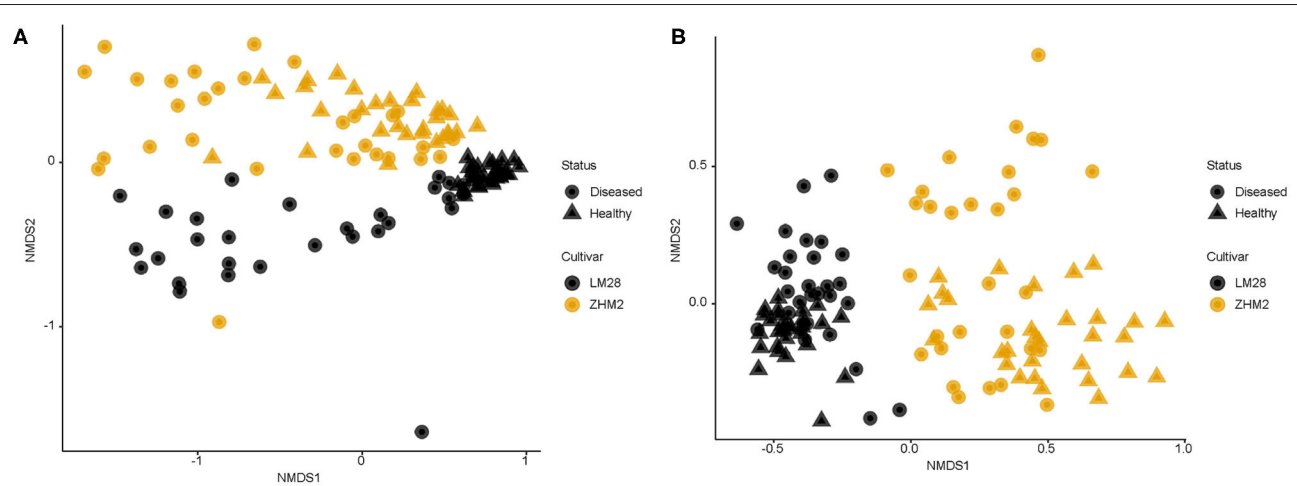

FIGURE 3 | Non-dimensional scaling analysis of bacterial **(A)** and fungal **(B)** community based on Bray–Curtis indices for rhizosphere soil samples collected from neighboring pairs of healthy and diseased (showing wilt symptoms) cotton plants of two cotton cultivars in two separated fields ∼300 m apart. 16S and ITS amplicon sequences were used to profile bacterial and fungal communities, respectively.

PC, more than half of its variability among samples was due to the differences between diseased and healthy plant samples (**Table 1**); cultivar and its interaction with plant wilt status each accounted for ∼10% of the total variability. The second PC was primarily affected by cultivars, accounting for 12.5% of the total variability. The third PC was mainly affected by within-field locations (namely, among pairs of plants) but accounting for only 3.8% of the total variability.

For fungal data, the first three PCs accounted for 19.7, 13.3, and 5.4% of the total variability, respectively. The effects of experimental variables on the first three PCs were similar to those for the bacterial community. Thus, the first and second PCs were primarily affected by plant diseased status and cultivar, respectively; nevertheless, the cultivar effect appeared to be more pronounced for the fungal than for the bacterial community (**Table 1**).

Comparison of Individual OTUs Between Diseased and Healthy Plant Samples

Rhizosphere Bacterial Community Composition

On cv. LM28, 1,543 bacterial OTUs passed the default DESeq2 filtering criteria and were subjected to statistical comparison. Of the 1,543 OTUs, 1,027 differed in their abundance between diseased and healthy plant samples. For 1,007 of the 1,027 OTUs, the abundance was less in the rhizosphere of diseased plants than healthy plants (**Supplementary Figure 4A**). For cv. ZHM2, only 1,243 bacterial OTUs passed the default DESeq2 filtering criteria. Of the 1,243 OTUs, 640 differed in their abundance between diseased and healthy plant samples. For 631 of the 640 OTUs, the abundance was less in the rhizosphere of diseased plants than healthy plants (**Supplementary Figure 4B**).

When both cultivars were analyzed together, 1,468 bacterial OTUs passed the default DESeq2 filtering criteria. Of the 1,468 OTUs, 1,050 differed in their abundance between diseased and healthy plant samples. For 1,035 of the 1,050 OTUs, the abundance was less in the rhizosphere of diseased plants than healthy plants (**Figures 4A,B**). The majority of these OTUs with reduced abundance in the diseased plants were shared between the two cultivars with only 25 and 8 unique to LM28 and ZHM2, respectively (**Figure 4B**). Except for Gammaproteobacteria, the relative abundance of almost all bacterial groups was reduced in the rhizosphere of diseased plants of both cultivars, including several well-known taxonomy groups containing beneficial microbes, such as *Bacilli* (Firmicutes) and Gemmatimonadetes (**Supplementary Figure 6**).

TABLE 1 | Percentage of the total variance in the first three principal components accounted by each term for bacterial and fungal rhizosphere communities of cotton plants.

Terms	Bacteria			Fungi		
	PC1	PC2	PC3	PC1	PC2	PC3
Cultivar [1]	10.4***	12.5***	0.1	20.0***	43.9***	0.4
Plant status [2]	51.5***	2.2***	<0.1	43.3***	9.4***	0.2
Location within cultivar	9.9	1.9	3.8***	19.1	5.1	18.3**
[1] × [2]	10.9***	1.8***	0.1*	0.4	2.7***	0.6*

Rhizosphere soils from paired of neighboring healthy and diseased plants of two cultivars were sampled and profiled with amplicon sequence for the 16S and ITS regions. Two cultivars were planted in two separate fields ~300 m apart.
**P < 0.05, **P < 0.01, ***P < 0.001 based on ANOVA.*

Rhizosphere Fungal Community Composition

On cv. LM28, only 1,142 fungal OTUs passed the default DESeq2 filtering criteria and were subjected to statistical comparison. In total, 597 OTUs differed in their abundance between diseased and healthy plant samples; for all these OTUs, the abundance was greater in the rhizosphere of diseased plants than healthy plants (**Supplementary Figure 5A**). For cv. ZHM2, only 1,272 fungal OTUs passed the default DESeq2 filtering criteria. Of the 1,272 OTUs, 738 differed in their abundance between diseased and healthy plant samples. For 736 of these 738 OTUs, the abundance was greater in the rhizosphere of diseased plants than healthy plants (**Supplementary Figure 5B**).

When both cultivars were analyzed together, 1,856 fungal OTUs passed the default DESeq2 filtering criteria. Of the 1,856 OTUs, 855 differed in their abundance between diseased and healthy plant samples. For all these 855 OTUs, the abundance was greater in the rhizosphere of diseased plants than healthy plants.

As with bacteria, the majority of those fungal OTUs with increased abundance in the rhizosphere of diseased plants were shared between the two cultivars with only 2 and 7 unique to LM28 and ZHM2, respectively (**Figure 4C**). Taxonomy heat trees (**Figure 5**) were constructed with Metacoder (Foster et al., 2017) for each cultivar to illustrate the differences in fungal abundances between healthy and diseased samples at specific taxonomy ranks. There were clear distinctions in the Tremellomycetes and a clade (Sordariales order) within the Sordariomycetes that had increased abundance in diseased plant samples over

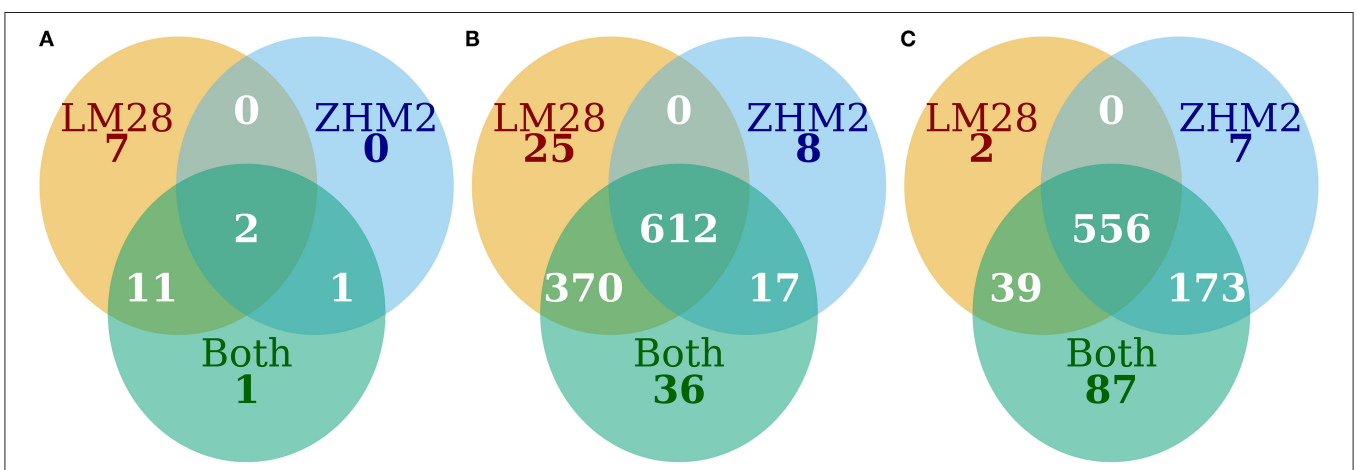

FIGURE 4 | The number of OTUs that differed ($P \leq 0.05$) in their abundance between diseased and healthy plant samples. **(A)** Bacteria increased in diseased samples, **(B)** bacteria decreased in diseased samples, and **(C)** fungi increased in diseased samples.

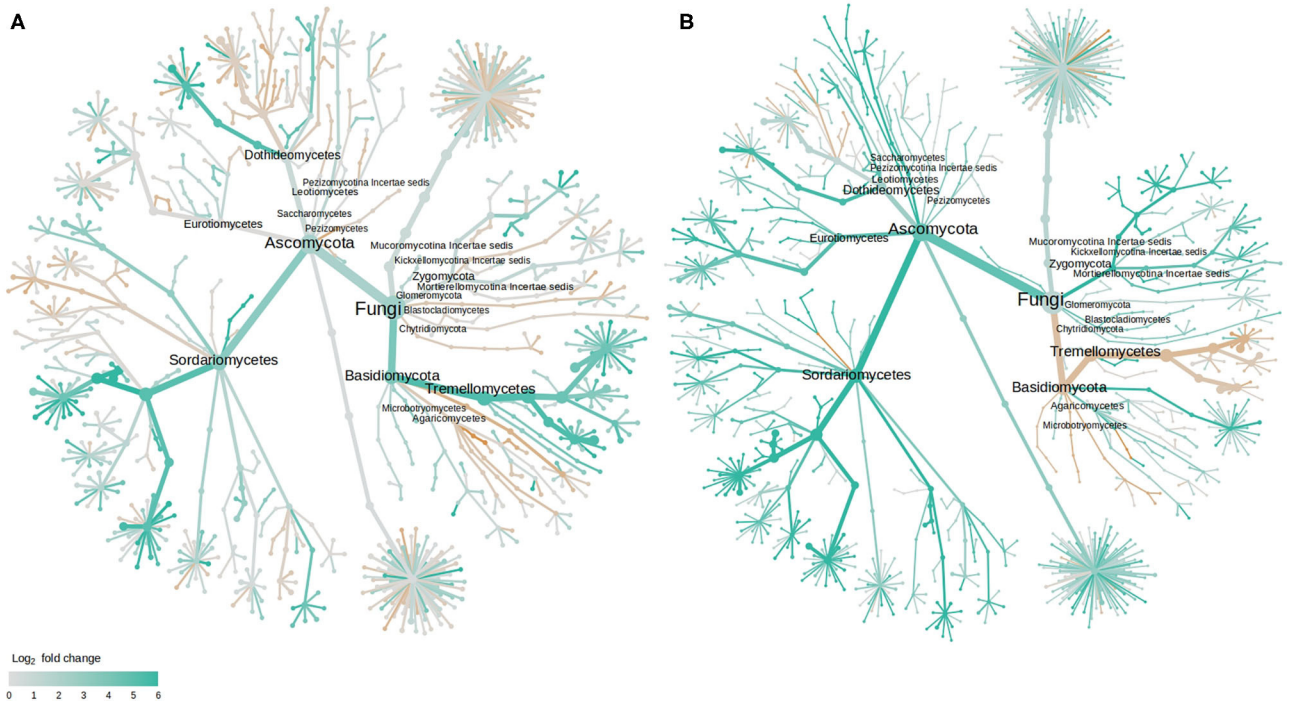

FIGURE 5 | Tree views of the differences in the fungal abundance in the rhizosphere of healthy and diseased cotton plants: **(A)** cv. LM28, and **(B)** cv. ZHM2; the difference in abundance is expressed as $\log_2$FoldChange, with values >0 indicating that the abundance of specific OTUs was greater in diseased samples than in healthy samples. Label and node represent the abundance of rhizosphere fungi at the class rank.

the healthy plants in cv. ZHM2 but not in LM28. Similar heat trees for bacterial OTUs showed very little difference (**Supplementary Figure 6**) between the two cultivars.

The fungal taxonomy was annotated using the online version of FUNGuild (http://www.stbates.org/guilds/app.php). Enrichment analysis (Fisher exact test with uncorrected *P*-values) of trophic level annotations of those OTUs with increased abundance in diseased samples indicated an increase in saprotrophs and a decrease in symbionts (**Table 2**). Enrichment of "Guilds" indicated a decrease in arbuscular mycorrhizal in cv. LM28 and an increase in several plant pathogen guilds (**Table 3**).

DISCUSSION

In the present study, similar to our previous finding (Wei et al., 2015), wilt severity increased significantly with the increasing *V. dahliae* inoculum density in the rhizosphere. However, the inoculum level explained only a very small proportion of the variability in observed wilt severities among plants, indicating that most differences in wilt severities are likely due to other factors rather than the differing inoculum densities.

Total bacterial biomass, as indicted by qPCR results, was significantly higher in the rhizosphere of healthy plants than that of diseased plants; the opposite was true for the total fungal biomass. Similar results in total bacteria were also observed for potato plants with high and low levels of potato common scab severity (Shi et al., 2019). Suppressive soil against *Fusarium* wilt has higher populations of bacteria than the wilt-conducive soil that has higher populations of fungi (Peng et al., 1999). The occurrence of cotton *Verticillium* wilt appears to be accompanied by increased fungal abundance in the rhizosphere. The ratio of total fungi with total bacteria in the rhizosphere of wilt cotton plants is much > 1; the opposite is true for the healthy plants. Therefore, the ratio of total fungal to total bacterial biomass can be considered as an indicator of cotton *Verticillium* wilt occurrence. This agrees with previous findings that the ratio of fungi to bacteria shows increasing trends in the soil of continuous cropping that leads to severe soilborne disease of *Panax notoginseng* (Dong et al., 2016). Interestingly, this ratio appears to be less variable for healthy plants than for diseased plants, suggesting that healthy plants are associated with stable fungal/bacterial communities.

Microorganisms are one indicator of soil health, particularly disease suppressiveness (Epelde et al., 2014; Ferris and Tuomisto, 2015; van Bruggen et al., 2015). High microbial diversity can improve community stability (Lefcheck et al., 2015; Delgado-Baquerizo et al., 2016). However, the relationship between microbial diversity and disease development varies with studies. For instance, a higher diversity in soil bacteria is associated with healthy plants in *P. notoginseng* (Wu et al., 2015) and cotton (Zhang et al., 2011), whereas the opposite was observed in tomato plants (Li et al., 2014). In the present study, higher bacterial alpha-diversity indices were found in healthy plants

TABLE 2 | Differences in the number of putative fungal functional groups (identified by FUNGuild "trophic level" annotations) in the rhizosphere of healthy and diseased cotton plants in two cultivars (LM28 and ZHM2).

Annotated		LM28			ZHM2			Both		
		Sig.	Odds ratio	*P*	Sig.	Odds ratio	*P*	Sig.	Odds ratio	*P*
Unknown	2,190	283	1.38	<0.001	394	1.22	0.004	462	1.21	0.003
Sap[a]	543	156	0.62	<0.001	190	0.63	0.000	199	0.7	<0.001
Path[b]	82	14	1.04	1.000	14	1.29	0.500	19	1.1	0.803
Sym[c]	83	5	2.96	0.010	9	2.03	0.039	12	1.77	0.070
Sap-Path	85	22	0.69	0.133	23	0.81	0.376	29	0.75	0.195
Sap-Sym	72	16	0.80	0.452	21	0.75	0.275	22	0.84	0.440
Path-Sym	26	6	0.77	0.618	6	0.95	0.820	7	0.95	0.830
Sap-Path-Sym	268	95	0.50	<0.001	79	0.75	0.030	105	0.65	0.001

Individual OTUs with significant differences in its abundance between diseased and healthy plants determined by DESeq2 analysis. Odds ratio of < 1 indicates a higher proportion of OTUs in the particular fungal functional group in the microbiome of diseased samples, and P-value indicates whether the odds ratio differs significantly from 1.0.
[a]Saprotroph, [b]Pathotroph, [c]Symbiotroph.

TABLE 3 | Differences in the number of putative fungal functional groups (identified by FUNGuild "trophic level" annotations) in the rhizosphere of healthy and diseased cotton plants in two cultivars (LM28 and ZHM2).

Annotated		LM28			ZHM2			Both		
		Sig.	Odds ratio	*P*	Sig.	Odds ratio	*P*	Sig.	Odds ratio	*P*
Undefined sap	433	143	0.54	<0.001	172	0.55	<0.001	178	0.62	<0.001
Plant path undefined sap	14	8	0.31	0.012	8	0.38	0.045	9	0.40	0.036
Dung sap undefined sap	5	4	0.22	0.035	4	0.27	0.062	4	0.32	0.090
Arbuscular mycorrhizal	71	5	2.52	0.035	9	1.73	0.140	12	1.51	0.215
Fungal parasite Undefined sap	133	52	0.45	<0.001	29	1.01	1.000	53	0.64	0.009
Endophyte fungal parasite plant path	9	5	0.32	0.048	5	0.39	0.152	5	0.46	0.178
Animal path endophyte Fungal parasite Plant path Wood sap	21	10	0.37	0.019	11	0.42	0.034	12	0.45	0.030
Endophyte Litter sap Soil sap undefined sap	39	15	0.46	0.020	19	0.45	0.009	20	0.50	0.014
Animal path Dung sap endophyte Epiphyte Plant sap Wood sap	19	10	0.34	0.008	12	0.35	0.008	12	0.40	0.022

Individual OTUs with significant differences in its abundance between diseased and healthy plants determined by DESeq2 analysis. Odds ratio of < 1 indicates a higher proportion of OTUs in the particular fungal functional group in the microbiome of diseased samples, and P-value indicates whether the odds ratio differs significantly from 1.0. Only guilds with P ≤ 0.05 in any of the contrasts are shown.

than in wilt diseased plants, but this is not true for fungal community. Higher diversity in soil bacteria is often associated with greater resistance to pathogens (Garbeva et al., 2004; Mallon et al., 2015). The increase of soil resistance/tolerance to pathogens may be related to the complexity of the interaction network of microorganisms in soil (Shi et al., 2019). Complex microbial community interaction can regulate the stability of the community (Eisenhauer et al., 2013), thus limiting pathogen increase.

Soilborne pathogens can have profound impacts on nutrient availability and plant root exudates in rhizosphere, which, in turn, may affect microbial communities (Cook et al., 1995). The present study showed large and consistent effects of a soilborne vascular disease on both fungal and bacterial communities in the rhizosphere of cotton plants. Rhizosphere microbiomes have been showed to differ between healthy and soilborne diseased plants (Mendes et al., 2011; Shanmugam et al., 2011; Zhang et al., 2011; Wu et al., 2015). However, a recent study showed that bacterial community in the geocaulosphere soil could be distinguished according to potato scab severity, but not in rhizosphere soil (Shi et al., 2019). This difference could be because that common scab of potato mainly invades through

tubers, whereas many other soilborne pathogens directly infect root systems. As a vascular pathogen of the cotton, *V. dahliae* infects roots and then colonizes vascular tissues, causing plant wilting, but usually does not cause root decay. However, fungal colonization in vascular tissues is expected to result in considerable changes in plant physiology and hence in root exudates. We thus speculate that it is the change in root exudates that may largely be responsible for the resulting differences in rhizosphere microbiomes between healthy and diseased plants.

In the present study, the differences in the rhizosphere microbiome between diseased and healthy plants within the same cultivars are much greater than differences between cultivars. Cultivar differences may be exaggerated as the two cultivars were grown separately in two neighboring fields and thus are confounded with the differences in microbial communities between the two fields, which is well-known (Edwards et al., 2015). Such a spatial effect was also illustrated by the significant differences between pairs of plants (representing different locations within a field). Precisely for this reason, we sampled scheme neighboring diseased and healthy plants of the same cultivar to minimize the compounding effects of disease phenotype and microbial variability in space. Thus, the differences in the rhizosphere microbial community between diseased and healthy plant within the pairs of plants are more likely to be directly related to the wilt development. The important question, however, remains whether the large community differences in the rhizosphere between diseased and healthy plants are a consequence of the infection by *V. dahliae* and/or subsequent wilt development that has affected root exudate composition leading to changes in rhizosphere microbiome.

Differences in abundance of individual OTUs between the diseased and healthy plants have a consistent pattern: nearly all those bacterial OTUs with differential abundance had much higher abundance in healthy plants than in diseased plants, and the opposite was true for the fungal OTUs. Many bacterial and some fungal groups have been shown to associate with cotton plant tolerance to development of wilt caused by *V. dahliae* (Wei et al., 2019). Many specific bacterial OTUs have higher abundance in rhizosphere of healthy plants than diseased plants. For instance, the abundance of Acidobacteria is reduced in rhizosphere of diseased plants, agreeing with a recent finding that the decrease of this phylum is linked to wilt development in olive, also caused by *V. dahliae* (Fernández-González et al., 2020). Furthermore, the abundance of several well-known taxonomy groups containing beneficial microbes, such as *Bacilli* (Firmicutes) and Gemmatimonadetes, was reduced in the rhizosphere of diseased plants, consistent with previous findings (Wei et al., 2019; Fernández-González et al., 2020). In contrast, the abundance of Gammaproteobacteria was increased in diseased plants in both cultivars. Gammaproteobacterial diversity and community members have been identified as potential health indicators (Köberl et al., 2017). For example, healthy banana (*Musa acuminata* L.) plants have increased presence of potentially plant-beneficial *Pseudomonas* and *Stenotrophomonas*, whereas diseased plants had a high level of Enterobacteriaceae known for their plant degradation ability (Köberl et al., 2017). Decreased abundance associated with diseased plants was also observed for arbuscular mycorrhizal fungi (AMF). AMF colonization may have increased the expression of pathogenesis-related genes and lignin synthesis-related genes more strongly, thus leading to induced resistance against *V. dahliae* in cotton (Zhang et al., 2018). In addition, AMF can also induce changes in the composition of cotton root exudates, contributing to bioactive effects against germination of *V. dahliae* conidia (Zhang et al., 2012a). Similarly, increased abundance of fungal pathogens in general together with reduced abundance of beneficial microbes may explain yield (mainly due to soilborne diseases) declining potential observed in continuous cotton monocropping systems (Wei and Yu, 2018).

The present study showed that a much greater proportion of rhizosphere microbial OTUs differed in their abundance between neighboring diseased and healthy plants of the same cultivars than between cultivars with differing susceptibility to *V. dahliae*. This may suggest that much of these differences between diseased and healthy plants may have resulted from postinfection or postdisease development as consequences of changes in root exudates and/or volatiles from plants associated with pathogen infection and subsequent disease development. Thus, rhizosphere of diseased plants has greater abundance of fungal saprophytes than healthy plants.

CONCLUSION

This study demonstrated that *V. dahliae* infection and subsequent disease development can lead to large changes in rhizosphere microbiomes in cotton. In addition to high *V. dahliae* inoculum density, a high fungal/bacterial biomass ratio in rhizosphere is an indicator of wilt disease development. Much of the increased fungal abundance in diseased roots is likely contributable to increased fungal saprophytes.

DATA AVAILABILITY STATEMENT

Raw sequence data reported in this paper have been deposited to the European Nucleotide Archive (ENA) under accession number PRJEB39152.

AUTHOR CONTRIBUTIONS

FW, XX, JP, and HZ planned, designed the research, and experiments. FW, HF, DZ, ZF, LZ, and YZ performed the experiments. FW, XX, and GD analyzed the data. FW and XX wrote the manuscript. FW acquired the funds for the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.618169/full#supplementary-material

Supplementary Figure 1 | Estimated *Verticillium dahliae* inoculum densities (CFU/g) in soil plotted against wilt scores of individual cotton plants.

Supplementary Figure 2 | Five examples of rarefaction curves for bacteria. All curves terminated at the number of sequences obtained in the examples.

Supplementary Figure 3 | Five examples of rarefaction curves for fungi. All curves terminated at the number of sequences obtained in the examples.

Supplementary Figure 4 | Plots of DeSeq2 analysis results for bacterial OTUs with significant ($P < 0.05$) differences between the healthy and diseased plants for **(A)** cv. LM28, **(B)** cv. ZHM2; mean expression is the average number of sequence reads for each OTU, and $\log_2$FoldChange is the $\log_2$ of the ratio in the number of sequence reads between the healthy and diseased plants. Positive $\log_2$FoldChange indicates that the relative abundance of specific OTUs was greater in the healthy plant samples than in diseased plant samples.

Supplementary Figure 5 | Plots of DeSeq2 analysis results for fungal OTUs with significant ($P < 0.05$) differences between the healthy and diseased plants for **(A)** cv. LM28, **(B)** cv. ZHM2; mean expression is the average number of sequence reads for each OTU and $\log_2$FoldChange is the $\log_2$ of the ratio in the number of sequence reads between the healthy and diseased plants. Positive $\log_2$FoldChange indicates that the abundance of specific OTUs was greater in the healthy plant samples than in diseased plant samples.

Supplementary Figure 6 | Tree views of the differences in bacterial abundance in the rhizosphere of healthy and diseased cotton plants: **(A)** cv. LM28, and **(B)** cv. ZHM2; the difference in abundance is expressed as $\log_2$FoldChange with values >0 indicating that the abundance of specific OTUs was greater in diseased samples than in healthy samples. Label and node represent the abundance of rhizosphere fungi at the class rank.

REFERENCES

Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., et al. (2015). Functional overlap of the arabidopsis leaf and root microbiota. *Nature* 528, 364–369. doi: 10.1038/nature16192

Benjamin, L. R., and Aikman, D. P. (1995). Predicting growth in stands of mixed species from that in individual species. *Ann. Bot.* 76, 31–41. doi: 10.1006/anbo.1995.1075

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., et al. (2014). Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642. doi: 10.1093/nar/gkt1244

Cook, R. J., Thomashow, L. S., Weller, D. M., Fujimoto, D. K., Mazzola, M., Bangera, G., et al. (1995). Molecular mechanisms of defense root disease by rhizobacteria against root disease. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4197–4201. doi: 10.1073/pnas.92.10.4197

Delgado-Baquerizo, M., Maestre, F. T., Reich, P. B., Jeffries, T. C., Gaitan, J. J., Encinar, D., et al. (2016). Microbial diversity drives multifunctionality in terrestrial ecosystems. *Nat. Commun.* 7, 1–8. doi: 10.1038/ncomms10541

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–930. doi: 10.1111/j.1654-1103.2003.tb02228.x

Dong, L., Xu, J., Feng, G., Li, X., and Chen, S. (2016). Soil bacterial and fungal community dynamics in relation to *Panax notoginseng* death rate in a continuous cropping system. *Sci. Rep.* 6:31802. doi: 10.1038/srep31802

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

Eisenhauer, N., Schulz, W., Scheu, S., and Jousset, A. (2013). Niche dimensionality links biodiversity and invasibility of microbial communities. *Funct. Ecol.* 27, 282–288. doi: 10.1111/j.1365-2435.2012.02060.x

Epelde, L., Becerril, J. M., Alkorta, I., and Garbisu, C. (2014). Adaptive long-term monitoring of soil health in metal phytostabilization: ecological attributes and ecosystem services based on soil microbial parametersxs. *Int. J. Phytoremediation* 16, 971–981. doi: 10.1080/15226514.2013.810578

FAO (1998). *World Reference Base for Soil Resources. World Soil Resources Report 84 FAO Vol. 88*. Rome: UN Food and Agriculture Organization.

Fernández-González, A. J., Cardoni, M., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Villadas, P. J., Fernández-López, M., et al. (2020). Linking belowground microbial network changes to different tolerance level towards *Verticillium* wilt of olive. *Microbiome* 8, 1–19. doi: 10.1186/s40168-020-0787-2

Ferris, H., and Tuomisto, H. (2015). Unearthing the role of biological diversity in soil health. *Soil Biol. Biochem.* 85, 101–109. doi: 10.1016/j.soilbio.2015.02.037

Fierer, N., Jackson, J. A., Vilgalys, R., and Jackson, R. B. (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* 71, 4117–4120. doi: 10.1128/AEM.71.7.4117-4120.2005

Finkel, O. M., Castrillo, G., Herrera Paredes, S., Salas González, I., and Dangl, J. L. (2017). Understanding and exploiting plant beneficial microbes. *Curr. Opin. Plant Biol.* 38, 155–163. doi: 10.1016/j.pbi.2017.04.018

Foster, Z. S. L., Sharpton, T. J., and Gru, N. J. (2017). Metacoder : An R package for visualization and manipulation of community taxonomic diversity data. *PLoS Comput. Biol.* 13, 1–15. doi: 10.1371/journal.pcbi.1005404

Garbeva, P., van Veen, J. A., and van Elsas, J. D. (2004). Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Annu. Rev. Phytopathol.* 42, 243–270. doi: 10.1146/annurev.phyto.42.012604.135455

Herlemann, D. P. R., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., and Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5, 1571–1579. doi: 10.1038/ismej.2011.41

Klosterman, S. J., Atallah, Z. K., Vallad, G. E., and Subbarao, K. V. (2009). Diversity, pathogenicity, and management of *Verticillium* species. *Annu. Rev. Phytopathol.* 47, 39–62. doi: 10.1146/annurev-phyto-080508-081748

Köberl, M., Dita, M., Martinuz, A., Staver, C., and Berg, G. (2017). Members of gammaproteobacteria as indicator species of healthy banana plants on *Fusarium* wilt-infested fields in Central America. *Sci. Rep.* 7, 1–9. doi: 10.1038/srep45318

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi: 10.1111/mec.12481

Lefcheck, J. S., Byrnes, J. E. K., Isbell, F., Gamfeldt, L., Griffin, J. N., Eisenhauer, N., et al. (2015). Biodiversity enhances ecosystem multifunctionality across trophic levels and habitats. *Nat. Commun.* 6:7936. doi: 10.1038/ncomms7936

Li, J. G., Ren, G., Di Jia, Z. J., and Dong, Y. H. (2014). Composition and activity of rhizosphere microbial communities associated with healthy and diseased greenhouse tomatoes. *Plant Soil* 380, 337–347. doi: 10.1007/s11104-014-2097-6

Mallon, C. A., van Elsas, J. D., and Salles, J. F. (2015). Microbial invasions: the process, patterns, and mechanisms. *Trends Microbiol.* 23, 719–729. doi: 10.1016/j.tim.2015.07.013

Martin, F. N. (2003). Development of alternatnarive strategies for management of soil borne pathogens currently controlled with methyl bromide. *Annu. Rev. Phytopathol.* 41, 325–350. doi: 10.1146/annurev.phyto.41.052002.095514

Mazzola, M., and Freilich, S. (2017). Prospects for biological soilborne disease control: application of indigenous versus synthetic microbiomes. *Phytopathology* 107, 256–263. doi: 10.1094/PHYTO-09-16-0330-RVW

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

Mendes, R., Kruijt, M., Bruijn, I., De, Dekkers, E., van der Voort, M., Bakker, P. A. H. M., et al. (2011). Deciphering the rhizosphere microbiome for diseasesuppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., et al. (2016). FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* 20, 241–248. doi: 10.1016/j.funeco.2015.06.006

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Peng, H. X., Sivasithamparam, K., and Turner, D. W. (1999). Chlamydospore germination and Fusarium wilt of banana plantlets in suppressive and conducive soils are affected by physical and chemical factors. *Soil Biol. Biochem.* 31, 1363–1374. doi: 10.1016/S0038-0717(99)00045-0

Shanmugam, V., Verma, R., Rajkumar, S., and Naruka, D. S. (2011). Bacterial diversity and soil enzyme activity in diseased and disease free apple rhizosphere soils. *Ann. Microbiol.* 61, 765–772. doi: 10.1007/s13213-010-0193-2

Shi, W., Li, M., Wei, G., Tian, R., Li, C., Wang, B., et al. (2019). The occurrence of potato common scab correlates with the community composition and function of the geocaulosphere soil microbiome. *Microbiome* 7, 1–18. doi: 10.1186/s40168-019-0629-2

Tilston, E. L., Deakin, G., Bennett, J., Passey, T., Harrison, N., O'Brien, F., et al. (2018). Candidate causal organisms for apple replant disease in the United Kingdom. *Phytobiomes J.* 2, 261–274. doi: 10.1094/PBIOMES-11-18-0050-R

van Bruggen, A. H. C., Sharma, K., Kaku, E., Karfopoulos, S., Zelenev, V. V., and Blok, W. J. (2015). Soil health indicators and *Fusarium* wilt suppression in organically and conventionally managed greenhouse soils. *Appl. Soil Ecol.* 86, 192–201. doi: 10.1016/j.apsoil.2014.10.014

van der Putten, W. H., Klironomos, J. N., and Wardle, D. A. (2007). Microbial ecology of biological invasions. *ISME J.* 1, 28–37. doi: 10.1038/ismej.2007.9

van Elsas, J. D., Garbeva, P., and Salles, J. (2002). Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. *Biodegradation* 13, 29–40. doi: 10.1023/A:1016393915414

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Wei, F., Fan, R., Dong, H., Shang, W., Xu, X., Zhu, H., et al. (2015). Threshold microsclerotial inoculum for cotton Verticillium wilt determined through wet-sieving and real-time quantitative PCR. *Phytopathology* 105, 220–229. doi: 10.1094/PHYTO-05-14-0139-R

Wei, F., Passey, T., and Xu, X. (2016). Effects of individual and combined use of bio-fumigation-derived products on the viability of *Verticillium dahliae* microsclerotia in soil. *Crop Prot.* 79, 170–176. doi: 10.1016/j.cropro.2015.09.008

Wei, F., Zhao, L., Xu, X., Feng, H., Shi, Y., Deakin, G., et al. (2019). Cultivar-dependent variation of the cotton rhizosphere and endosphere microbiome under field conditions. *Front. Plant Sci.* 10, 1–15. doi: 10.3389/fpls.2019.01659

Wei, Z., and Yu, D. (2018). Analysis of the succession of structure of the bacteria community in soil from long-term continuous cotton cropping in Xinjiang using high-throughput sequencing. *Arch. Microbiol.* 200, 653–662. doi: 10.1007/s00203-018-1476-4

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). Am-plification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protoc.* 18, 315–322. doi: 10.1016/B978-0-12-372180-8.50042-1

Wu, Z., Hao, Z., Zeng, Y., Guo, L., Huang, L., and Chen, B. (2015). Molecular characterization of microbial communities in the rhizosphere soils and roots of diseased and healthy *Panax notoginseng*. *Antonie Van Leeuwenhoek* 108, 1059–1074. doi: 10.1007/s10482-015-0560-x

Xiong, W., Li, R., Ren, Y., Liu, C., Zhao, Q., Wu, H., et al. (2017). Distinct roles for soil fungal and bacterial communities associated with the suppression of vanilla *Fusarium* wilt disease. *Soil Biol. Biochem.* 107, 198–207. doi: 10.1016/j.soilbio.2017.01.010

Xu, X., Passey, T., Wei, F., Saville, R., and Harrison, R. J. (2015). Amplicon-based metagenomics identified candidate organisms in soils that caused yield decline in strawberry. *Hortic. Res.* 2:15022. doi: 10.1038/hortres.2015.22

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-marcell, J., Lax, S., et al. (2015). The soil microbiome influences grapevine-associated microbiota. *mBio* 6, 1–10. doi: 10.1128/mBio.02527-14

Zhang, G., Raza, W., Wang, X., Ran, W., and Shen, Q. (2012a). Systemic modification of cotton root exudates induced by arbuscular mycorrhizal fungi and *Bacillus vallismortis* HJ-5 and their effects on Verticillium wilt disease. *Appl. Soil Ecol.* 61, 85–91. doi: 10.1016/j.apsoil.2012.02.003

Zhang, J., Sanogo, S., Flynn, R., Baral, J. B., Bajaj, S., Hughs, S. E., et al. (2012b). Germplasm evaluation and transfer of *Verticillium* wilt resistance from Pima (*Gossypium barbadense*) to upland cotton (*G. hirsutum*). *Euphytica* 187, 147–160. doi: 10.1007/s10681-011-0549-0

Zhang, Q., Gao, X., Ren, Y., Ding, X., Qiu, J., Li, N., et al. (2018). Improvement of *Verticillium* wilt resistance by applying arbuscular mycorrhizal fungi to a cotton variety with high symbiotic efficiency under field conditions. *Int. J. Mol. Sci.* 19:241. doi: 10.3390/ijms19010241

Zhang, Y., Du, B. H., Jin, Z., Li, Z., Song, H., and Ding, Y. Q. (2011). Analysis of bacterial communities in rhizosphere soil of healthy and diseased cotton (*Gossypium* sp.) at different plant growth stages. *Plant Soil* 339, 447–455. doi: 10.1007/s11104-010-0600-2

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REVIEW
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Benefits to Plant Health and Productivity From Enhancing Plant Microbial Symbionts

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Plants exist in close association with uncountable numbers of microorganisms around, on, and within them. Some of these endophytically colonize plant roots. The colonization of roots by certain symbiotic strains of plant-associated bacteria and fungi results in these plants performing better than plants whose roots are colonized by only the wild populations of microbes. We consider here crop plants whose roots are inhabited by introduced organisms, referring to them as Enhanced Plant Holobionts (EPHs). EPHs frequently exhibit resistance to specific plant diseases and pests (biotic stresses); resistance to abiotic stresses such as drought, cold, salinity, and flooding; enhanced nutrient acquisition and nutrient use efficiency; increased photosynthetic capability; and enhanced ability to maintain efficient internal cellular functioning. The microbes described here generate effects in part through their production of Symbiont-Associated Molecular Patterns (SAMPs) that interact with receptors in plant cell membranes. Such interaction results in the transduction of systemic signals that cause plant-wide changes in the plants' gene expression and physiology. EPH effects arise not only from plant-microbe interactions, but also from microbe-microbe interactions like competition, mycoparasitism, and antibiotic production. When root and shoot growth are enhanced as a consequence of these root endophytes, this increases the yield from EPH plants. An additional benefit from growing larger root systems and having greater photosynthetic capability is greater sequestration of atmospheric CO_2. This is transferred to roots where sequestered C, through exudation or root decomposition, becomes part of the total soil carbon, which reduces global warming potential in the atmosphere. Forming EPHs requires selection and introduction of appropriate strains of microorganisms, with EPH performance affected also by the delivery and management practices.

Keywords: holobiont, endophyte, plant, bacteria, fungi

INTRODUCTION

Plants, like other so-called higher organisms, do not exist as entities unto themselves. They are biotic systems which consist of the plant plus innumerable microorganisms, the plant microbiome. This review considers plant-associated bacteria and fungi, focusing on those that internally colonize plant roots as microbial endophytes. Plants, together with their associated microbiomes, function as complex multi-species entities, referred to in the literature as holobionts (Margulis and Fester, 1991). The association can be detrimental to the plant if pathogens predominate, or it can be neutral. More often, it results in plants having better health, growth, and performance. We have previously discussed endophytic root colonization and the resulting symbiotic increases in plants' capabilities using the concept of Enhanced Plant Holobionts (EPHs; Harman and Uphoff, 2019).

A number of mechanisms are involved in producing these effects, particularly plant-microbial interactions, but there are also various microbe-microbe interactions. Because they are not widely known, we are particularly interested here in the microbial production of organic molecules that interact with plant cell membranes and induce system-wide changes in plant physiology, altering both plant gene and protein expression. These molecules we refer to as Symbiont-Associated Molecular Patterns (SAMPs; Harman and Uphoff, 2019). This terminology is consistent with the scientific literature on plant pathogens that already uses the terms Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs); see, for example, Malik et al. (2020). As a consequence of certain plant-microbial interactions, plants' photosynthesis can be enhanced, and there can be induced improvements in internal cellular environments. A result of these effects is having significantly larger EPH roots and shoots with higher crop yields.

By enhancing yields, EPHs can contribute to maintaining food security and reducing hunger in the world. These goals will become more challenging in future decades as still-rising human populations must be supported from a diminishing natural resource base that will be further constrained by the changing climate. EPHs can help to mitigate this as increased plant photosynthesis with greater root growth can increase carbon sequestration from the atmosphere and larger root systems will increase carbon stores in the soil. Increases in soil carbon (SC), especially in soil organic matter (SOM), will improve soil health and fertility which are associated with greater plant health, crop yields, and ultimately human health.

Following this introduction, Section 2 (entitled the functioning of enhanced plant holobionts) describes in general terms the physical interactions of symbiotic microbes with plants, and especially the colonization of internal plant organs and the nature of the endophytic associations. Section 3 (mechanisms for enhanced plant holobionts) reviews mechanisms by which endophytes react with plants to protect plants' health and support their growth, robustness, and productivity. Section 4 (biochemical and genetic effects associated with EPHs) discusses the biochemical effects associated with SAMPs and their gene and protein regulation, improvements in photosynthetic efficiency, and effects of internal cellular functioning.

Section 5 (benefits conferred on plants) considers benefits that this symbiotic association confers on plants, including the control of biotic stresses, including disease, insect pests, and nematodes, as well as the mitigation of abiotic stresses such as drought, salt, and adverse temperatures. Section 6 (agricultural and societal benefits) discusses higher-level agricultural and societal benefits, including improvements in soil health, enhancing sustainable food production, and creating environmental benefits such as carbon sequestration and storage, which can help to slow global warming by capturing and removing greenhouse gases such as CO_2 from the atmosphere.

Section 7 (management and delivery systems for EPHs) addresses important aspects of the delivery and management of microbial agents, including the application of exogenous inoculum and the mobilization of existing soil populations. Section 8 (enhancing microbial endowments with changes in crop and soil management) then goes into management systems such as no-till cultivation that can have beneficial effects on the soil biota, and the system of rice intensification for which we have experimental evidence confirming the practicality of EPHs when modifying crop management. Section 9 summarizes the various components that contribute to the creation and cultivation of EPHs and to the benefits that these confer at both micro and macro levels. Section 9 is a summary of the paper.

Numerous fungi and bacteria may provide beneficial effects including endophytic fungi (*T. afroharzianum, viride, atroviride, virens, reesei* and others; Woo et al., 2014; Harman and Uphoff, 2019; Ikram et al., 2019), in addition to other endophytic fungi such as strains of *Aspergillus niger* (Ismail et al., 2020), *Penicillium roqueforti* (Ikram et al., 2018), *Aspergillus terreus* (Khushdil et al., 2019) *Yarriwia lipolytica* (Farzana et al., 2019), as well as *Piriformaspora indica* (Gill et al., 2016) *Penicillium citrinum* and *Aspergillus terreus* (Waqas et al., 2015), and bacteria such as *Pseudomonas* (Pieterse et al., 2014), *Bacillus* (Kloepper et al., 2004) and Rhizobiacae (Chi et al., 2005). It is important to note that, while many fungi and bacteria are endophytic and provide benefits to plants, microbial effects on plants are highly specific to individual strains. Just because one strain benefits certain plants, does not mean that all members of the same genus or species of microbes will have the same effects.

THE FUNCTIONING OF ENHANCED PLANT HOLOBIONTS

Microbial Enhancement of Plants

Many bacteria and fungi are beneficial to plants in various ways:

- By improving their resistance to diseases and pests;
- By mitigating abiotic stresses such as drought, salt, and adverse temperatures;
- By improving plants' nutritional status through better acquisition of nutrients from the soil, enhancing supply of nutrients such as through the fixation of nitrogen, and better nutrient-use efficiency;
- By enhancement of plants' photosynthetic capability; and

- By maintaining internal cellular environments that are more conducive for the functioning of critical plant metabolic processes.

These services generally result in better growth of plants' shoots and roots and thus in higher yields, especially under adverse conditions.

Numerous bacteria and fungi are known to improve plant performance, among them bacteria in the genus or families *Pseudomonas, Bacillus*, and Rhizobiacae, and fungi such as *Trichoderma* and *Piriforma*. Other microbes may also induce similar benefits, but the groups mentioned here are the best documented, most studied, and most widely used. For a comprehensive listing of beneficial microbes, see (Copping, 2004). The most effective strains of either fungi or bacteria are usually ones that colonize the plant roots endophytically.

Endophytic Root, Leaf, and Branch Colonization

Many of the most beneficial microbes live within the internal space of plant roots. In at least one case, they take up residence anywhere in the plant where they are applied, not limited to the roots. In two other cases, once established in the roots, the microbes become systemic throughout the plant. The beneficial species that are considered in this review do not cause disease or other deleterious effects. Not all, but many microorganisms living within plant organs and tissues have beneficial effects on plants' growth, health, and productivity. Their symbiotic services to plants are similar to those that myriad microbes in the human microbiome confer on our species. These endophytes when introduced purposefully to augment whatever microbial populations exist naturally in the plants' microbiomes create what we refer to as EPHs.

Colonization patterns differ as shown in **Figure 1**. As noted above, the beneficial effects are always strain-specific, and no generalizations can be made regarding specific taxa since a given strain or species may have members that are highly beneficial, while other members of the same species may not confer advantages. Some genera contain species whose colonization confers advantages to the plants that harbor them, while other genera, e.g., *Pseudomonas, Fusarium*, and *Rhizoctonia*, include both pathogens and beneficial organisms. Some strains of *Pseudomonas* can be beneficial symbionts for certain plants, while other strains cause disease in both animals and plants.

Among the more complex interactions are those involving bacteria in the Rhizobiacae family of protobacteria. In leguminous plant species, these bacteria infect plant roots and form nitrogen-fixing nodules, structures composed of both plant and bacterial cells as shown in **Figures 1C,D**.

Conversely, some of these same bacteria which fix nitrogen in the roots of clover plants can infect the roots of cereal plans like wheat or rice and become systemic throughout these plants (**Figure 1A**; Chi et al., 2005). Little is known about the extent to which such systemic colonization occurs in other plants.

Vascular-arbuscular mycorrhizal (VAM) fungi also form complex plant root-microbial structures. These fungi interact with plant roots to form arbuscules within the roots that are highly efficient in transferring nutrients such as phosphorus from the VAM to the plant and of nutrients from the plant to the VAM in return, as seen in **Figure 1B**.

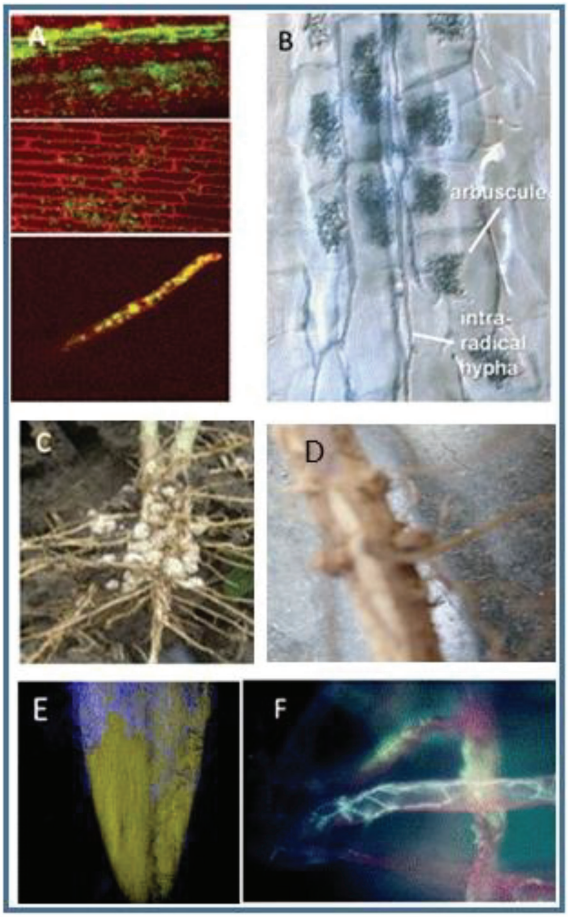

FIGURE 1 | Examples of endophytic colonization by different bacteria or fungi. **(A)** Endophytic colonization of rice by *Rhizobium leguminosarum bv. Trifoli* in rice. The photomicrograph shows the bacteria in the stems after the bacterium was applied to seeds. From (Chi et al., 2005). The journal does not require persmission to use materials. **(B)** Photograph of mycorhizzae labeled to show arbucules and hyphae within roots, from (Schussler, 2009), used with permission of the author. **(C)** Nodules on soybean roots. Used courtesy of Advanced Biological Marketing. **(D)** Photograph of nodules on soybean roots. Photo by the first author. **(E)** A photomicrograph of fluorescent imaging of a corn root showing growth of *Trichoderma* on the surface. Spores of the fungus were added to seeds, and the growth shown was taken 2 days later. Photograph courtesy of Advanced Biological Marketing. **(F)** Fluorescent photomicrograph of *Trichoderma* growing in root hairs. From (Harman et al., 2004a), used with permission of the journal.

Some strains of *Trichoderma* become endophytes and colonize the cortical cells of roots. **Figures 1E,F** show this interaction. If specific strains of *Trichoderma* are applied to seeds, spores of the fungi germinate very rapidly, and within a few days hyphae, branching filaments, grow from the seed onto the emerging radicle (**Figure 1E**). The fungi then colonize the roots (**Figure 1F**). Other strains of *Trichoderma*, however, are unable to colonize the rhizosphere or to become endophytic (Harman and Uphoff, 2019).

In certain plants, strains of *Trichoderma* become systemic, living throughout the plant, while in other plants these same strains are confined to the roots (Bae et al., 2011). The location of endophytes is therefore plant-specific. Other fungi may become endophytic in any part of the plant where they are inoculated. For example, *Clonostachys rosea* can colonize plant tissues including leaves, with the result of controlling certain plant diseases that affect the leaves (Shafia et al., 2001; Xue et al., 2014).

Chemical crosstalk between roots and root colonizing microbes is required for symbiotic relationships to occur. With maize, roots and *A. nominus* require indole acetic acid and flavonoids to establish the relationship (Mehmood et al., 2020). Similar crosstalk occurs to establish the relationship with legumes and Rhizobiacae and with mycorrhizae and the numerous plants they colonize. These signaling pathways developed first with mycorrhizae and the legume-Rhizobiacae developed similar interactions (Genre and Russo, 2016). Other relationships, such as those between *Fusarium* and legumes employ elements of these same pathways (Skiada et al., 2020).

MECHANISMS FOR ENHANCED PLANT HOLOBIONTS

Mycoparasitism

The ability of certain fungi to colonize and parasitize other fungi and Oomycetes has been known for almost 90 years. This capability of fungi does not per se lead to the formation of holobionts, but it is included here for reasons of completeness. In addition, some fungi that are mycoparasiitic are endophytes that form holobiontic associations with plants.

The events that occur in mycoparasitism have been well documented in interactions of *Trichoderma* with other fungi, including pathogens. The interactions can be complex. At least *in vitro*, *Trichoderma* can be grown in a directed fashion to attack targeted fungi (Chet et al., 1981). This is facilitated by the ability of the mycoparasite to secrete small amounts of an exochitinase that releases carbohydrates; *Trichoderma* sensing these follows this trail of nutrients to the target fungi (Brunner et al., 2003). Once *Trichoderma* comes into contact with the target fungus, it engages in interaction that results, in some cases, with the coiling response noted above.

Once in contact with the target fungus, *Trichoderma* can penetrate its internal lumen and establishes an internal infection (Harman et al., 2004a). This is facilitated by the ability of *Trichoderma* to produce a potent array of fungal cell-wall-degrading enzymes (**Figure 1D**) and also various antibiotics, many of which exhibit synergistic fungitoxicity. Scores of genes, enzymes, and metabolites are known to be involved in this interaction (Harman et al., 2004a; Lorito et al., 2010). In some cases, mycoparasitism is essential and sufficient for biocontrol.

For example, strains of *Ampelomyces* are parasitic on powdery mildews (Kiss et al., 2004), and since they are obligate pathogens on mildews, this mechanism must be the operative one. *Coniothyrium minitans* is a commercial product for control of sclerotia-producing fungal pathogens. *C. minitans* parasitizes the sclerotia which contain food reserves for the pathogen, and over time this reduces the pathogenic populations, resulting in successful control (Whipps and Lumsden, 1991; Whipps, 1997; Gwynn, 2014).

Production of Antibiotics

Numerous metabolites are produced by bacteria and fungi that are toxic to particular plant pathogens and pests. For example, certain strains of *Agrobacterium* that do not give rise to galls in plants have the ability to control other strains of the bacteria that do. This effect is associated with the microbe's production of agrocin, a biochemical that is highly toxic to tumorigenic bacterial strains (Burr and Reid, 1994). It cannot be ascertained whether this toxin is solely responsible for the observed biocontrol ability, however, since other factors such as competition for infection sites could be operative.

Similarly, control of the take-all disease in wheat caused by *Guamannomyces tritici* can be controlled by the addition of strains of *Pseudomonas fluorscens* that produce phenazine antibiotics (Fravel, 1988; Weller, 1988). This bacterium was obtained from soils that became suppressive to the disease following years of wheat monoculture. This microorganism can create a soil in which the disease is reduced to levels where crop damage is greatly reduced (Cook, 2017).

In another case, we searched for strains of *Trichoderma* that could control the *Phytophthora* disease caused by pathogenic Oomycetes. Hundreds of strains were screened, first *in vitro*, and then by plant assays. The only strain identified with an ability to control this pathogen was a strain of *T. virens* that produced the antibiotic gliotoxin (Smith et al., 1990). This antibiotic is extremely toxic to zoospores of the pathogen (Wilcox et al., 1992).

This strain was isolated from a soil that had been periodically cultivated with peas for over a century. The root rot caused by the water mold *Aphanomyces euteiches* f. sp. *pisi* was not present in this soil, although earlier reports indicated that in the pathogen had caused very damaging root rot in this same area. The pathogen *A. euteiches* is a relative to the plant-damaging Oomycete *Phytophthora* and both produce motile zoospores, so the gliotoxin produced by *T. virens* is extremely toxic to both.

In the cases mentioned above, the antibiotics are clearly associated with disease suppression. However, it should not be assumed that this antibiotic production is the only mechanism involved in disease control. Many or most microorganisms produce at least some antibiotic substances. While their antibiotics may be involved in biocontrol, in most cases other mechanisms may also be involved, as discussed in later sections.

Antifungal Enzymes

Earlier, we mentioned fungitoxic enzymes from *Trichoderma* that are involved in mycoparasitism. In addition, various antibiotics that are produced have antifungal synergy with certain enzymes. The diversity of these enzymes and their functions is remarkable. There are multiple classes of enzymes that degrade chitin, cellulose, proteins, and other polymers

found in nature. Beyond this, there are numerous separate enzymes for each functional classification, and the numbers of active enzymes and combinations are very large.

For example, a search for *Trichoderma*-produced chitinases in the Uniprot database gave 1,090 entries (https://www.uniprot.org/uniprot/?query=trichoderma+chitinase&sort=score–chitinases are one of several classes of cellulose-degrading enzymes), while a similar search for the broader category of *Trichoderma* cellulases gave 9,559. Some of these are from different strains that produce similar enzymes, but a perusal of this database demonstrates that many are different enzymes produced by beneficial microorganisms, having, e.g., dissimilar molecular weights.

Competition

For many years, this has been considered one of the three main mechanisms for microbial disease control, along with antibiosis and mycoparasitism. It is obvious that competition occurs widely and can never be excluded as a mechanism of biocontrol, but it is hard to prove or disprove. In at least one case, the control of aflatoxin by heavy application of atoxigenic strains of *Aspergillus flavus*, competition is clearly the main factor. The atoxigenic strains compete with the one that produces aflatoxin, and this prevents accumulation of levels of this highly-toxic metabolite that are harmful to animals, including humans (Garber and Cotty, 1997).

Competition for infection sites by nonharmful strains can be expected to exclude harmful strains or species that are deleterious. It is thus difficult to rule competition in or out, since many cases of biocontrol may include competition.

Siderophores

Siderophores are a class of microbial metabolites that sequester iron, binding to it very tightly and making it difficult for other microbes with a lower affinity for iron to compete with those that are producing siderophores with a very strong binding of iron (Trapet et al., 2016). Because iron under many soil conditions is poorly soluble, plants and other organisms in soil have evolved mechanisms to acquire iron under these conditions, either through the production of siderophores or through acidification or the immediate area in proximity to the organism. Organisms that produce siderophores with a very high affinity to iron can compete successfully with other organisms (Trapet et al., 2016).

Siderophores from fluorescent *Pseudomonas* strains (e.g., pyoverdines and pseudobactins) are among the strongest iron-binding compounds known. They can solubilize iron in the soil and to transport it back into the microorganism, which enables it to obtain this vital compound for its metabolism. In so doing, it scavenges iron away from other microbes, and this is an important mechanism in the competitive battle with other organisms that would occupy this niche.

This capability can be demonstrated through several methods, such as comparing the Fe-EDTA or EDDHA (Scher and Baker, 1982; Hubbard et al., 1983). These compounds chelate iron and make it available to plants and microorganisms. If the synthetic compound reverses the biological effect, this is evidence of the role of the siderophore in that effect. This approach was taken in both of the citations just given. The effect may be either beneficial or detrimental. The strong effect of the *Pseudomonas* siderophore has been shown to be important in biocontrol of *Fusarium* wilt (Scher and Baker, 1982).

However, competition for iron prevented biocontrol of damping-off diseases by a strain of *Trichoderma* in some New York soils that had low levels of available iron; however, it was effective in Colorado, where Fe carbonates were more available. In the NY soils, biocontrol could be accomplished in the presence of synthetic iron-chelating compounds. A search for *Trichoderma* strains in NY soils that could be effective in biocontroil resulted in the identification and selection of a strain that provided biocontrol and which was found to produce siderophores of its own (Hubbard et al., 1983). These two examples demonstrate the importance of this mechanism.

Induced Systemic Resistance

The previous sections have described biocontrol by the action of one organism on another. However, it is becoming increasingly clear that a great deal of the control of diseases and pests, as well as control of abiotic stresses, is a result of the effects of the microbial agent on the plant, changing gene and protein expression that results in benefits from developing EPHs.

We noted above, for example, that *T. virens* produces the antibiotic gliotoxin. For many years this antibiotic was assumed to be responsible for the *Trichoderma's* ability to control seedling diseases of cotton (Howell, 2003). However, we now know that antibiotic production was not the primary mechanism of control in this case. Studies in which mutants of *T. virens* were produced have shown varying capacities for biocontrol, mycoparasitism, and antibiotic production. The only property correlated with the ability of *T. virens* to control *R. solani*, which causes sheath blight in rice, for example, was the *Trichoderma's* ability to induce plant resistance to the pathogen, as there was no correlation of biocontrol with either antibiotic production or mycoparasitism (Howell et al., 2000; Harman et al., 2004a).

Evidence of induced resistance includes the ability to control abiotic or biotic stresses at a distance from where the agent is located (Harman et al., 2004a). For example, many organisms are restricted to plant roots, but induce plant resistance to foliar diseases. An example of shown in **Figure 2** in the photograph of corn leaves, where *T. afroharzianum* was either applied or not applied to cpheorn seeds. The fungus is restricted to growth only in the roots, but it induces plant resistance to anthracnose on its leaves. In the photo, the two upper leaves were from a plant whose seeds had been treated, while on the lower leaves, no treatment was applied. Clearly, much less disease occurred on leaves from treated plants, thus demonstrating the concept of protection at plant sites distant from the actual location of the fungus.

Other evidence comes from genetic studies. For example, the gene NPR1 is essential to functioning of both Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR). These are two separate biochemical pathways by which each kind of resistance is induced. In studies with strains of the plant *Arabidopsis* that were deficient in the gene encoding

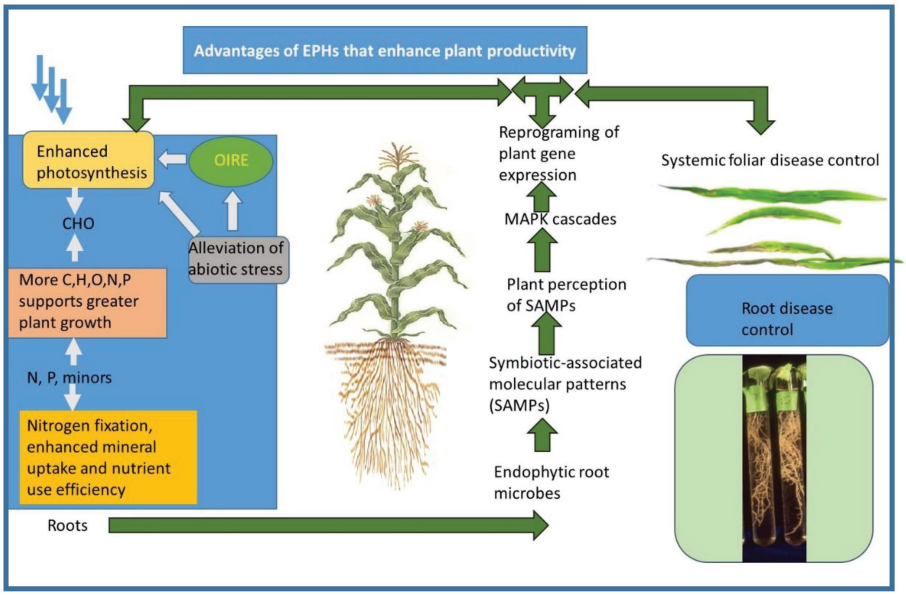

FIGURE 2 | A synoptic diagram of the interactions of plants and endophytes that result in the formation of EPHs. A similar diagram was presented in (Harman and Uphoff, 2019), which is an open access journal. The photograph of corn leaves is from (Harman et al., 2004a) and is used with permission of the journal.

this factor, *Trichoderma* did not control the fungal pathogen *Pythium* while strains with functional NPR1 were well-protected by the fungus (Shoresh et al., 2010).

However, these strains are also potent inducers of systemic resistance in plants (Pieterse et al., 2014), so attribution of causation is not unambiguous.

These two examples (many others could be cited) demonstrate that effects on plants of beneficial microbes occur as a consequence of microbial effects on plants, rather than just their effects on plant pathogens as provided in section 5. Most of the studies demonstrating this effect began to be published in the early 2000s cf. (Harman et al., 2004a). Older literature frequently ascribed the benefits of microbial agents to other mechanisms, but this was based on imcomplete knowledge. The ability of endophytic microbes to directly affect plants is the primary element contributing to the superiority of EPHs. To understand this, we turn to biochemistry.

BIOCHEMICAL AND GENETIC EFFECTS ASSOCIATED WITH EPHs

Production and Description of SAMPs

In almost all cases, the induction of systemic effects which protect plants from pathogenic losses is mediated by the production of signaling molecules by endophytic microbes that interact with plant cell membranes. This results in signal transduction that occurs, in many cases, through mitogen-activated protein (MAP) kinases (Shoresh et al., 2010). Two principal pathways for effects on plants are the ISR and SAR mentioned above. ISR uses jasmonic acid as a principal signaling molecule, while SAR relies on salicylic acid. There is a considerable amount of cross-talk between these two pathways, and the interactions and triggering systems have been provided diagrammatically elsewhere (Harman et al., 2004a; Pieterse et al., 2014).

As indicated in those and other references, wounding of plants by injury or insects, infection by pathogenic microorganisms, and especially the effects of endophytic beneficial organisms all result in induction of beneficial systemic effects as does the alleviation of the negative effects of abiotic stresses.

The signaling compounds produced by beneficial organisms are numerous and varied. The primary elicitors for Rhizobiace and AMFs are chitooligosaccarides, and these are part of the common symbiotic pathway (Gough and Cullimore, 2011; Genre and Russo, 2016). The elicitor in *P. indica* is cellotriose (Johnson et al., 2018). *Trichoderma* uses a number of different compounds that trigger systemic activity including sulfur-rich proteins including SM1 (Djonovic et al., 2007) and certain hydrophobins (Ruoccco et al., 2015). These proteins contain a hydrophobic and a hydrophilic portion that enables them to insert themselves into cell membranes. These elicited responses frequently act by signal transduction (Shoresh et al., 2006).

Surfactins from *Bacillus subtlis* also have hydrophobic and hydrophilic portions of the molecule, and they too induce systemic reactions in plants. Both can be characterized by Ca^{++} bursts in the plant cells to which they are applied (Jourdan et al., 2009). There may be small-molecular-weight volatile compounds such as 1-octen-3-ol (Harman et al., 2018) and 6-pentyl-α-prone (Lorito et al., 2010). Other non-volatile small-molecular-weight compounds such as koningic acid, also known as heptelidic acid, act as elicitors of systemic plant responses (Lorito et al., 2010) is required for the systemic response of *T. virens* (Djonovic et al., 2007). Any of the

small molecular compounds used alone can elicit systemic responses in plants (Harman and Uphoff, 2019).

Interaction of SAMPs With Cellular Membranes

Signal transduction leads to systemic responses in plants (Ward and Schroeder, 1977; Shoresh et al., 2006). The amphoteric molecules (those with both hydrophobic and hydophilic regions) such as the proteins and surfactins just mentioned interact with cellular membranes through the production of pores in the membranes that open channels for ion influx and efflux and also trigger Ca^{++} bursts and H_2O_2 production that are characteristic of resistance responses.

This in turn leads to signal transduction that leads to systemic responses (Shoresh et al., 2010). This transduction is indispensable for the systemic response that EPHs induce, eliciting resistance to biotic and abiotic stresses. Signal transduction is induced by the interactions of SAMPs produced by the microorganism, as described earlier. This in turn leads to signal transduction which leads to systemic responses (Shoresh et al., 2010). This transduction is indispensable for the systemic response that EPHs induce, eliciting resistance to biotic and abiotic stresses. Signal transduction is induced by the interactions of SAMPs produced by the microorganism, as described earlier (Harman and Uphoff, 2019). In addition to the SAMPs described in that reference, plant hormones such as salicylic acid and jasmonic acid can act a elicitors of plant responses, In addition, the methylated forms can also be involved in systemic transport throughout the plant (Morita et al., 2009; Shah and Zeier, 2013).

Signal Transduction and Systemic Changes in Plant Genes and Proteins

As mentioned above, systemic changes in plant gene and protein expression require signal transduction, at least in some cases, through the action of MAP kinases (Shoresh et al., 2006). This results in changes in plant genes and proteins, frequently as up-regulation. There are many thousands of proteins and genes that are known to be up-regulated. For example, a study of microbial inoculation of rice identified 2,414 transcripts that were differentially expressed in plants whose roots had been endophytically colonized by *Trichoderma*, thereby affecting plant growth and health (Doni et al., 2019a; Harman et al., 2019). These differently-expressed genes included genes directly involved in photosynthesis (335 transcripts), in the synthesis of photosynthetic products, and in the protection and maintenance of cellular functions (Doni et al., 2019a; Harman et al., 2019). Other examples are provided in section 8.

An important consideration in this up-regulation are the energy costs to the plant. The pathways involved require both energy and resources such as fixed carbon, nitrogen, and other components that also are necessary for plant growth and development (Shoresh and Harman, 2008). As part of meeting the needs for both plant growth and effective responses to stresses, and to economize on their expenditure of resources, plants employ a process called gene priming. In this process, a stimulus such as either stress or a response to plant endophytes induces an initial response in the plant which then establishes processes whereby proteins and other elements can be produced more rapidly and in larger quantity in the future. A plant that is primed by previous experience or by induced resistance can make responses that are quicker and stronger than would otherwise occur (Conrath et al., 2015).

This process may take the form of some modification of histones surrounding DNA or DNA methylation which are part of the plant's regulatory machinery (Jaskiewicz et al., 2011a,b). These changes may be long-lasting and may even persist in the next generation. They are an efficient mechanism to minimize the cells' energy costs while still permitting rapid responses by the plant to stressors or other demands, while preserving energy and resources for plant growth when these are not required by environmental or other internal requirements (see section 5). This reprogramming of gene expression is shown pictorially (**Figure 2**).

BENEFITS CONFERRED ON PLANTS

Control of Biotic Stresses Including Diseases, Insect Pests, and Nematodes

Conventional agriculture practices use chemical biocides in controlling plant diseases and pests. Unfortunately, this approach induces resistance in pathogens and pests during long-term use (Mahmood et al., 2016). The excessive use of biocides in agriculture can also cause undesirable effects on humans and the natural environment (Heong et al., 2015). The use of EPHs is an underutilized opportunity for sustaining high agricultural production with lower negative impacts (Harman and Uphoff, 2019). Several endophytes belonging to different genera are involved in eliciting induced systemic resistance in plants and reported to be an effective tool for the biological control of certain plant pathogens and pests (Compant et al., 2019; Doni et al., 2019a).

The development of high-throughput molecular techniques such as proteomics, genomics, metagenomics and metabolomics for microbe isolation and characterization have allowed for the identification of a variety of bacteria and fungi able to act beneficially when proliferating in the soil for disease control and plant-growth promotion. Multi-omics has also advanced our ability to strategically select for microorganisms that have unique host site-specific qualities that function in biological disease or pest control (Bonanomi et al., 2018).

P. roqueforti and *T. reesei* produced numerous substances that had broad-spectrum activity against several bacterial plant pathogens, as well as antioxidative compounds (Ikram et al., 2019). *P. citrinum* and *A. terrus* are endophytes that secrete gibberelins were evaluated for the ability to protect against stem rot caused by *Scleerotium rolfsii*. These endophytes reduced disease. The authors concluded that the endophytes reprogramed the plants involved in systemic plant defense reactions (Waqas et al., 2015).

Application of microbes as biocontrol agents in the field can have many benefits without requiring extensive investment in plant breeding and genetics (Harman et al., 2019).

These beneficial microbes can provide benefits through various mechanisms such as competing with pathogens or by directly antagonizing plant pathogens by producing certain antibacterial or antifungal compounds (Ab Rahman et al., 2018); induction of plant genes and biochemical pathways that provide greater resistance to disease and pest through induced systemic resistance (Harman et al., 2019); modulation of the signaling and metabolism of reactive oxygen species (ROS) as a key mechanism to counteract microbial antagonism (Nath et al., 2016); changing microbial balance in the rhizosphere in favor of beneficial microorganisms, thus suppressing a broad spectrum of diseases and pests (Siddiqui, 2005).

The *Trichoderma* spp. controls plant diseases through various mechanisms, e.g., mycoparasitism and production of antibiotics. However, more recent studies have indicated that a primary method of pathogen control occurs through the ability of the *Trichoderma* spp. to reprogram plants' gene expressions so as to lead to the induction of systemic resistance (Shoresh et al., 2010; Poveda et al., 2019). For example, *T. asperellum* has been able to control white rot disease caused by *Sclerotium cepivorum* in onion plants by up-regulating the expression of biocontrol-related genes such as *AcPR1, AcPAL1, AcLOX1*, and *AcEIN3* (Rivera-Mendez et al., 2020).

The ability of *T. asperellum* to control white rot is only a subset of its capabilities. They can control disease, and have other benefits, including enhancement of growth, yield, and physiological parameters (Rivera-Mendez et al., 2020). Moreover, a study in inoculated rice seedlings revealed that *T. asperellum* can up-regulate numerous genes that were involved in plant defense responses and in systemic acquired resistance (SAR) signaling (Doni et al., 2019a). An abundance of up-regulated genes that relate to defense response and SAR signaling is an indication of greater plant ability to resist biotic stresses.

Some endophytic fungi, particularly *Beauveria bassiana*, have demonstrated antagonistic activity against numerous insect pests and arthropods (Mascarin and Jaronski, 2016). A recent study has indicated that *B. bassiana* could endophytically colonize pecan seedlings by seed soaking, seed coating, and soil drenching. The establishment of endophytic *B. bassiana* in pecan plants led to a protection against two pecan aphids in particular, namely *Melanocallis caryaefoliae* and *Monellia caryella* (Ramakuwela et al., 2020).

Moreover, the presence of *B. bassiana* in maize plants has reduced the survival and fecundity of the aphid *Sitobion avenae*, an important pest in agricultural systems (Mahmood et al., 2019). It has been suggested that protective alkaloids developed by the plant in response to endophytic fungal colonization could play an important role in protecting plants against chewing insects (Gange et al., 2019). In addition to producing protective alkaloids, endophytes promote plant immunity against chewing insects by enhancing endogenous defense responses mediated by the jasmonic acid (JA) pathway (Bastias et al., 2017).

Arbuscular mycorrhizal fungi (AMF), which are endosymbionts, are effective biocontrol agents against several soil-borne plant pathogens. AMF are obligate root symbionts that can offer many benefits and protect their host plant against pathogen infections (Schouteden et al., 2015). For example, the biocontrol efficacy of AMF against common bean root rot caused by *Fusarium solani* f. sp. *Phaseoli* has showed that plants grown with symbiotic AMF exhibited greater resistance against the pathogen compared to untreated plants (Eke et al., 2016).

The results also revealed that total soluble phenols, flavonoids contents, and the defense enzyme phenylalanine ammonia lyase (PAL) were increased with AMF colonization, indicating an enhancement of the plant immune system against *F. solani* f. sp. *Phaseoli* (Eke et al., 2016). A transcriptomic analysis of soybean plantlets in symbiosis with AMF versus non-AMF soybean plantlets following infection by *F. virguliforme* showed a comprehensive view of changes in gene expression, confirming an alteration of gene-expression patterns by AMF. There were 1,768 differentially expressed genes (DEGs) in the AMF-colonized plants compared to 967 DEGs in the noncolonized plants. Major transcriptional changes corresponded to defense response-related genes belonging to secondary metabolism, stress, and signaling categories (Marquez et al., 2019).

Finally, several plant growth-promoting bacteria (PGPR) also possess traits that make them well suited as biocontrol and growth-promoting agents. As a consequence, plants treated with PGPR may be larger and healthier and have greater yields than plants without such treatment (Mhatre et al., 2019). *Pseudomonas* and *Bacillus* species are two PGPR species that are capable of protecting *Solanum lycopersicum* against bacterial wilt disease. These PGPRs produced biocatalysts for plant-growth promotion and regulated pathogen-related genes such as *PR-la* and *PAL* (Durairaj et al., 2018).

Canola plants inoculated with *P. chlororaphis* showed greater protection against the necrotrophic fungus *Sclerotinia sclerotiorum*. The *P. chlororaphis* treatment resulted in structural and metabolic changes, and it prevented the accumulation of ROS in the leaf of canola plants. Global transcriptional analysis showed a significant up-regulation of genes associated with SAR mechanisms, ROS signaling, and chloroplast integrity (Duke et al., 2017). In addition, *P. chlororaphis* was capable of suppressing *S. sclerotiorum* by secreting the antibiotics pyrrolnitrin and phenazine, degradative enzymes, and siderophores (Nandi et al., 2017).

Microorganisms have developed mechanisms that increase their host fitness and provide a more stable ecological community by working effectively against a broad spectrum of pathogens and pests and for alleviation of the adverse effects of plant stress, which is discussed in the next section.

Alleviation of Abiotic Stresses

On a daily basis, plants are exposed to numerous harmful environmental conditions that significantly affect their growth (Shahid et al., 2018). EPHs are reported to have substantial impacts on plants by enabling plants to grow consistently faster and more uniformly whether the stress was drought, salt, cold or flooding (Cheng et al., 2019). Over the years, some EPHs, especially fungal endophytes, have been widely regarded to be an important tool that can mitigate plant stress through various cellular plant processes (Harman, 2011; Rodriguez et al., 2019).

Drought Stress

Drought stress, one of the most destructive abiotic stresses, has increased in intensity over the past decades, endangering the world's food security. Currently, 90% of global water consumption is used for agriculture, and water constraints are expected to intensify in the future (Huang et al., 2019). Numerous microbes can confer fitness benefits to plants, including increased tolerance to drought stress (Rodriguez and Redman, 2005).

There are various agronomic mechanisms that explain microbes' contributions to reducing the impacts of drought: alterations in root architecture that result in improved water use efficiency; increases in the synthesis of osmolytes, particularly proline; increases in antioxidant enzymes that scavenge for ROS; manipulation of phytohormones; and modifying plant gene regulation (Ngumbi and Kloepper, 2016).

Root colonization by *Pseudomonas chlororaphis* induces systemic drought tolerance in *Arabidopsis thaliana* by increasing transcript accumulation from genes associated with defense, response to ROS, and phytohormone-responsiveness, accompanied by decreases in transcription factors associated with ethylene and abscisic acid signaling (Cho et al., 2013). *P. simiae* promoted growth and protected soybean plants from damage from drought stress, mainly manifested as changes in the gene expression profiles of drought stress-responsive genes such as *DREB2A, EREB, GOLS* and *P5CS*, and secondarily as modulation of phytohormonal signaling altered plant physiology and morphology (Vaishnav and Choudhary, 2019).

Inoculation of wheat with *Trichoderma* enhances drought tolerance through induced changes in stomatal conductance, net photosynthesis, and chlorophyll fluorescence. The inoculation also decreases proline, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2), while increasing total phenolics and plant capacity to scavenge ROS (Shukla et al., 2015). *Trichoderma* inoculation in rice plants results in significant increases in plant height, total dry matter and chlorophyll content (Doni et al., 2014). Other studies have demonstrated the ability of *Trichoderma* to improve drought tolerance in rice by modulating proline, superoxide dismutase, and lipid peroxidation products and altering the transcript level of dehydrins-related genes such as *LOC_Os01g50700, LOC_Os02g44870*, and *LOC_Os11g26750* (Shukla et al., 2015). Further, *Trichoderma* has improved the ability of tomato plants to neutralize damaging ROS when exposed to a water deficit. The ability of tomato plants to protect themselves from oxidative damage was accompanied by the up-regulation of genes encoding antioxidant enzymes that significantly reduce the glutathione and ascorbate contents (Mastouri et al., 2012).

The application of *Alternaria*, a fungal endophyte, conferred drought tolerance on tomato plants. Endophyte-colonized plants exposed to drought had higher root and shoot biomass, better water-use efficiency, and higher photosynthetic efficiency, and they also recorded lower ROS than non-colonized plants (Azad and Kaminskyj, 2016). Moreover, application of *Curvularia protuberata* has been found to reduce rice plants' water consumption by 20–30% and increase their growth rate, reproductive yield, and biomass when exposed to drought stress (Redman et al., 2011). These different findings indicate that symbiotic endophytes can be useful in mitigating the impacts of drought stress on plants, which will unfortunately increase with climate change.

Salt Stress

Salinity is another major abiotic stress that limits the growth and productivity of plants in arid and semiarid environments (Thu et al., 2017). To cope with salt stress, plants deploy a variety of traits to control cell functioning and development that rely on signal perception, signal integration, and processing plant systems (Hanin et al., 2016). ROS are overproduced in plants when they are exposed to abiotic stresses such as salinity. However, we must bear in mind that despite their toxicity, ROS are also regulators of growth, development, and defense pathways, so their elimination is not desirable (Farooq et al., 2019). Balancing the generation and reduction of ROS is a crucial process in plants (Rodriguez and Redman, 2005).

Many studies have reported microbes' abilities to induce salt stress in plants by modulating ROS production (Harman and Uphoff, 2019). Inoculation of chickpea plants with *Bacillus subtilis* under saline conditions increased plant biomass and photosynthetic pigments, while reducing the levels of ROS and lipid peroxidation. *B. subtilis* has also been found to decrease Na accumulation while enhancing the N, K, Ca, and Mg content in the plants (Abdallah et al., 2018). *B. amyloliquefaciens* inoculation of rice plants under salt stress increased rice plant growth and the expression of several stress related genes such as *NADP-Me2, EREBP, SOSI, BADH, SERK1, GIG*, and *SAPK4* (Nautiyal et al., 2013).

In another study, barley plants previously inoculated with *Piriformospora indica* have been shown to increase resistance of salt stress. *P. indica* appeared to confer tolerance to this stress, at least partly, through the up-regulation of ascorbate and antioxidant enzymes (Baltruschat et al., 2008). Similarly, colonization of tomato plants by *P. indica* increased the expression of an important gene in leaves, namely LeNHX1, one of a family of genes responsible for removing sodium from cells. Further, *P. indica* also increased levels of antioxidant enzyme activity, offering further protection (Abdelaziz et al., 2019).

Enterobacter spp. have improved vegetative growth and alleviated salt stress in tomato plants by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole-3-acetic acid (IAA) which resulted in increased fresh weight, dry weight, and plant height. At the molecular level, treatment with *Enterobacter* spp. has increased the expression of salt-stress-related genes such as *P5CS1, P5CS2, MPK3,* and *MPK6* (Kim et al., 2014). *Dietzia natronolimnaea STR1* protected wheat plants from salt stress by stimulating the expression of *TaST* (a salt stress-induced gene), and *SOS1* and *SOS4* (salt-sensitive-pathway-related genes), accompanied with enhanced gene expression of various antioxidant enzymes such as *APX, MnSOD, CAT, POD, GPX*, and *GR* (Bharti et al., 2016).

Y. lipolytica minimized the effects of salt on corn growth and development through the plant hormones absicic acid and indole acetic acid, indole-3-acetmimide and flavonoids. Treated plants had higher levels of chlorophyll, carotenes, and the antioxidants perioxidase and catalase (Farzana et al., 2019).

Cold and Heat Stress

Cold stress also severely hampers the reproductive development of plants and will result in significant agricultural losses. The major negative effect of cold stress is that it induces severe membrane damage in plants (Yadav et al., 2010). To improve cold stress in plants, cold-tolerant microorganisms such as *Trichoderma* spp., *Burkholderia* spp., and *Pseudomonas* spp. are usually used (Mishra et al., 2011). Inoculation of *B. phytofirmans* increased grapevine growth and starch, proline, and phenolic contents at a low temperature (Barka et al., 2006). With wheat plants, bacterization with *Pseudomonas* spp. under cold-induced stress significantly increased growth, total chlorophyll, anthocyanin, total phenolics, and starch content, and decreased the Na^+/K^+ ratio, and electrolyte leakage compared to non-bacterized control (Mishra et al., 2009).

Pseudomonas spp. were also reported to mitigate impacts of cold stress in tomato plants by reducing membrane damage and ROS levels, improved antioxidant activity in leaf tissues, and altering expression of cold acclimation genes *LeCBF1* and *LeCBF3* (Subramanian et al., 2015). Furthermore, endophytic *T. harzianum* increased the tolerance of tomato plants under chilling stress by reducing the lipid peroxidation rate and electrolyte leakage while increasing leaf water content and proline accumulation. *T. harzianum* was also able to alter the expression of transcription factor *NAC1* and dehydrin *TAS14* genes, leading to cold tolerance in tomato plants (Ghorbanpour et al., 2018). *A. niger* enhanced heat tolerance of soybean and sunflower by control of ROS and peroxidative damage. This organism also increased plant growth and enhanced chlorophyll levels (Ismail et al., 2020).

Flooding Stress

During complete submergence, plants experience a strong decline in their photosynthesis rate and carbon availability. This is due to a lack of light or a reduced rate of CO_2 diffusion, and to impaired respiration through reduced O_2 availability (Perata et al., 2011). To survive in such conditions, microorganisms come up with a variety of physiological, biochemical, and molecular mechanisms to protect plants from this adverse condition.

Beneficial microorganisms induce plant growth and counteract flooding stress through production of ACC deaminase, discussed in section "salt stress." Under flooded conditions, plant roots become hypoxic. In response to oxygen-limitation, the enzyme ACC synthase is synthesized in roots. Because there is limited oxygen, ACC cannot be converted into ethylene. Subsequently, the unused ACC is transferred to the shoots where oxygen is available, and ACC can be converted into ethylene. However, the overproduction of ethylene by plants results in their wilting, necrosis and chlorosis.

One way that beneficial microorganisms contribute to mitigate flooding-stress conditions is by their producing ACC deaminase that can convert ACC into α-ketobutyrate and ammonia, thus reducing the levels of ethylene (Tewari and Arora, 2016). In addition, beneficial microorganisms such as *Klebsiella variicola* was reported to mitigate the effect of flooding stress in plants by increasing plants' adventitious roots. An increase in adventitious root growth encourages plant survival when the level of oxygen in the root zone is decreased (Kim et al., 2017). Maintaining proper shoot and root development especially under flooding stress is very essential for sustainable growth and development of plants. This is just one of many well-documented mechanisms by which the symbiotic relationship between plants and microorganisms works to the advantage of both.

Alleviation of Environmental Pollutants

One other stress that is alleviated by microorganisms is heavy metal stress. *Staphlococcus arlettae* alleviated chromate toxicity to sunflowers by suppressing the uptake and reducing the hexavalent form to the less toxic trivalent form (Qadir et al., 2020). Beneficial effects do not always occur. In commercial plantings of poinsettia, a report was received that said that in the presence of *T. afroharzium* T22, all the plants died, while in its absence, plants were healthy. It was discovered that the medium in which the plants were grown was sewage sludge with high levels of chromium. *T. afroharzianum* increased uptake of the element to toxic levels (observations of the first author).

The ability of a gibberellin-producing endophytic strain of *Penicillium janthinellum* in a tomato with tolerance to aluminum toxicity. Plant cell membranes were less damaged by application of the endophyte or exogenous gibberellic acid. Salicylic acid was upregulated by the presence of the fungus and It was concluded that either application of gibberellin or the endophyte counteracted the adverse effects of aluminum toxicity (Khan et al., 2015).

Amelioration of other environmental toxicants may be accomplished by the use of endophytic fungi. Cyanides are frequently present in mine tailings. Endophytic *T. afroharzianum* is able to take up and degrade metallocyanides. The polyphenols are toxicants produced in olive-processing systems and are toxic. If olive waste water is diluted and aerated, *Trichoderma* spp. degraded the polyphenols and appear to be a useful method to cleanse olive oil waste waters (Harman et al., 2004b).

Soils polluted by oily wastes are a serious problem. Researchers in Canada utilized *Pseudomonas* strains including *P. putida* which colonize plant roots. Treatment with the bacteria enabled plants to grow through enhancing stress resistance by mechanisms already described. This permitted roots to grow and provide a habitat for other microorganisms to proliferate. These other microflora degraded the hydrocarbons and thereby removed the pollution problem (Gurska et al., 2009). This system has been used commercially in Ontario. Research by the first author has been able to achieve this amelioration of oil pollution of soil by the use of *T. afroharzianum*.

Improvements in Internal Cellular Environments Allowing Efficient Functioning

An important component of higher-level functioning leading to EPHs is the maintenance of internal cellular processes critical for plant growth, development and induced resistance to both biotic abiotic stresses is the avoidance of damaging ROS by gene and protein upregulation of the pathways for alleviating and mitigating their damaging effects (see the preceding divisions

of this section). This is a feature of many endophytic microorganisms discussed in this paper. Earlier in this review, we discussed the damage that ROS cause to cellular functioning, and these are described in (Nath et al., 2016). Since the mid-90s, many microbes have been shown to protect plants from ROS. These include mycorrhizae (Mo et al., 2016), *P. indica* (Sherameti et al., 2008), *Trichoderma* (Sherameti et al., 2008; Mastouri et al., 2012), and *Azospirillum* and *Rhizobium* (Fukami et al., 2018). In all of these cases, plants under stress were protected from damage against ROS. A very strong evidence of the capability of *Trichoderma afroharzianum* was provided by experiments in which tomato seedlings were exposed to the herbicide Paraquat™, which kills plants by inducing very strong ROS production to lethal levels. Inoculation of the seedlings overcame this damage (Mastouri et al., 2012). Alleviation of ROS damage in plants is accompanied by overexpression of the genes and proteins involved in managing ROS stress and was described in the earlier divisions of this section.

Thus, these endophytes are able to create an internal environment that maintains a cellular environment that is conducive to efficient operation of photosynthesis and all the other metabolic processes necessary for plant growth and amelioration of stresses. We have coined the term Optimized Internal Redox Environment (OIRE; Harman and Uphoff, 2019). This is a very important component of the beneficial effects of these beneficial organisms.

Improvements in Photosynthesis

The previous section described maintenance of cellular functions, including photosynthesis. However, photosynthesis is actually increased and improved by endophytes, which contribute to formation of EPHs. Within the genus *Trichoderma*, the presence of endophytes increases levels of photosynthetic pigments, which increase levels of proteins critical to photosynthesis (De Palma et al., 2016; Vitti et al., 2016; Pelhivan et al., 2017; Fu et al., 2018). Such changes are not restricted to *Trichoderma; Rhizobium* also demonstrates such effects (Chi et al., 2005). These beneficial effects are documented more fully in a paper devoted to this topic (Harman et al., 2019). Enhancement of photosynthesis is a very important attribute of endophytes that increase plant growth and limit adverse effects of biotic and abiotic stresses and that contribute to the formation of EPHs.

AGRICULTURAL AND SOCIETAL BENEFITS

Meeting Food Demand and Reducing World Hunger

The global population is increasing rapidly and is predicted to be roughly 9 billion by the middle of this century. Concurrently, global agriculture is facing increasing competition for land, water, and energy in food production (Godfray et al., 2010). At the same time, the agricultural sector must cope with increasing temperatures, more erratic rainfall, more frequent droughts, rising sea-levels due to melting snow, and pest and pathogen pressure in crops around the globe (Wheeler and Braun, 2013). Importantly, despite a doubling of the global population in the past half century, the extent of global hunger has been reduced (Godfray et al., 2010). This was mainly due to contributions from the Green Revolution which increased crop productivity by the introduction of high yielding varieties, new irrigation schemes, and a broad range of agrochemical inputs to supply nutrients and manage pests, weeds, and pathogens. However, negative impacts of the Green Revolution started coming to the surface at the latter decades of 20th century in terms of soil degradation, environmental imbalance, and the emergence of chemical-resistant biotypes in pests, pathogens, and weeds.

In any case, global food requirements in the 2050s are expected to be double those of 2005. Unfortunately, annual productivity gains have been decelerating over the past two decades, and crop production area has been decreasing due to industrialization, desertification, and urbanization, which further constrains future food production. Therefore, there is urgent need for new initiatives to mitigate these challenges to the global food supply. Much evidence shows that careful selection and augmentation of efficient soil microbial strains is a potentially sustainable solution for raising agricultural production and reducing hunger globally. As mentioned previously, soil microbes play vital roles in helping crops to cope with multiple stresses induced by climate change such as higher temperature, more flooding and drought, and containing pests and pathogens. Soil microbes can also help to rehabilitate degraded soils by improving soil nutrient availability, suppressing soil pathogens, cleansing the soil of pollution and heavy metal contaminants, and preventing soil erosion.

Environmental Benefits

Long-term monoculture production systems are one of the major factors contributing to soil degradation and contamination with inorganic pesticides, chemicals, and herbicide. Depending upon their concentration, soil contaminants can have destructive consequences on soil ecosystems, on the abundance and diversities of soil microbial communities, and on soil health. Soil microorganisms are not only crucial in crop production and soil health, but are also becoming important tools for cleaning up environmental pollutants (Morris et al., 2011). The process is known as bioremediation, where microorganisms are enlisted for their ability to degrade harmful chemicals and heavy metal contaminants in the soil by changing them into less harmful materials. Bioremediation using soil microbes is achieved by augmenting populations of selected microbial strains with high degradation abilities at the site of contaminant.

Several beneficial microbes have been reported to have high bioremediation potential for chemical pesticides, petroleum hydrocarbons, and heavy metal contaminants in the soil. Microbial services for soil rehabilitation are less costly, easier to employ, and more environmentally friendly than other methods now used such as the "muck, suck, and truck" approach. Bacterial and fungi species employed for bioremediation include *Trichoderma* species which are capable of degrading a wide range of polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, phenanthrene, chrysene, pyrene, and benzene (Zafra and Cortés-Espinosa, 2015; Malla et al., 2018).

Alleviation of Greenhouse Gases

It is well-established that microorganisms play critical roles in determining the concentrations of greenhouse gases such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) in the atmosphere (Singh et al., 2010). Furthermore, use of chemical fertilizers in agriculture increases the greenhouse gas emission in three ways: (i) a huge amount of fossil fuels are utilized in the production of chemical fertilizers which is responsible for the emission of GHGs; (ii) fossil fuels are also used for the transportation and application of fertilizers and pesticides; and (iii) chemical fertilizers provide the substrates for methanogenesis and denitrifying bacteria which are responsible for the emission of methane, carbon dioxide, and nitrous oxide from soil into the atmosphere.

On the other hand, the application of beneficial microbes as biofertilizers, bio-stimulants, or biocontrol agents reduces the need for agrochemicals including fertilizers. Also, it reduces the habitat and sustenance for methanogens and denitrifying bacteria by competition for space, nutrition, and ecological niches in the soil, thereby reducing microbial sources of GHG emissions. Furthermore, if beneficial microbes such as *Trichoderma* are combined with conservation agriculture practices such as minimum tillage and with SRI as discussed in the next section, emission of GHGs can be reduced at the same time agricultural production is enhanced (Hawken, 2017).

For example, minimum tillage operations favor microbial communities dominated by fungi which decrease the microbial decomposition and respiration induced by soil disturbance during plowing. It has been reported that this could lead to sequestration of as much as 55 Pg organic carbon in the surface soil (Conant et al., 2003; Singh et al., 2010). SRI promotes alternate drying and wetting practice in paddy irrigation instead of continuous flooding, which is the usual practice in conventional rice farming. Continuous flooding supports the methanogenic activity of bacteria which emit large amounts of methane gas into the atmosphere. Flooded paddies are major contributors to GHG emissions from agriculture which account for around 11% of global anthropogenic methane emission (Stocker et al., 2013).

In China, a microbial preparation used with tea plants in their cultivation reduced emissions of N_2O by 33.1–71.8%, while yield was increased by 16.2–62.2% relative to standard use of synthetic N fertilizers. These indicated once again that microbial enhancement of crop holobionts can reduce GHG emission from agriculture with favorable impact for less global warming with a gain rather than a sacrifice in production.

MANAGEMENT AND DELIVERY SYSTEMS FOR EPHs

The previous sections review the potential of various microorganisms to improve plant productivity. However, results may be inconsistent and suboptimal unless the organisms are properly used. This includes appropriate means of delivery to maximize and ensure that the organisms are able to colonize, protect and enhance plant growth and development. In addition to delivery systems, the way that microbial formulations are assembled and delivered can make a large difference in whether or not the organisms are able to provide the benefits anticipated. These are formidable challenges for their successful use.

Delivery

The major challenges to be dealt with in the commercialization of microbial agents for improving crop production are inconsistent efficacy of the biological material, having a delivery system that can maintain the viability of the material, and ensuring appropriate physiological conditions of the microbial inoculants during transportation and handling (Arora et al., 2016). For the success of any microbial-based products in agriculture, selection of suitable delivery systems and formulation processes are equally important as the selection of effective microbial strain(s) (Zayed, 2016).

Based on considerations of survivability, mode of action, and local climatic conditions, there are several types of delivery systems for microbial inoculants. These can be directly applied to seeds by seed inoculation, to the roots by root dip methods, by drenching of young plants, to the plant and soil through drip irrigation or flooding, or to growing plants by foliar spray (**Figure 3**; Gašić and Tanović, 2013).

The basic characteristics of an effective delivery system include easy mode of delivery; enhancement of target activities with effective concentration; little or no ecotoxicity; time controlled release; solubility, stability, and effectiveness (Vejan et al., 2019). In the last several decades, exogenous application to plants, seeds or soil with microbial products has mostly been in the forms of powder, granules, slurry, or liquid. The dispersal system should provide the nutrients necessary for the microbial strains and protect them from desiccation, supporting release of the microbial cells as soon as this is required (Schoebitz et al., 2013).

Previously, the peat, clay, and liquid-based delivery systems were well-established; however, peat and clay-based carriers have proven difficult to sterilize, and they have a high chance of external contamination, plus low stability and viability during storage and handling. Similarly, liquid-based formulations may not be sufficiently protective to bacterial products during storage, transportation, and application into the soil. Stable liquid formulations are easier to achieve with spore-formers such as fungal spores and with gram-positive bacteria that form endospores; they are more difficult with gram-negative bacteria that lack resistant spore types.

Commercial practice usually requires that preparations have at least a year of shelf life without refrigeration or other special handling. Furthermore, suspension-based inoculations have several other demerits, such as settling of the microbial inoculants, blockage of spray nozzles during the application, and abiotic stresses affecting the viability of the spores (Bateman et al., 2007). It is essential to prepare formulations that meet the criteria of shelf life and ease of use if microbial formulations are to be accepted in large-scale commercial agriculture.

Recent advances in science have focused on developing various encapsulation technologies to deliver the microbial products more effectively and cheaply in the farming system. The processes of lengthening the cell viability of microbes by

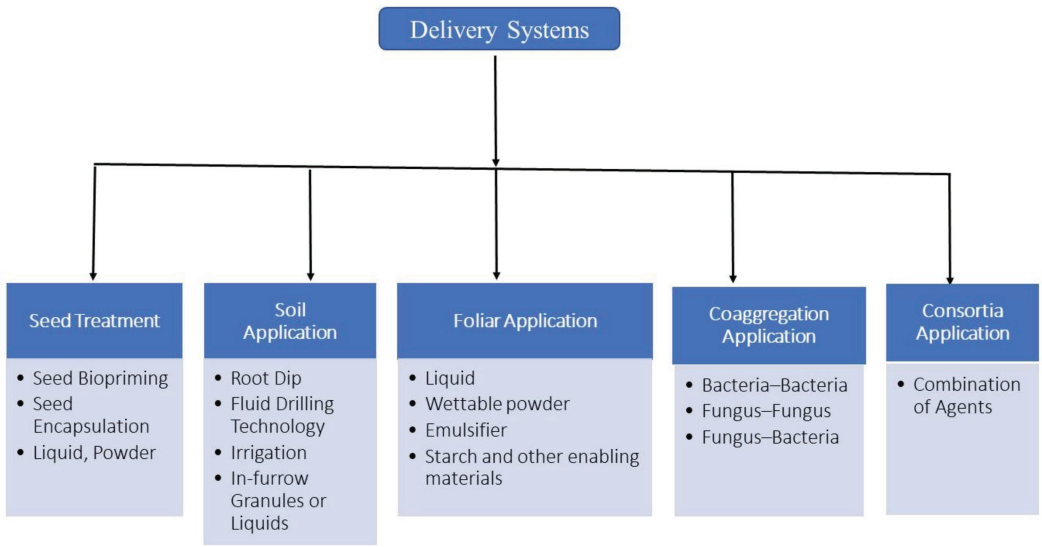

FIGURE 3 | A diagram of different delivery systems employed in agriculture.

entrapping or coating them within polymeric materials that are permeable to nutrients, gases and metabolites is known as encapsulation. Encapsulation has been divided into two categories based on the bead size: (i) macro encapsulation (a few mm to cm), and (ii) microencapsulation (1–1,000 μm; John et al., 2011).

Synthetic polymer, biopolymer, and different kinds of organic/inorganic materials are used in encapsulating microbial cells for agricultural use. The three major benefits related to encapsulation technology compared to the traditional delivery system include: (i) enhanced efficacy due to increased surface area; (ii) enhanced microbial activity due to higher penetration efficiency; and (iii) higher dispersion on plant surfaces due to smaller particle size (Sasson et al., 2007). Encapsulation may also increase stability and shelf life and may be essential if non-spore forming organisms are used (Harman and Custis, 2006).

Bioformulation improves the performance of microorganisms by preserving them and delivering them to their targets more reliably. (Stephens and Rask, 2000) proposed that good microbial carriers are characterized by their effectiveness in supplying the right number of viable cells in excellent physiological conditions, and at an appropriate stage or time. The carrier materials that increase the storability of the products generally include fine clay, peat, talc, lignite, alginate, polyacrylamide beads, starch, diatomaceous earth, vermiculite, cellulose (carboxymethyl cellulose), and polymers, especially xanthan gum (Digat, 1991; Harman and Custis, 2006). Furthermore, in various local conditions, charcoal, farmyard manure, vermicompost, compost, bagasse, press mud, and sterilized grains such as rice, corn, millet and barley have also been successfully used as carriers for different kinds of microorganisms (Connick et al., 1991; Schisler et al., 2004; Pitt and Hocking, 2006; Dorner, 2009; Harman et al., 2010).

Microbial inoculants can be formulated as dust, seed dressing powder, micro-granules, water dispersal, wettable powder, emulsifiers, suspension concentrates, oil dispersion, emulsions, capsule suspension, and ultra-volume formulations (Knowles, 2006). Granular formulations protect microorganisms from ultraviolet light. A recently-developed soybean oil-based formulation increases the half-life of the conidia and the performance of *T. asperellum*. The oil-based formulation has more benefits than a water-based formulation because it remains in contact with the plant surface for a longer time, evaporates much less, and can also be applied as an emulsion (Batta, 2007).

Some researchers (Xue, 2002) have expressed concerns regarding inconsistent results from the use of single strains and have proposed using multiple strains or consortia of microbes, so there has been interest among researchers in using multiple species or strains of beneficial microbes in aggregation or in consortium form to exploit the potential synergy between them. Bioformulation based on consortia of beneficial microbes should have more impact in farming systems due to utilization of all possible mechanisms of action inherent in the different strains within the consortium. Application of combinations of strains resulted in higher levels of disease suppression compared to applying individual strains (Meyer and Roberts, 2002; Roberts et al., 2005).

In the senior author's experience, single strains or at most three strains can be effective if the strains are carefully selected, if the formulations are appropriate, and if they are used with appropriate management systems. Limiting the number of strains is essential if registration is required, since most registration requirements include toxicology evaluations that are very time-consuming and expensive. Full registration packets typically require several tens of thousand dollars for each strain, and if multiple strains are used the total cost can exceed a million dollars. Regardless of the strain(s), delivery system, or formulation used, it is essential that any product or strategy be tested rigorously to ensure reliable results in the field.

Application of Exogenous Inoculum

Obviously, the selection of effective microbial strains is critical in bio-based product development. These strains should be sufficiently competitive with indigenous soil microbial populations to survive, as well as being compatible with other inoculants and native microorganisms along with their colonization efficiency in plant rhizoplane (Berendsen et al., 2012). It is well-established that exogenous inoculation of beneficial microorganisms in soil increases plant growth, promotes plant nutrient uptake, enhances plants' tolerance to biotic and abiotic stresses, and suppresses plant diseases (Roiger and Jeffers, 1991). Due to the dynamic and self-sustaining propensities of microorganisms, many strains or species can establish themselves well in the soil so that there would not be a need for repeated applications (Lucas et al., 1990).

As mentioned already, microbial species and strains have differential potentialities to serve the plant communities within and with which they reside, utilizing different mechanisms and colonizing different niches in the rhizosphere and rhizoplane regions. Many will inhabit the plants' endospheres. The carefully-selected application of microbial strains can fill vacant niches in the soil and establish communication with plants and other strains differently than do the indigenous microbes that are already present in the soil. Some microbial strains can vigorously thrive in more variable environments than can others. These can quickly capture spaces and nutrients in the plant rhizosphere, making these resources unavailable to potential pathogenic invaders.

The effects from exogenous application of plant microbes are not always consistent across fields and crop species. However, research has been undertaken focusing on how to prepare and adapt the rhizosphere environment for colonization of specific exogenously-applied microbial strains by rhizosphere engineering (Ryan et al., 2009), and on how to deliver them more effectively for establishment. Plant microbial colonization is also greatly affected by indigenous microbes, by the soil's physiochemical environment, and by plant species and even genotypes within the crop species. Thus, the application of beneficial microbes requires an understanding of which microbial strains are more suited for which soil quality, which farming system, or even which crop genotypes, and consideration of which plant species and genotype-specific strains might respond more favorably to inoculation (Siddiqui and Shaukat, 2003).

Host variation along with plant variety will affect a plant's response to beneficial microorganisms (Smith and Goodman, 1999). These authors further elaborated that hosting beneficial microbes in any plant species is influenced by that plant's genes. (Tucci et al., 2011) have reported differential rates of gene expression after the inoculation of *Trichoderma* strains in different varieties of tomato. Thus, understanding the roles of individual plant genotypes in supporting or impeding plant-microbe interactions is a necessary step in the success of any beneficial microbes.

Mobilization of Existing Soil Populations

Agricultural soil is a vast reservoir of microbial biomass and diversity. It is estimated that 1 g of rhizosphere soil contains more than 100 species of microbes and 10^8–10^{11} viable cells (Berendsen et al., 2012). Furthermore, plant rhizosphere and rhizoplane shelter different microbiomes with a diversity of microbial traits that is highly relevant to plant growth and health. The proper management and enhancement of these existing populations of soil microbes may provide excellent services for plant growth and help plants cope with multiple stresses, thereby reducing the need for use of agrochemicals (Saleem et al., 2019).

High tillage, the use of pesticides and chemical fertilizers, continuous monoculture, flood irrigation, reduced crop rotation, and lack of organic matter in the soil all negatively impact the soil microbial population and its diversity. On the other hand, cover cropping, crop rotation, intercropping, minimum tillage, system of rice intensification methods, the addition of organic manure, and organic amendments to the soil all support microbial and microbiome diversity and population. The addition of microbial probiotics, biochar, and organic manure with these different agronomic practices has been reported to increase microbial abundance and activity in the soil. Thus, it is possible to increase the efficacy of indigenous microbial populations by complementary crop and soil management practices and by purposeful enhancement of the soil biota (Pang et al., 2017; Meng et al., 2019).

ENHANCING MICROBIAL ENDOWMENTS WITH CHANGES IN CROP AND SOIL MANAGEMENT

The System of Rice Intensification

Rice is the staple food for more than 50% of the global population. A rice production system known as the system of rice intensification developed in Madagascar and validated in over 60 countries is currently gaining currency because of its higher productivity per seed grain, per drop of water, per unit area of land, and per unit cost of production. SRI relies on alternate wetting and drying (AWD) of rice paddies rather than on their continuous flooding, and it controls weeds by actively aerating the upper layer of soil around plants by use of a rotary weeder, rather than by using herbicides. It enhances soil nutrients by the addition of organic matter rather than with chemical fertilizer (Thakur et al., 2010; Thakur and Uphoff, 2017). AWD by itself boosts water use efficiency in rice production (Carrijo et al., 2017). The set of recommended practices raise factor productivity in addition to lowering water requirements (Stoop et al., 2002).

These SRI effects have been reported in over 1,000 papers and are already being achieved by around 20 million farmers using its methods in Asia, Africa and Latin America (SRI-Rice, n.d.). The productivity gains are achieved not by making changes in crop genetic potential and applying external agrochemical inputs but by making synergistic changes in soil, crop, and water management practices to create a more favorable environment for beneficial microbes such as mycorrhizal fungi and phosphorus-solubilizing microbes.

Continuous flooding has deleterious effect on rice plant physiology and anatomy as well as suppression of aerobic soil microbes. For example, flooding has deleterious effects on rice root systems by deforming cells in their cortex to create aerenchyma (air pockets) which affect the transport of water and nutrients (Kirk and Bouldin, 1991). Under flooded soil conditions, lower metabolism and ion transport reduces the growth of rice roots and canopy (Barison and Uphoff, 2011). Further, depletion of oxygen in the bulk soil of flooded rice paddies enhances methanogenic archaea rather than aerobic plant growth-promoting microorganisms (Liesack et al., 2000) and methanotrophs, that consume methane produced by methanogens (Rajkishore et al., 2013).

The effects of inoculating SRI-grown rice plants with *Trichoderma* have been evaluated in Nepal to assess possible synergy between SRI's crop management methods and enhancement of the plants with beneficial endophytes, thereby creating EPHs (Khadka and Uphoff, 2019). Trials were conducted in Nepal with two rice varieties, one improved and the other local. It was found with two seasons of trials that there was a 75% average increase in grain yield from the two varieties when rice seedlings were treated with a native isolate of *T. asperellum* before they were transplanted and raised in a field managed organically according to SRI-recommended practices, compared with farmers' usual practices, reduced or including inorganic fertilization. There was a 58% average increase in yield from these varieties managed with SRI methods but with no microbial inoculation; and the increase from plants inoculated with *Trichoderma* but managed otherwise with conventional methods was 67%. The latter methods included higher plant density, flooding of fields, and inorganic fertilization. These results were not surprising since growing rice under aerobic soil conditions is more favorable for microbes such as *Trichoderma* than is hypoxic flooded soil.

There was a 58% average increase in yield from these varieties when they were managed with SRI methods but no microbial inoculation. However, yield was further increased by inoculation with *Trichoderma*. Among all the replicated trials, the yield was highest (6.5 t ha^{-1}) when inoculation was combined with SRI methods (more aerobic soil, more organic matter). These results should not be surprising since SRI soil conditions are more favorable for aerobic microbes such as *Trichoderma* than is hypoxic flooded soil with no enhancement of soil organic matter.

Research in Malaysia found similar results with a significant increase in seedling growth, germination rate, seed vigor index, and leaf chlorophyll content when rice plants grown under SRI management were inoculated with *T. asperellum* sp. SL2 compared to their cultivation with conventional methods plus inoculation with *T. asperellum* (Doni et al., 2017, 2019b). In further research, it was determined that there was greater net photosynthesis, more internal CO_2 concentration, greater water use efficiency, plant height, tiller number, root length, and fresh root weight in rice plants that were inoculated with *T. asperellum* compared to uninoculated plants (Doni et al., 2019b). This research confirmed that EPH effects can be heightened by the use of conducive crop management practices.

No-Till With Cover Crops and Other Management Practices

There is growing interest in alternative cropping methods not only to reduce farmers' costs of production but also to increase their yields and reduce adverse environmental impacts of farming, particularly soil erosion and agrochemical contamination of soil and water. What is known as conservation agriculture combines reduced mechanical tillage such as no-tillage or minimum tillage, plus crop residue retention on the soil, the use of cover crops, and crop rotation. It has been reported that about 13% of global croplands, 200 million ha in 80 countries, are covered by conservation tillage (Kassam et al., 2014).

Both cover crops and reduced tillage practices improve soil systems' physical and chemical properties, which has a positive impact in the biological properties of soil. Reduced tillage improves the soil's structure through better aggregation, soil organic matter, water infiltration, water-holding capacity, and less soil erosion. Cover crops cultivation increase nutrient inputs through crop residue decomposition, biological N fixation, and root exudates, both for succeeding crops and microbial communities (Schmidt et al., 2018). This enables cover crops to enhance microbial abundance and microbial diversity. Further, broader metabolic capacities of microbes have been reported in cover crop-cultivated fields compared to non-cover crop-cultivated fields that enhance the fungal populations in the soil (Schmidt et al., 2018).

Conservation tillage greatly influences the soil microclimate, dissemination and decomposition of crop residues, and nutrient recycling. All of these changes have positive effects in soil microbial diversity and abundance (Li et al., 2019). It has been reported that conservation tillage conditions the soil pH to make it more suitable for crop growth and microbial diversity and population, which also enhances the fungal hyphal networks in the soil. Higher antibiosis abilities of endophytic and root-zone bacteria, both within and around roots, have been reported with crop rotation and conservation tillage (Peterson et al., 1993). Furthermore, several reports are available that the population, diversity and root colonization rates of arbuscular mycorrhizal fungi (AMF) were consistently reduced by the tillage operation in soil (Säle et al., 2015; Jesus et al., 2016; Schmidt et al., 2018) Enhancement of this symbiotic fungus in crop plant roots is a good strategy for enhancing crop supply of nutrients such as phosphorus and for giving the plants more resilience to drought stress.

SUMMARY

This paper offers a comprehensive review of the knowledge and practice for using endophytic microorganisms to enhance and maintain more beneficial systems of crop production. Most of the cases reported involve endophytic bacteria and fungi

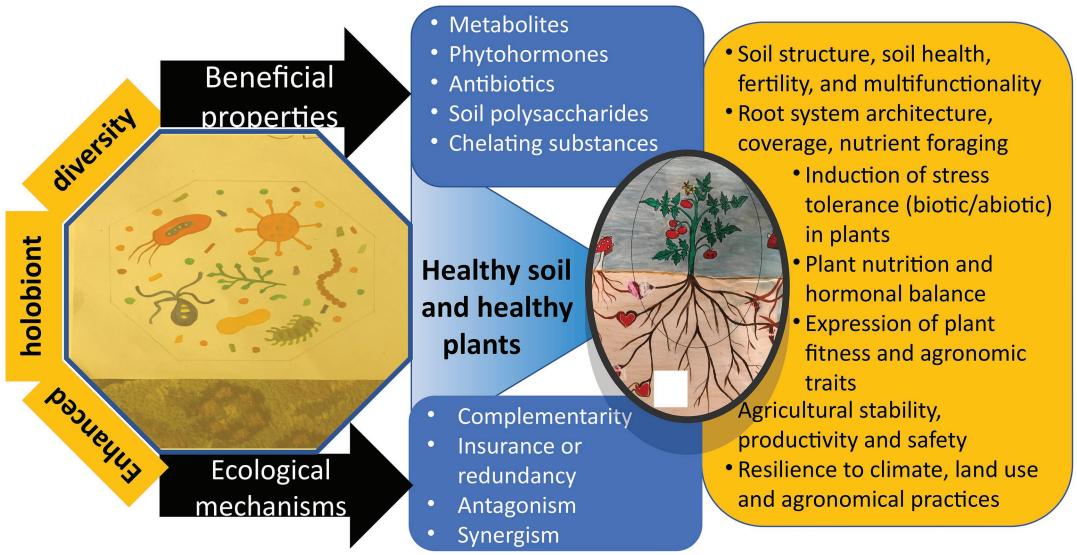

FIGURE 4 | Summary of the various components and management systems that interact to provide optimal performance of plant agriculture.

symbiotically affecting the functioning of plants, but in some instances beneficial microorganisms' principal means of benefitting agriculture and the environment are by other mechanisms such as competition and mycoparasitism.

The review considered first different patterns of endophytic root colonization (**Figure 1**). Then the next section dealt with mechanisms by which microbes have beneficial effects on crop plant performance. **Figure 2** provided a synoptic view of the mechanisms that can be found in nature. Colonization of the interior of plants, particularly their roots, can induce systemic reactions in the whole plant that give it more resistance or tolerance to pests and pathogens, that enhance photosynthesis, or that improve plant functioning at the cellular level.

Beneficial endophytes produce SAMPs that interact with cell membranes and result in signal transduction *via* MAP kinases. This results in higher expression of many proteins and genes, including those that give rise to coordinated improvements in disease suppression and to increased resistance to abiotic stresses such as drought, salt, flooding, and adverse temperatures.

These systemic responses also improve and maintain photosynthesis. This together with improved nutrient efficiency and acquisition, including N_2 fixation, provide the essential components for plant growth and development. Improving photosynthesis is critically important. Plants require the energy and fixed carbon compounds to support both the numerous changes involved is systemic resistance systems and to provide the wherewithal for plant growth and development.

Provision of beneficial endophytes by itself cannot provide total reliability and results. There need to be management practices and good delivery methods that ensure viability and efficacy of applications. The components of effective delivery systems include effective seed treatments or soil applications, and effective organisms that must be selected from either single strains or consortia of different strains since only a few strains or species can enter into mutually-beneficial relationships with plants (**Figure 3**). Management strategies that can enhance the abundance and diversity of beneficial microbial populations include conservation agriculture farming systems, System of Rice Intensification practices, and the management of native populations to strengthen the effectiveness of soil microbial populations. No-till and minimum-till systems can be part of soil management and are particularly effective if accompanied by cover cropping or other methods of maintaining soil coverage with vegetation or other organic materials. There can, however, be disadvantages. For example, leaving corn residue on soil surfaces may provide a source of inoculum of pathogenic *Fusarium* for the next year's crop. In addition, heavy plant residues may make till and cultivation difficult or impossible.

These changes in crop and soil management improve soil health and increase the organic matter in soils (**Figure 4**). With these enhancements, the prospects for meeting the world's food needs are greatly improved, along with the opportunities to avoid the worst consequences of climate change by abating greenhouse gas emissions. Given the great challenges and uncertainties that we presently face, there are no guarantees of success. But understanding and taking advantage of the potency of some of the world's tiniest organisms to make our agriculture more sustainably productive could improve the future for farmers and consumers and provide some simple, low-cost tools for alleviating some of the largest and most basic problems confronting our society.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

REFERENCES

Abdallah, E. F., Alqarawi, A. A., Hashem, A., Radhakrishnan, R., Al-Huqail, A. A., Al-Otibi, F. O., et al. (2018). Endophytic bacterium *Bacillus subtilis* (BERA 71) improves salt tolerance in chickpea plants by regulating the plant defense mechanisms. *J. Plant Interact.* 13, 37–44. doi: 10.1080/17429145.2017.1414321

Abdelaziz, M. E., Abdelsattar, M., Abdeldaym, E. A., Atia, M. A. M., Mahmoud, A. W. M., Saad, M. M., et al. (2019). *Piriformospora indica* alters Na^+/K^+ homeostasis, antioxidant enzymes and LeNHX1 expression of greenhouse tomato grown under salt stress. *Sci. Hortic.* 256:108532. doi: 10.1016/j.scienta.2019.05.059

Ab Rahman, S. F. S., Singh, E., Pieterse, C. M., and Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Sci.* 267, 102–111. doi: 10.1016/j.plantsci.2017.11.012

Arora, N. K., Mehnaz, S., and Balestrini, R. (2016). *Bioformulations: For sustainable agriculture*. New Dehli: Springer, India.

Azad, K., and Kaminskyj, S. (2016). A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. *Symbiosis* 68, 73–78. doi: 10.1007/s13199-015-0370-y

Bae, H., Roberts, D. P., Lim, H. -S., Strem, M., Park, S. -C., Ryu, C. -M., et al. (2011). Endophytic *Trichoderma* isolates from tropical environments delay disease and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Mol. Plant Microbe Interact.* 24, 336–351. doi: 10.1094/MPMI-09-10-0221

Baltruschat, H., Fodor, J., Harrach, B. D., Niemczyk, E., Barna, B., Gullner, G., et al. (2008). Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol.* 180, 501–510. doi: 10.1111/j.1469-8137.2008.02583.x

Barison, J., and Uphoff, N. (2011). Rice yield and its relation to root growth and nutrient-use efficiency under SRI and conventional cultivation: an evaluation in Madagascar. *Paddy Water Environ.* 9, 65–78. doi: 10.1007/s10333-010-0229-z

Barka, A. E., Nowk, J., and Clement, C. (2006). Enhancement of chilling resistance of inoculated grapevine plantlets with plant growth promoting rhizobacteria *Burkholderia phytofermans* strain PsJN. *Appl. Environ. Microbiol.* 72, 7246–7252. doi: 10.1128/AEM.01047-06

Bastias, D. A., Martínez-Ghersa, M. A., Ballaré, C. L., and Gundel, P. E. (2017). Epichloë fungal endophytes and plant defenses: not just alkaloids. *Trends Plant Sci.* 22, 938–948. doi: 10.1016/j.tplants.2017.08.005

Bateman, R. P., Matthews, G. A., and Hall, F. R. (2007). "Ground-based application equipment" in *Field manual of techniques in invertebrate pathology*. Springer, 73–98.

Batta, Y. A. (2007). Biocontrol of almond bark beetle (Scolytus amygdali Geurin-Meneville, Coleoptera: Scolytidae) using *Beauveria bassiana* (Bals.) Vuill. (Deuteromycotina: Hyphomycetes). *J. Appl. Microbiol.* 103, 1406–1414. doi: 10.1111/j.1365-2672.2007.03369.x

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Bharti, N., Pandey, S. S., Barnawal, D., Patel, V. K., and Kalra, A. (2016). Plant growth promoting rhizobacteria *Dietzia natronolimnaea* modulates the expression of stress responsive genes providing protection of wheat from salinity stress. *Sci. Rep.* 6, 1–16. doi: 10.1038/srep34768

Bonanomi, G., Lorito, M., Vinale, F., and Woo, S. L. (2018). Organic amendments, beneficial microbes, and soil microbiota: toward a unified framework for disease suppression. *Annu. Rev. Phytol.* 56, 1–20. doi: 10.1146/annurev-phyto-080615-100046

Brunner, K., Peterbauer, C. K., Mach, R. L., Lorito, M., Zeilinger, S., and Kubicek, C. P. (2003). The Nag1 *N*-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. *Curr. Genet.* 43, 289–295. doi: 10.1007/s00294-003-0399-y

Burr, T. J., and Reid, C. L. (1994). Biological control of grape crown gall with non-tumorigenic agrobacterium vitis strain F2/5. *Am. J. Enol. Vitic.* 45, 213–219.

Carrijo, D. R., Lundy, M. E., and Linquist, B. A. (2017). Rice yields and water use under alternate wetting and drying irrigation: a meta-analysis. *Field Crops Res.* 203, 173–180. doi: 10.1016/j.fcr.2016.12.002

Cheng, Y. T., Zhang, L., and He, S. Y. (2019). Plant-microbe interactions facing environmental challenge. *Cell Host Microbe* 26, 183–192. doi: 10.1016/j.chom.2019.07.009

Chet, I., Harman, G. E., and Baker, R. (1981). *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microb. Ecol.* 7, 29–38. doi: 10.1007/BF02010476

Chi, F., Shen, S. -H., Cheng, H. -P., Jing, Y. -X., Yanni, Y. G., and Dazzo, F. B. (2005). Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Appl. Environ. Microbiol.* 71, 7271–7278. doi: 10.1128/AEM.71.11.7271-7278.2005

Cho, S. M., Kang, B. R., and Kim, Y. C. (2013). Transcriptome analysis of induced systemic drought tolerance elicited by *Pseudomonas chlororaphis* O6 in *Arabidopsis thaliana*. *Plant Pathol. J.* 29, 209–220. doi: 10.5423/PPJ.SI.07.2012.0103

Compant, S., Samad, A., and Faist, H. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004

Conant, R. T., Smith, G. R., and Paustian, K. (2003). Spatial variability of soil carbon in forested and cultivated sites: implications for change detection. *J. Environ. Qual.* 32, 278–286. doi: 10.2134/jeq2003.2780

Connick, W. J. J., Daigle, D. J., and Quimby, P. C. Jr. (1991). An improved invert emulsion with high water retention for mycoherbicide delivery. *Weed Technol.* 5, 442–444. doi: 10.1017/S0890037X00028402

Conrath, U., Beckers, G. J. M., Langenbach, C. J. G., and Jaskiewicz, M. R. (2015). Priming for enhanced defense. *Ann. Rev.Phytopathol.* 53, 97–119. doi: 10.1146/annurev-phyto-080614-120132

Cook, R. J. (2017). *Untold stories*. St Paul: American Phytopathological Society.

Copping, L. G. (2004). *The manual of biocontrol agents. 3rd Edn*. Alton, Hampshire, UK: British Crop Protection Council.

De Palma, M., D'agostino, N., Proiett, S., Bertini, L., Lorito, M., Ruocco, M., et al. (2016). Suppression subtractive hybridization analysis provides new insights into the tomato (*Solanum lycopersicum* L.) response to the plant probiotic microorganism *Trichoderma longibrachiatum* MK1. *J. Plant Physiol.* 190, 79–94. doi: 10.1016/j.jplph.2015.11.005

Digat, B. (1991). A new encapsulation technology for bacterial inoculants and seed bacterization. *Bulletin OILB SROP*.

Djonovic, S., Vargas, W. A., Kolomiets, M. V., Horndeski, M., Weist, A., and Kenerley, C. M. (2007). A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for systemic resistance in maize. *Plant Physiol.* 145, 875–889. doi: 10.1104/pp.107.103689

Doni, F., Fathurrahman, F., Mispan, M. S., Suhaimi, N. S. M., Yussof, W. M. W., and Uphoff, N. (2019a). Transciptome profiling of rice seedlings inoculated with symbiotic fungus *Trichoderma asperellum* SL2. *J. Plant Growth Reg.* 38, 1507–1515. doi: 10.1007/s00344-019-09952-7

Doni, F., Isahak, A., Zain, C. R. C. M., and Yusoff, W. M. W. (2014). Physiological and growth response of rice plants (*Oryza sativa* L.) to *Trichoderma* spp. inoculants. *AMB Express* 4:45. doi: 10.1186/s13568-014-0045-8

Doni, F., Mispan, M. S., Suhalmi, M. S. M., Ishad, N., and Uphoff, N. (2019b). Roles of microbes in supporting sustainable rice production using the system of rice intensification. *Appl. Environ. Microbiol.* 103, 5131–5142. doi: 10.1007/s00253-019-09879-09879-9

Doni, F., Zain, C. R. C. M., Isahak, A., Fathurrahman, F., Sulaiman, N., Uphoff, N., et al. (2017). Relationships observed between *Trichoderma* inoculation and characteristics of rice grown under system of rice intensification (SRI) vs. conventional methods of cultivation. *Symbiosis* 72, 45–59. doi: 10.1007/s13199-016-0438-3

Dorner, J. W. (2009). Development of biocontrol technology to manage aflatoxin contamination in peanuts. *Peanut Sci.* 36, 60–67. doi: 10.3146/AT07-002.1

Duke, K. A., Becker, M. G., Girard, I. J., Millar, J. L., Fernando, W. D., Belmonte, M. F., et al. (2017). The biocontrol agent pseudomonas chlororaphis PA23 primes *Brassica napus* defenses through distinct gene networks. *BMC Genomics* 18:467. doi: 10.1186/s12864-017-3848-6

Durairaj, K., Velmurugan, P., Vedhanayakisri, K. A., Chang, W. -S., Senthilkumar, P., Choi, K. -M., et al. (2018). Molecular and phenotypic characterization of pathogenic fungal strains isolated from ginseng root rot. *Physiol. Mol. Plant Pathol.* 104, 141–146. doi: 10.1016/j.pmpp.2018.09.006

Eke, P., Chatue, G. C., Wakam, L. N., Kouipou, R. M. T., Fokou, P. V. T., and Boyom, F. F. (2016). Mycorrhiza consortia suppress the fusarium root rot (*Fusarium solani* f. sp Phaseoli) in common bean (*Phaseolus vulgaris* L.). *Biol. Control* 103, 240–250. doi: 10.1016/j.biocontrol.2016.10.001

Farooq, M. A., Niazi, A. K., Akhtar, J., Farooq, M., Souri, Z., Karimi, N., et al. (2019). Acquiring control: the evolution of ROS-induced oxidative

stress and redox signaling pathways in plant stress response. *Plant Physiol. Biochem.* 141, 353–369. doi: 10.1016/j.plaphy.2019.04.039

Farzana, G. J., Muhammad, H., Hussain, A., Gul, J., Amjad, I., Aman, K., et al. (2019). An endophytic isolate of the fungus *Yarrowia lipolytica* produce metabolites that ameliorate the negative impact of salt stress of the physiology of maize. *BMC Microbiol.* 19:3. doi: 10.1186/s12866-018-1374-6

Fravel, D. R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annu. Rev. Phytol.* 26, 75–91. doi: 10.1146/annurev.py.26.090188.000451

Fu, J., Wang, Y. -F., Liu, Z. -H., Li, Z. -T., and Yang, K. -J. (2018). *Trichoderma asperellum* alleviates the effects of saline-alkaline stress on maize seedlings via the regulation of photosynthesis and nitrogen metabolism. *Plant Growth Regul.* 85, 363–374. doi: 10.1007/s10725-018-0386-4

Fukami, J., de la Osa, C., Javier, O. F., Megias, M., and Hungria, M. (2018). Co-inoculation of maize with *Azospirillum brasilense* and *Rhizobium tropici* as a strategy to mitigate salinity stress. *Funct. Plant Biol.* 45, 328–339. doi: 10.1071/FP17167

Gange, A. C., Koricheva, J., Currie, A. F., Jaber, L. R., and Vidal, S. (2019). Meta-analysis of the role of entomopathogenic and unspecialized fungal endophytes as plant bodyguards. *New Phytol.* 223, 2002–2010. doi: 10.1111/nph.15859

Garber, R. K., and Cotty, P. J. (1997). Formation of sclerotia and aflatoxins in developing cotton bolls infected by the S strain of Aspergillus flavus and potential for biocontrol with an atoxigenic strain. *Phytopathology* 87, 940–945. doi: 10.1094/PHYTO.1997.87.9.940

Gašić, S., and Tanović, B. (2013). Biopesticide formulations, possibility of application and future trends. *Pesticidi i fitomedicina* 28, 97–102. doi: 10.2298/PIF1302097G

Genre, A., and Russo, G. (2016). Does a common pathway transduce symbiotic signals in plant-microbe interactions? *Front. Plant Sci.* 7:96. doi: 10.3389/fpls.2016.00096

Ghorbanpour, A., Salimi, A., Ghanbary, M. A. T., Pirdashti, H., and Dehestani, A. (2018). The effect of *Trichoderma harzianum* in mitigating low temperature stress in tomato (*Solanum lycopersicum* L.) plants. *Sci. Hortic.* 230, 134–141. doi: 10.1016/j.scienta.2017.11.028

Gill, S. S., Gill, R., Trivedi, D. K., Anjum, N. A., Sharma, K. K., Ansari, M. W., et al. (2016). *Piriformospora indica*: potential and significance in plant stress tolerance. *Front. Microbiol.* 7:332. doi: 10.3389/fmicb.2016.00332

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., et al. (2010). Food security: the challenge of feeding 9 billion people. *Science* 327, 812–818. doi: 10.1126/science.1185383

Gough, C., and Cullimore, J. (2011). Lipo-chitooligosaccharide signaling in endosymbiotic plant-microbe interactions. *Molec. Plant-Microbe Interact.* 24, 867–878. doi: 10.1094/MPMI-01-11-0019

Gurska, J., Wang, W., Gerhardt, K. E., Khalid, A. M., Isherwood, D. M., Huang, X. -D., et al. (2009). Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste. *Environ. Sci. Technol.* 43, 4472–4479. doi: 10.1021/es801540h

Gwynn, R. L. (ed.) (2014). *The manual of biocontrol agents. 5th Edn.* Alton Hamshire, UK: British Crop Protection Council.

Hanin, M., Ebel, C., Ngom, M., Laplaze, L., and Masmoudi, K. (2016). New insights on plant salt tolerance mechanisms and their potential use for breeding. *Front. Plant Sci.* 7:1787. doi: 10.3389/fpls.2016.01787

Harman, G. E. (2011). Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. *New Phytol.* 189, 647–649. doi: 10.1111/j.1469-8137.2010.03614.x

Harman, G. E., Cadle-Davidson, M., and Nosir, W. (2018). Patent application WO2017192117A1. Highly effective and multifunctional microbial compositions and uses. USA patent application.

Harman, G. E., and Custis, D. (2006). US Patent 9,090,884. Formulations of viable microorganisms and their method of use PCT WO 2007030557 patent application WO 2007030557.

Harman, G. E., Doni, F., Khadka, R. B., and Uphoff, N. (2019). Endophytic strains of *Trichoderma* increase plants' photosynthetic capability. *J. Appl. Microbiol.* doi: 10.1111/jam.14368 [Epub ahead of print]

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004a). *Trichoderma* species---opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Harman, G. E., Lorito, M., and Lynch, J. M. (2004b). Uses of *Trichoderma* spp. to remediate soil and water pollution. *Adv. Appl. Microbiol.* 56, 313–330. doi: 10.1016/S0065-2164(04)56010-0

Harman, G. E., Obregón, M. A., Samuels, G. J., and Lorito, M. (2010). Changing models of biocontrol in the developing and developed world. *Plant Dis.* 94, 928–939. doi: 10.1094/PDIS-94-8-0928

Harman, G. E., and Uphoff, N. (2019). Advantages and methods of using symbiotic microbes to enhance plant agriculture and the environment. *Scientifica* 2019:9106395. doi: 10.1155/2019/9106395

Hawken, P. (2017). *Drawdown*. New York: Penguin Books.

Heong, K. L., Escalada, M. M., Van Chien, H., and Reyes, J. H. D. (2015). "Are there productivity gains from insecticide applications in rice production?" in *Rice Planthoppers*. eds. K. L. Heong, J. A. Cheng and M. M. Escalada (Dordrecht: Springer), 179–189.

Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* 87, 4–10. doi: 10.1094/PDIS.2003.87.1.4

Howell, C. R., Hanson, L. E., Stipanovic, R. D., and Puckhaber, L. S. (2000). Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. *Phytopathology* 90, 248–252. doi: 10.1094/PHYTO.2000.90.3.248

Huang, Z., Hejazi, M., Tang, Q., Vernon, C. R., Liu, Y., Chen, M., et al. (2019). Global agricultural green and blue water consumption under future climate and land use changes. *J. Hydrol.* 574, 242–256. doi: 10.1016/j.jhydrol.2019.04.046

Hubbard, J. P., Harman, G. E., and Hadar, Y. (1983). Effect of soilborne *Pseudomonas* sp. on the biological control agent, *Trichoderma hamatum*, on pea seeds. *Phytopathology* 73, 655–659. doi: 10.1094/Phyto-73-655

Ikram, M., Ali, N., Jan, G., Hamayun, M., Jan, F. G., and Iqbal, A. (2019). Novel antimicrobial and antioxidative activity by endophytic *Penicillium roqueforti* and *Trichoderma reesei* isolated from *Solanum surattense*. *Acta Physiol. Plant.* 41:164. doi: 10.1007/s11738-019-2957-z

Ikram, M., Ali, N., Jan, G., Jan, F. G., Rahman, I. U., Iqbal, A., et al. (2018). IAA producing fungal endophyte *Penicillium roqueforti* Thom., enhances stress tolerance and nutrients uptake in wheat plants grown on heavy metal contaminated soils. *PLoS One* 13:e0208150. doi: 10.1371/journal.pone.0208150

Ismail, Hamayun, M., Hussain, A., Iqbal, A., Khan, S. A., and Lee, I. -J. (2020). *Aspergillus niger* boosted heat stress tolerance in sunflower and soybean via regulating their metabolic and antioxidant system. *J. Plant Interact.* 15, 223–232. doi: 10.1080/17429145.2020.1771444

Jaskiewicz, M., Conrath, U., and Peterhaensel, C. (2011a). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* 12, 50–55. doi: 10.1038/embor.2010.186

Jaskiewicz, M., Peterhansel, C., and Conrath, U. (2011b). Detection of histone modifications in plant leaves. *Jove-J. Visualized Exp.* 55:e3096. doi: 10.3791/3096

Jesus, E. D. C., Liang, C., Quensen, J. F., Susilawati, E., Jackson, R. D., Balser, T. C., et al. (2016). Influence of corn, switchgrass, and prairie cropping systems on soil microbial communities in the upper Midwest of the United States. *GCB Bioenergy* 8, 481–494. doi: 10.1111/gcbb.12289

John, R. P., Tyagi, R. D., Brar, S. K., Surampalli, R. Y., and Prévost, D. (2011). Bio-encapsulation of microbial cells for targeted agricultural delivery. *Crit. Rev. Biotechnol.* 31, 211–226. doi: 10.3109/07388551.2010.513327

Johnson, J. M., Thuerich, J., Petutschnig, E. K., Altschmied, L., Meichsner, D., Sherameti, I., et al. (2018). A poly(A) ribonuclease controls the cellotriose-based interaction between *Piriformospora indica* and its host *Arabidopsis*. *Plant Physiol.* 176, 2496–2514. doi: 10.1104/pp.17.01423

Jourdan, E., Henry, G., Duby, F., Dommes, J., Barthelemy, J., Thonart, P., et al. (2009). Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. *Mol. Plant-Microbe Interact.* 22, 456–468. doi: 10.1094/MPMI-22-4-0456

Kassam, A., Friedrich, T., Shaxson, F., Bartz, H., Mello, I., Kienzle, J., et al. (2014). The spread of conservation agriculture: policy and institutional support for adoption and uptake. *Field Actions Sci. Rep.* 7.

Khadka, K. B., and Uphoff, N. (2019). *Trichoderma* seedling treatment with system of rice intensification management and with conventional management of transplanted rice. *Peer J.* 7:e5877. doi: 10.7717/peerj.5877

Khan, L., Waqas, M., Hussain, J., Al-Harrasi, A., Hamayun, M., and Lee, I. -J. (2015). Phytohormones enabled endophytic fungal symbiosis improve aluminum phytoextraction in tolerant *Solanum lycopersicum*: an examples of *Penicillium janthinellum* LK5 and comparison with exogenous GA(3). *J. Hazard. Mater.* 295, 70–78. doi: 10.1016/j.jhazmat.2015.04.008

Khushdil, F., Jan, F. G., Jan, G., Hamayun, M., Iqbal, H., and A., and Bibi, N., (2019). Salt stress alleviation in *Pennisetum glaucum* through secondary

metabolites modulation by *Aspergillus terreus*. *Plant Physiol. Biochem.* 144, 127–134. doi: 10.1016/j.plaphy.2019.09.038

Kim, K., Jang, Y. J., Lee, S. M., Oh, B. T., Chae, J. C., and Lee, K. J. (2014). Alleviation of salt stress by Enterobacter sp. EJ01 in tomato and *Arabidopsis* is accompanied by up-regulation of conserved salinity responsive factors in plants. *Mol. Cell* 7, 109–117. doi: 10.14348/molcells.2014.2239

Kim, A. Y., Shazad, R., Kang, S. M., Seo, C. W., Park, Y. G., Park, H. J., et al. (2017). IAA-producing Klebsiella variicola AY13 reprograms soybean growth during flooding stress. *J. Crop. Sci. Biotechnol.* 20, 235–242. doi: 10.1007/s12892-017-0041-0

Kirk, G. J. D., and Bouldin, D. R. (1991). "Speculations on the operation of the rice root system in relation to nutrient uptake" in *Simulation and systems analysis for rice production.* eds. F. W. T. Penning de Vries, H. H. van Laar, and M. J. Kropff (Wageningen, Netherlands: Pudoc), 195–203.

Kiss, L., Russell, J. C., Szentivanyi, O., Xu, X., and Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonists of powdery mildew fungi. *Biocontrol Sci. Tech.* 14, 635–651. doi: 10.1080/09583150410001683600

Kloepper, J. W., Ryu, C. M., and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94, 1259–1266. doi: 10.1094/PHYTO.2004.94.11.1259

Knowles, A. (2006). *Adjuvants and additives*. (London: T&F Informa UK Ltd).

Li, Y., Li, Z., Cui, S., Jagadamma, S., and Zhang, Q. (2019). Residue retention and minimum tillage improve physical environment of the soil in croplands: a global meta-analysis. *Soil Tillage Res.* 194:104292. doi: 10.1016/j.still.2019.06.009

Liesack, W., Schnell, S., and Revsbech, N. P. (2000). Microbiology of flooded rice paddies. *FEMS Microbiol. Rev.* 24, 625–645. doi: 10.1111/j.1574-6976.2000.tb00563.x

Lorito, M., Woo, S. L., Harman, G. E., and Monte, E. (2010). Translational research on *Trichoderma*: from 'omics to the field. *Annu. Rev. Phytopathol.* 48, 395–417. doi: 10.1146/annurev-phyto-073009-114314

Lucas, J. A., Greer, G., Oudemans, P. V., and Coffey, M. D. (1990). Fungicide sensitivity in somatic hybrids of *Phytophthora capsici* obtained by protoplast fusion. *Physiol. Mol. Plant Pathol.* 36, 175–187. doi: 10.1016/0885-5765(90)90105-7

Mahmood, I., Imadi, S. R., Shazadi, K., Gul, A., and Hakeem, K. R. (2016). "Effects of pesticides on environment" in *Plant, soil and microbes*. eds. K. R. Hakeem, M. S. Akhtar and S. N. A. Abdullah (Switzerlad: Spriner), 253–269.

Mahmood, Z., Steenberg, T., Mahmood, K., Labouriau, R., and Kristensen, M. (2019). Endophytic Beauveria bassiana in maize affects survival and fecundity of the aphid *Sitobion avenae*. *Biol. Control* 137:104017. doi: 10.1016/j.biocontrol.2019.104017

Malik, N. A. A., Kumar, I. S., and Nadarajah, K. (2020). Elicitor and receptor molecules: orchestrators of plant defense and immunity. *Int. J. Mol. Sci.* 21:5463. doi: 10.3390/ijms21155463

Malla, M. A., Dubey, A., Yadav, S., Kumar, A., Hashem, A., and Abd-Allah, E. F. (2018). Understanding and designing the strategies for the microbe-mediated remediation of environmental contaminants using omics approaches. *Front. Microbiol.* 9:1132. doi: 10.3389/fmicb.2018.01132

Margulis, L., and Fester, R. (1991). *Symbiosis as a source of evolutionary innovation*. Boston: MIT Press, 470.

Marquez, N., Giachero, M. L., Gallou, A., Debat, H. J., Declerck, S., and Ducasse, D. A. (2019). Transcriptome analysis of mycorrhizal and nonmycorrhizal soybean plantlets upon infection with *Fusarium virguliforme*, one causal agent of sudden death syndrome. *Plant Pathol.* 68, 470–480. doi: 10.1111/ppa.12964

Mascarin, G. M., and Jaronski, S. T. (2016). The production and uses of *Beauveria bassiana* as a microbial insecticide. *World J. Microbiol. Biotechnol.* 32:177. doi: 10.1007/s11274-016-2131-3

Mastouri, F., Bjorkman, T., and Harman, G. E. (2012). *Trichoderma harzianum* strain T22 enhances antioxidant defense of tomato seedlings and resistance to water deficit. *Mol. Plant-Microbe Interact.* 25, 1264–1271. doi: 10.1094/MPMI-09-11-0240

Mehmood, A., Hussain, A., Irshad, M., Hamayun, M., Iqbal, A., Tawab, A., et al. (2020). Yucasin and cinnamic acid inhibit IAA and flavonoids biosynthesis minimizing interaction between maize and endophyte *Aspergillus nomius*. *Symbiosis* 81, 149–160. doi: 10.1007/s13199-020-00690-z

Meng, L., Sun, T., Li, M., Saleem, M., Zhang, Q., and Wang, C. (2019). Soil-applied biochar increases microbial diversity and wheat plant performance under herbicide fomesafen stress. *Ecotoxicol. Environ. Saf.* 171, 75–83. doi: 10.1016/j.ecoenv.2018.12.065

Meyer, S. L. F., and Roberts, D. P. (2002). Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. *J. Nematol.* 34, 1–8.

Mhatre, P. H., Karthik, C., Kadirvelu, K., Divya, K. L., Venkatasalam, E. P., Srinivasan, S., et al. (2019). Plant growth promoting rhizobacteria (PGPR): A potential alternative tool for nematodes bio-control. *Biocatal. Agric. Biotechnol.* 17, 119–128. doi: 10.1016/j.bcab.2018.11.009

Mishra, P. K., Bisht, S. C., Bisht, J. K., and Bhatt, J. C. (2011). "Cold-tolerant PGPRs as bioinoculants for stress management" in *Bacteria in agrobiology: Stress management*. ed. D. K. Maheshwari (Berlin: Springer), 95–118.

Mishra, P. K., Bisht, S. C., Ruwari, P., Selvakumar, G., and Bisht, J. K. (2009). Enhancement of chilling tolerance of inoculated wheat seedlings with cold tolerant plant growth promoting Pseudomonads from N. W. Himalayas. *Arch. Microbiol.* 193, 497–513.

Mo, Y., Wang, Y., Yang, R., Zheng, J., Liu, C., Li, H., et al. (2016). Regulation of plant growth, photosynthesis, antioxidation and osmosis by an arbuscular mycorrhizal fungus in watermelon seedlings under well-watered and drought conditions. *Front. Plant Sci.* 7:644. doi: 10.3389/fpls.2016.00644

Morita, M., Shitan, N., Sawada, K., Van Montagu, M. C. E., Inze, D., Rischer, H., et al. (2009). Vacuolar transport of nicotine is mediated by a multidrug and toxic compound extrusion (MATE) transporter in *Nicotiana tabacum*. *Proc. Nati. Acad. Sci. USA* 106, 2447–2452. doi: 10.1073/pnas.0812512106

Morris, C. E., Sands, D. C., Bardin, M., Jaenicke, R., Vogel, B., Leyronas, C., et al. (2011). Microbiology and atmospheric processes: research challenges concerning the impact of airborne micro-organisms on the atmosphere and climate. *Biogeosciences* 8, 17–25. doi: 10.5194/bg-8-17-2011

Nandi, M., Selin, C., Brawerman, G., Fernando, W. D., and Kievit, T. (2017). Hydrogen cyanide, which contributes to *Pseudomonas chlororaphis* strain PA23 biocontrol, is upregulated in the presence of glycine. *Biol. Control* 108, 47–54. doi: 10.1016/j.biocontrol.2017.02.008

Nath, M., Bhatt, D. D., Prasad, R., Gill, S. S., Anjum, N. A., and Tuteja, N. (2016). Reactive oxygen species generation-scavenging and signaling during plant-Arbuscular Mycorrhizal and *Piriformospora indica* interaction under stress condition. *Front. Plant Sci.* 7:1574. doi: 10.3389/fpls.2016.01574

Nautiyal, C. S., Srivastava, S., Chauhan, P. S., Seem, K., Mishra, A., and Sopory, S. K. (2013). Plant growth-promoting bacteria *Bacillus amyloliquefaciens* NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress. *Plant Physiol. Biochem.* 66, 1–9. doi: 10.1016/j.plaphy.2013.01.020

Ngumbi, E., and Kloepper, J. (2016). Bacterial-mediated drought tolerance: current and future prospects. *Appl. Soil Ecol.* 105, 109–125. doi: 10.1016/j.apsoil.2016.04.009

Pang, G., Cai, F., Li, R., Zhao, Z., Li, R., Gu, X., et al. (2017). Trichoderma-enriched organic fertilizer can mitigate microbiome degeneration of monocropped soil to maintain better plant growth. *Plant Soil* 416, 181–192. doi: 10.1007/s11104-017-3178-0

Pelhivan, N., Yesilyurt, A. M., Durmas, N., and Karaoglu, S. A. (2017). *Trichoderma lixii* ID11D seed biopriming mitigates dose dependent salt toxicity in maize. *Acta Physiol. Plant.* 39, 79–91. doi: 10.1007/s11738-017-2375-z

Perata, P., Armstrong, W., and Voesenek, L. A. C. J. (2011). Plants and flooding stress. *New Phytol.* 190, 269–273. doi: 10.1111/j.1469-8137.2011.03702.x

Peterson, T. A., Blackmer, T. M., Francis, D. D., and Schepers, J. S. (1993). "Using a chlorophyll meter to improve N management" in *NebGuide. D-13, Fertiltiy.* Cooperative Extension, Institute of Agriculture and Natural Resources, University of Nebraska-Lincoln.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Pitt, J. I., and Hocking, A. D. (2006). Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. *Mycopathologia* 162, 233–243. doi: 10.1007/s11046-006-0059-0

Poveda, J., Hermosa, R., Monte, E., and Nicolás, C. (2019). The *Trichoderma harzianum* Kelch protein ThKEL1 plays a key role in root colonization and

the induction of systemic defense in Brassicaceae plants. *Front. Plant Sci.* 10:1478. doi: 10.3389/fpls.2019.01478

Qadir, M., Hussain, A., Hamayun, M., Shah, M., Iqbal, A., Husna, et al. (2020). Phytohormones producing rhizobacterium alleviates chromium toxicity in *Helianthus annuus* L. by reducing chromate uptake and strengthening antioxidant system. *Chemosphere* 258:127386. doi: 10.1016/j.chemosphere.2020.127386

Rajkishore, S. K., Doraisamy, P., Subramanian, K. S., and Maheswari, M. (2013). Methane emission patterns and their associated soil microflora with SRI and conventional systems of rice cultivation in Tamil Nadu, India. *Taiwan Water Conserv.* 61, 126–134.

Ramakuwela, T., Hatting, J., Bock, C., Vega, F. E., Wells, L., Mbata, N., et al. (2020). Establishment of *Beauveria bassiana* as a fungal endophyte in pecan (*Carya illinoinensis*) seedlings and its virulence against pecan insect pests. *Biol. Control* 140:104102. doi: 10.1016/j.biocontrol.2019.104102

Redman, R. S., Kim, Y. O., Woodward, C. J. D. A., Greer, C., Espino, L., Doty, S. L., et al. (2011). Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. *PLoS One* 6:e14823. doi: 10.1371/journal.pone.0014823

Rivera-Mendez, W., Obregon, M., Moran-Diez, M. E., Hermosa, R., and Monte, E. (2020). Trichoderma asperellum biocontrol activity and induction of systemic defenses against *Sclerotium cepivorum* in onion plants under tropical climate conditions. *Biol. Control* 141:104145. doi: 10.1016/j.biocontrol.2019.104145

Roberts, D. P., Lohrke, S. M., Meyer, S. L. F., Buyer, J. S., Bowers, J. H., Baker, C. J., et al. (2005). Biocontrol agents applied individually and in combination for suppression of soilborne diseases of cucumber. *Crop Protection* 24, 141–155. doi: 10.1016/j.cropro.2004.07.004

Rodriguez, R., Baird, A., Cho, S., Gray, Z., Groove, R. E., Harto, R., et al. (2019). "Programming plants for climate resilience through Symbiogenics" in *Seed Endophytes*. eds. S. Verma and J. J. White (Cham: Springer), 127–137.

Rodriguez, R., and Redman, R. (2005). Balancing the generation and elimination of reactive oxygen species. *Proc. Natl. Acad. Sci. U. S. A.* 102, 3175–3176. doi: 10.1073/pnas.0500367102

Roiger, D. J., and Jeffers, S. N. (1991). Evaluation of *Trichoderma*-SPP for biological control of phytophthora crown and root rot of apple seedlings. *Phytopathology* 81, 910–917. doi: 10.1094/Phyto-81-910

Ruoccco, M., Lanzuise, S., Lombardi, N., Woo, S. L., Vinale, F., Marra, R., et al. (2015). Multiple roles and effects of novel *Trichoderma* hydrophobin. *Mol. Plant Microbe Interact.* 28, 167–179. doi: 10.1094/MPMI-07-14-0194-R

Ryan, P. R., Dessaux, Y., Thomashow, L. S., and Weller, D. M. (2009). Rhizosphere engineering and management for sustainable agriculture. *Plant Soil* 321, 363–383. doi: 10.1007/s11104-009-0001-6

Säle, V., Aguilera, P., Laczko, E., Mäder, P., Berner, A., Zihlmann, U., et al. (2015). Impact of conservation tillage and organic farming on the diversity of arbuscular mycorrhizal fungi. *Soil Biol. Biochem.* 84, 38–52. doi: 10.1016/j.soilbio.2015.02.005

Saleem, M., Hu, J., and Jousset, A. (2019). More than the sum of its parts: microbiome biodiversity as a driver of plant growth and soil health. *Ann. Rev. Ecol. Evolut. System.* 50, 145–168. doi: 10.1146/annurev-ecolsys-110617-062605

Sasson, Y., Levy-Ruso, G., Toledano, O., and Ishaaya, I. (2007). "Nanosuspensions: emerging novel agrochemical formulations" in *Insecticides design using advanced technologies*. Springer, 1–39.

Scher, F. M., and Baker, R. (1982). Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. *Phytopathology* 72, 1567–1573. doi: 10.1094/Phyto-72-1567

Schisler, D. A., Slininger, P. J., Behle, R. W., and Jackson, M. A. (2004). Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology* 94, 1267–1271. doi: 10.1094/PHYTO.2004.94.11.1267

Schmidt, R., Gravuer, K., Bossange, A. V., Mitchell, J., and Scow, K. (2018). Long-term use of cover crops and no-till shift soil microbial community life strategies in agricultural soil. *PLoS One* 13:e0192953. doi: 10.1371/journal.pone.0192953

Schoebitz, M., López, M. D., and Roldán, A. (2013). Bioencapsulation of microbial inoculants for better soil–plant fertilization. A review. *Agron. Sustain. Dev.* 33, 751–765. doi: 10.1007/s13593-013-0142-0

Schouteden, N., De Waele, D., Panis, B., and Vos, C. M. (2015). Arbuscular mycorrhizal fungi for the biocontrol of plant-parasitic nematodes: a review of the mechanisms involved. *Front. Microbiol.* 6:1280. doi: 10.3389/fmicb.2015.01280

Schussler, A. (2009). Struktur, funktion and Okologie der abruskalaren mycorrhizae. *Rundgersprache der Komission fur Onkologie* 37, 97–108.

Shafia, A., Sutton, J. C., Yu, H., and Fletcher, R. A. (2001). Influence of preinoculation light intensity on development and interactions of *Botrytis cinerea* and *Clonostachys rosea* in tomato leaves. *Can. J. Plant Pathol.* 23, 346–357. doi: 10.1080/07060660109506955

Shah, J., and Zeier, J. (2013). Long-distance communication and signal amplification in systemic acquired resistance. *Front. Plant Sci.* 4:30. doi: 10.3389/fpls.2013.00030

Shahid, M., Pinelli, E., and Dumat, C. (2018). Tracing trends in plant physiology and biochemistry: need of databases from genetic to kingdom level. *Plant Physiol. Biochem.* 127, 630–635. doi: 10.1016/j.plaphy.2018.04.030

Sherameti, I., Tripathi, S., Varma, A., and Oelmueller, R. (2008). The root-colonizing endophyte *Pirifomospora indica* confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. *Mol. Plant-Microbe Interact.* 21, 799–807. doi: 10.1094/MPMI-21-6-0799

Shoresh, M., Gal-On, A., Liebman, D., and Chet, I. (2006). Characterization of a mitogen-activated protein kinase gene from cucumber required for *Trichoderma*-conferred plant resistance. *Plant Physiol.* 142, 1169–1179. doi: 10.1104/pp.106.082107

Shoresh, M., and Harman, G. E. (2008). The relationship between increased growth and resistance induced in plants by root colonizing microbes. *Plant Signal. Behav.* 3, 737–739. doi: 10.4161/psb.3.9.6605

Shoresh, M., Mastouri, F., and Harman, G. E. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi: 10.1146/annurev-phyto-073009-114450

Shukla, N., Awasthi, R. P., Rawat, L., and Kumar, J. (2015). Seed biopriming with drought tolerant isolates of *Trichoderma harzianum* promote growth and drought tolerance in *Triticum aestivum*. *Annals Appl. Biol.* 166, 171–182.

Siddiqui, Z. A. (ed.) (2005). "PGPR: prospective biocontrol agents of plant athogens" in *PGPR: Biocontrol and biofertilization*. (Dordrecht: Springer), 111–142.

Siddiqui, I. A., and Shaukat, S. S. (2003). Plant species, host age and host genotype effects on *Meloidogyne incognita* biocontrol by *Pseudomonas fluorescens* strain CHA0 and its genetically-modified derivatives. *J. Phytopathol.* 151, 231–238. doi: 10.1046/j.1439-0434.2003.00716.x

Singh, B. K., Bardgett, R. D., Smith, P., and Reay, D. S. (2010). Microorganisms and climate change: terrestrial feedbacks and mitigation options. *Nat. Rev. Microbiol.* 8, 779–790. doi: 10.1038/nrmicro2439

Skiada, V., Avramidou, M., Bonfante, P., Genre, A., and Papadopoulou, K. K. (2020). An endophytic Fusarium-legume association is partially dependent on the common symbiotic signalling pathway. *New Phytol.* 226, 1429–1444. doi: 10.1111/nph.16457

Smith, K. P., and Goodman, R. M. (1999). Host variation for interactions with beneficial plant-associated microbes. *Annu. Rev. Phytopathol.* 37, 473–491. doi: 10.1146/annurev.phyto.37.1.473

Smith, V. L., Wilcox, W. F., and Harman, G. E. (1990). Potential for biological control of Phytophthora root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathology* 80, 880–885. doi: 10.1094/Phyto-80-880

Stephens, J. H. G., and Rask, H. M. (2000). Inoculant production and formulation. *Field Crop Res.* 65, 249–258. doi: 10.1016/S0378-4290(99)00090-8

Stocker, T. F., Qin, D., Plattner, G. -K., Tignor, M., Allen, S. K., Boschung, J., et al. (2013). Climate change 2013: The physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change.

Stoop, W. A., Uphoff, N., and Kassam, A. H. (2002). Research issues raised for the agricultural sciences by the system of Rice intensification (SRI) from Madagascar: opportunities for improving farming systems for resource-limited farmers. *Agric. Syst.* 71, 249–274. doi: 10.1016/S0308-521X(01)00070-1

Subramanian, P., Mageswari, A., Kim, K., Lee, Y., and Sa, T. (2015). Psychrotolerant endophytic *Pseudomonas* sp. strains OB155 and OS261 induced chilling resistance in tomato plants (*Solanum lycopersicum* Mill.) by activation of their antioxidant capacity. *Mol. Plant-Microbe Interact.* 28, 1073–1081. doi: 10.1094/MPMI-01-15-0021-R

Tewari, S., and Arora, N. K. (2016). "Soybean production under flooding stress and its mitigation using plant growth-promoting microbes" in *Environmental Stresses. Soybean Production. Vol. 2.* ed. M. Miransari (Oxford, United Kingdom: Academic Press), 23–40.

Thakur, A. K., and Uphoff, N. T. (2017). How the system of rice intensification can contribute to climate-smart agriculture. *Agron. J.* 109, 1163–1182. doi: 10.2134/agronj2016.03.0162

Thakur, A. K., Uphoff, N., and Antony, E. (2010). An assessment of physiological effects of system of rice intensification (SRI) practices compared to recommended rice cultivated practices in India. *Exp. Agric.* 46, 77–98. doi: 10.1017/S0014479709990548

Thu, T. T. P., Yasui, H., and Yamakawa, T. (2017). Effects of salt stress on plant growth characteristics and mineral content in diverse rice genotypes. *Soil Sci. Plant Nutr.* 53, 264–273. doi: 10.1080/00380768.2017.1323672

Trapet, P., Avoscan, L., Klinguer, A., Pateyron, S., Citerne, S., Chervin, C., et al. (2016). The *Pseudomonas fluorescens* siderophore pyoverdine weakens *Arabidopsis thaliana* defense in favor of growth in iron-deficient conditions. *Plant Physiol.* 171, 675–693. doi: 10.1104/pp.15.01537

Tucci, M., Ruocco, M., Masi, L. D., Palma, M. D., and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12, 341–354. doi: 10.1111/j.1364-3703.2010.00674.x

Vaishnav, A., and Choudhary, D. K. (2019). Regulation of drought-responsive gene expression in *Glycine max* L. Merrill is mediated through *Pseudomonas simiae* strain AU. *J. Plant Growth Regul.* 38, 333–342. doi: 10.1007/s00344-018-9846-3

Vejan, P., Khadiran, T., Abdullah, R., Ismail, S., and Dadrasnia, A. (2019). Encapsulation of plant growth promoting Rhizobacteria—prospects and potential in agricultural sector: a review. *J. Plant Nutr.* 42, 1–24. doi: 10.1080/01904167.2019.1659330

Vitti, A., Pellegrini, E., Nali, C., Lovelli, S., Sofo, A., Valerio, M., et al. (2016). *Trichoderma harzianum* T-22 induces systemic resistance in tomato infected by cucumber mosaic virus. *Front. Plant Sci.* 7:1520. doi: 10.3389/fpls.2016.01520

Waqas, M., Khan, A. L., Hamayun, M., Shahzad, R., Kang, S. -M., Kim, J. -G., et al. (2015). Endophytic fungi promote plant growth and mitigate the adverse effects of stem rot: an example of *Penicillium citrinum* and *Aspergillus terreus*. *J. Plant Interact.* 10, 1–18. doi: 10.1080/17429145.2015.1079743

Ward, J. M., and Schroeder, J. J. (1977). "Roles of ion channels in initation of signal transduction in higher plants" in *Signal transduction in plants. MCBU molecular and cell biology update*. ed. P. Aducci (Basel: Birkhäuser).

Weller, D. M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* 26, 379–407.

Wheeler, T., and Braun, J. V. (2013). Climate change impacts on global food security. *Science* 341, 508–513. doi: 10.1126/science.1239402

Whipps, J. M. (1997). Developments in the biological control of soil-borne plant pathogens. *Adv. Bot. Res.* 26, 1–134.

Whipps, J. M., and Lumsden, R. D. (1991). Biological control of *Pythium* species. *Biocontrol Sci. Tech.* 1, 75–90. doi: 10.1080/09583159109355188

Wilcox, W. F., Harman, G. E., and Dipietro, A. (1992). Effect of gliotoxin on growth, sporulation and zoospore motility of seven *Phytophthora* spp. in vitro. *Phytopathology* 82, 1121.

Woo, S. J., Ruocco, M., Vinale, F., Marra, R., Lombardi, N., Pascale, A., et al. (2014). *Trichoderma*-based and their widespread use in agriculture. *Open Mycology J.* 8, 71–126. doi: 10.2174/1874437001408010071

Xue, A. G. (2002). Gliocladium roseum strains useful for the control of fungal pathogens in plants. USA patent application 6, 495:133.

Xue, A. G., Chen, Y. H., Sant'anna, S. M. R., Voldeng, H. D., Fedak, G., Savard, M. E., et al. (2014). Efficacy of CLO-1 biofungicide in suppressing perithecial production by *Gibberella zeae* on crop residues. *Can. J. Plant Pathol.* 36, 161–169. doi: 10.1080/07060661.2014.881920

Yadav, S. K., Kachhwaha, S., and Kothari, S. L. (2010). Comparison of in vitro regeneration efficiency of leaf explants in response to different cytokinins and assessment of genetic uniformity of regenerated plants of *Solanum surattense* Burm.F. *African J. Biotech.* 9, 8991–8997.

Zafra, G., and Cortés-Espinosa, D. V. (2015). Biodegradation of polycyclic aromatic hydrocarbons by *Trichoderma* species: a mini review. *Environ. Sci. Pollut. Res.* 22, 19426–19433. doi: 10.1007/s11356-015-5602-4

Zayed, M. S. (2016). "Advances in formulation development technologies" in *Microbial inoculants in sustainable agricultural productivity*. eds. P. S. Dhananjaya, S. Pratap, D. P. Singh, B. S. Harikesh and R. Prabha (New Dehli: Springer, India).

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Bioprospecting of Beneficial Bacteria Traits Associated With Tomato Root in Greenhouse Environment Reveals That Sampling Sites Impact More Than the Root Compartment

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Tomato is subject to several diseases that affect both field- and greenhouse-grown crops. To select cost-effective potential biocontrol agents, we used laboratory throughput screening to identify bacterial strains with versatile characteristics suitable for multipurpose uses. The natural diversity of tomato root–associated bacterial communities was bioprospected under a real-world environment represented by an intensive tomato cultivation area characterized by extraseasonal productions in the greenhouse. Approximately 400 tomato root–associated bacterial isolates, in majority Gram-negative bacteria, were isolated from three compartments: the soil close to the root surface (rhizosphere, R), the root surface (rhizoplane, RP), and the root interior (endorhizosphere, E). A total of 33% of the isolates produced siderophores and were able to solubilize phosphates and grow on NA with 8% NaCl. A total of 30% of the root-associated bacteria showed antagonistic activity against all the tomato pathogens tested, i.e., *Clavibacter michiganesis* pv. *michiganensis*, *Pseudomonas syringae* pv. *tomato*, *Pseudomonas corrugata* and *Xanthomonas euvesicatoria* pv. *perforans*, and *Fusarium oxysporum* f. sp. *lycopersici*. We found that the sampling site rather than the root compartment of isolation influenced bacterial composition in terms of analyzed phenotype. This was demonstrated through a diversity analysis including general characteristics and PGPR traits, as well as biocontrol activity *in vitro*. Analysis of 16S rRNA gene (rDNA) sequencing of 77 culturable endophytic bacteria that shared multiple beneficial activity revealed a predominance of bacteria in Bacillales, Enterobacteriales, and Pseudomonadales. Their *in vitro* antagonistic activity showed that *Bacillus* species were significantly more active than the isolates in the other taxonomic group. *In planta* activity against phytopathogenic bacteria of a subset of *Bacillus* and *Pseudomonas* isolates was also assessed.

Keywords: microbiome, tomato, PGPR, BCA, endorhizosphere

INTRODUCTION

Tomato is one of the most widely grown vegetables and represents a major agricultural industry, with a global production of more than 180 million tons in 2018[1]. It is one of the vegetables that is most consumed in the world (second to potatoes) and is also one with the most beneficial effects on human health (He et al., 2006). Plant diseases seriously impact tomatoes in several geographical areas worldwide. At least 140 viral species have been reported, some of which have emerged in greenhouse grown tomato plants (Moriones and Verdin, 2020). Several bacterial species were described causing leaf spots, vascular diseases and roots (Catara and Bella, 2020). In addition, intensive greenhouse cropping systems have greatly facilitated the development of fungal and fungal-like diseases (Bardin and Gullino, 2020).

The intensive management required to mitigate serious economic losses has encouraged the search of alternative approach for the control of tomato diseases, including the use of biological control agents (BCAs) (Singh et al., 2017).

Instead of an independent entity, according to the most recent definition, the plant is regarded as a holobiont or "super organism" that is integrated with the microorganisms associated with it (microbiota) and their genetic information (often referred to as the microbiome) (Vandenkoornhuyse et al., 2015). The microbiome is involved in multiple plant functions, ranging from nutrition to resistance to biotic and abiotic factors (Hardoim et al., 2008; Mendes et al., 2011). The productivity, vigor, and resistance of the plant is therefore not only the direct consequence of the genetic makeup of the plant itself, but also of its microbiome or set of microorganisms (Philippot et al., 2013; Berg et al., 2016).

There is a relatively large body of information on the tomato microbiome as many studies have explored the mechanisms of microorganism selection in different compartments of the plants, also identifying beneficial microorganisms and potential candidates for biological control. Metagenomic studies based on amplicon sequencing have identified the microbial communities associated with different tomato plant organs (Ottesen et al., 2013). An interesting gradient with regard to the distance of each plant part from the soil has been observed as microbial diversity decreases as the distance from the soil increases (Ottesen et al., 2013; Dong et al., 2019). The most attention has been paid to the rhizosphere where there is a highly active microbial interaction as exudates released by plant roots are the main food source for microorganisms and a driving force for their population density and activities (Raaijmakers et al., 2009; Bulgarelli et al., 2013). A subset of rhizospheric microorganisms penetrates the plant roots and colonizes the endosphere (horizontal transmission) (Compant et al., 2010). Vertical transmission of bacterial endophytes via seeds has also been reported in different crops (Truyens et al., 2015; Cavazos et al., 2018; Rezki et al., 2018). These endophytes reside within plants with no obvious negative effects on the host, contributing to their growth and development and the ability to adapt to adverse conditions (Vandenkoornhuyse et al., 2015; Sinno et al., 2020). Tomato rhizosphere and endorhizosphere microbial communities have been investigated according to soil characteristics (Poli et al., 2016), genotypes (French et al., 2020; Taffner et al., 2020), crop management (Allard et al., 2016), rootstocks (Poudel et al., 2019), and soilborne pathogen infections (Li et al., 2014; Tian et al., 2015; Larousse et al., 2017). Overall, the results suggest that the tomato endophytic microbiome is mainly horizontally transferred from the soil environment (Poli et al., 2016; Chialva et al., 2018), but also vertically transmitted via seeds from where it can be transmitted to the subsequent plant generation (Bergna et al., 2018). Culture-dependent methods have been used to study microbial communities of the tomato root environment, mainly aimed at selecting plant growth-promoting rhizobacteria (PGPRs) and biocontrol agents (Abbamondi et al., 2016; Abdeljalil et al., 2016; Tian et al., 2017; Attia et al., 2020; Saqib et al., 2020). Microorganisms may have a neutral, pathogenic, or beneficial interaction with their host plant, and together with plant pathogens, beneficial microorganisms in the plants can interact in different ways with the plant (Raaijmakers et al., 2009). The main roles of beneficial microorganisms are biostimulation (or phytostimulation), i.e., the direct promotion of plant growth by the production of phytohormones (Bloemberg and Lugtenberg, 2001); biofertilization (Bashan, 1998), i.e., the promotion of plant growth generated by the microorganisms that facilitate accessibility to essential nutrients or increase the supply of nutrients to the plant; and biocontrol activity, i.e., the ability to control plant pathogens [biological control agents (BCAs)] through the competition for space and nutrients, the production of antibiotic substances, or the induction of resistance mechanisms (Bloemberg and Lugtenberg, 2001; Heimpel and Mills, 2017). Bacteria that share at least two of these mechanisms of action are known as PGPRs (Glick, 1995). The use of microorganisms, alone or combined in consortia, is foreseen as a method to positively modify the plant microbiome in order to improve the quantity and quality of agricultural crops. It has shown great potential as a low-environmental-impact alternative to agrochemicals and fertilizers (Ciancio et al., 2016; Woo and Pepe, 2018; Compant et al., 2019).

Microbiome studies based on metagenomics have greatly contributed to the understanding of the complex network established between the tomato rhizosphere and its microbiota. However, the information gained to date mainly refers to identify the microbiota, understanding where they come from and what the main driving conditions are that modify the microbiota but not what their actual role is. To date, cultivation-dependent methods have been used to isolate and characterize bacterial isolates from tomato plants exhibiting appreciable PGPR and BCA capabilities (Enya et al., 2007; Amaresan et al., 2012; Xu et al., 2014; Abbamondi et al., 2016; Romero et al., 2016; Tian et al., 2017; Attia et al., 2020; Saqib et al., 2020).

Vacheron et al. (2016) and Besset-Manzoni et al. (2019) used a systematic sampling method for comparing bacterial populations on maize and wheat, respectively, we adopted a similar approach in the current study. A similar approach was also used by Lemanceau et al. (1995) that demonstrated that *Pseudomonas* communities of the root compartments are

[1]http://faostat.fao.org, accessed September 20, 2020.

influenced by plant species (flax and tomato). While they faced the problem with biochemical features and genotyping aimed at taxonomic resolution of the problem (nowadays approached by metabarcoding), we focused on the phenotyping of the beneficial traits. This approach was applied within the framework of a project on tomato microbiota aimed at the selection of bacterial isolates to be used in microbial consortia for seed or plantlet bacterization in the nursery. We investigated the diversity of the cultivable bacterial population associated with the tomato root environment. In addition, we particularly focused on bacterial endophytes in terms of being beneficial biocontrol agents. Samples were collected from farms from a restricted area specialized in the intensive cultivation of tomato under a greenhouse environment in Ragusa province (Sicily). This is the principal production area in Italy that uses greenhouses covered by plastic films, with more than half of the national tomato production. This area is characterized by sandy soil, high salinity conditions, and favorable climatic conditions that permit extraseasonal productions (up to two cycles a year), above all of cherry tomato typologies.

The main findings of our work were as follows: (i) cultivable bacterial population sizes in the root are higher in the rhizosphere and in the rhizoplane than in the endosphere compartment; (ii) the site of isolation (i.e., farm and agricultural conditions) rather than the root compartment drives the phenotypic characteristic of bacterial populations; (iii) efficient cultivable bacteria from tomato endorhizosphere belong to Bacillales, Pseudomonadales, and Enterobacteriales order; (iv) *Bacillus* species are significantly more effective in inhibiting tomato plant pathogens *in vitro*; (v) preliminary *in vivo* results showed that some *Pseudomonas* and *Bacillus* isolates from endorhizosphere may protect tomato plants against plant pathogenic bacteria and thus deserve further investigation.

MATERIALS AND METHODS

Sampling of Tomato Root–Associated Bacteria

Tomato plants (*Solanum lycopersicum* L.) were grown in unheated greenhouses on four farms located in an area devoted to greenhouse vegetable production in Ragusa province (Sicily, Italy). The positions, soil proprieties, and genotypes are shown in **Table 1**. Plants were grown in agricultural soil and watered by drip irrigation, following standard agronomical practices. Five healthy plants from each farm were randomly selected from the central rows of each greenhouse, and their associated root material was collected at the fruiting stage, in March 2018. Plant stems were cut 30 cm above the root collar, and the five root systems were placed in a plastic bag and immediately transferred to the laboratory in a cooler. The samples were preserved at 4°C and processed within 24 h.

Tomato root–associated bacteria were isolated from three compartments: the soil close to the root surface (rhizosphere, R), the root surface (rhizoplane, RP), and the root interior (endorhizosphere, E). Samples were processed according to the protocol described by Normander and Prosser (2000) and Wieland et al. (2001), with some modifications, as follows:

- *Rhizosphere (R)*: Roots were shaken carefully to remove non-adhering soil. Five grams of soil adhering to the roots was manually collected and transferred in sterile 50 mL centrifuge tubes containing 20 mL of sterile saline buffer (0.85% NaCl) and then mixed thoroughly by vortex for 2 min.
- *Rhizoplane (RP)*: Roots (approximately 5 g), from which the rhizospheric soil had been dislodged, were soaked in 20 mL of sterile saline buffer (0.85% NaCl) and mixed thoroughly by vortex for 5 min.
- *Endorhizosphere (E)*: After treatment for rhizoplane bacteria extraction, roots (approximately 5 g) were sterilized with 75% ethanol (2 min), 50% sodium hypochlorite solution (2 min), and ethanol 75% (1 min) and rinsed five times in sterile distilled water (SDW). Sterility was assessed by placing the sterilized roots on potato dextrose agar (PDA, Oxoid, Milan, Italy) at 27°C for 4–7 days. A lack of bacterial growth ensured the sterility of the root surfaces. The roots were then homogenized with a sterile pestle and mortar in 20 mL of sterile saline buffer (0.85% NaCl).

Culturable Bacterial Population Sizes

Serial 10-fold dilutions in sterile saline buffer (0.85% NaCl) were prepared from each extract (R, RP, and E), and 0.1 mL of each dilution was plated onto the following media: plate count agar (Lickson, Palermo, Italy), supplemented with cycloheximide (100 $mg{\cdot}mL^{-1}$) to isolate and quantify the cultivable fast-growing bacteria; King's medium B agar, supplemented with cycloheximide (100 $mg{\cdot}mL^{-1}$) to count the fluorescent *pseudomonads* (King et al., 1954). In order to isolate spore-forming bacteria, each extract was heat-treated (90°C) for 10 min and mixed by vortex for 1 min (Janštová and Lukášová, 2001; Manzum and Al Mamun, 2018), and after serial 10 dilutions, 0.1 mL of suspensions were plated onto nutrient agar (NA; Oxoid, Milan, Italy) with cycloheximide (100 $mg{\cdot}mL^{-1}$). For each compartment, dilution, and medium, three replicates were performed. The inoculated plates were incubated at 27°C for 48–72 h. The number of bacterial colony-forming units (CFUs) was then counted by visual observation, and selected colonies were isolated in pure culture. The culturable population of tomato-associated bacteria was expressed as the log of the number of CFUs per gram of soil (rhizosphere) or of roots (rhizoplane and endorhizosphere).

The root-associated bacteria were selected from plates containing 30–300 colonies, i.e., typically 10^{-2} endorhizosphere (1:100) and 10^{-5} rhizosphere and rhizoplane dilutions (1:100,000). Bacterial colonies were selected according to their macromorphological diversity (size, color, and morphology of the colony), streaked twice on PDA medium, and checked for purity. After 24 h of incubation, single colonies of the

TABLE 1 | Data on sampling sites and number of bacterial isolates from the tomato root environment.

	Farm 1	Farm 2	Farm 3	Farm 4
Position				
Locality	Ispica (RG, Italy)	Ispica (RG, Italy)	Ragusa (Italy)	Vittoria (RG, Italy)
Geographic coordinates	36°42′35.62″ N 14°57′36.13″ E	36°42′59.08″ N 14°58′59.98″ E	36°51′3.24″ N 14°27′41.40″ E	36°56′40.49″ N 14°23′42.37″ E
Soil properties				
Soil texture	Sandy clay calcareous loamy	Sandy calcareous	Sandy calcareous	Sandy calcareous loamy
Organic matter (%)	1.93	2.1	1.07	2.5
Ph	7.57	7.72	7.71	7.7
Electrical conductivity (mmhos cm^{-1})	2.85	8.45	2.13	3.52
P (mg kg^{-1})	102	655	135	155
Zn (mg kg^{-1})	1.9	11.6	6.9	5.9
Mn (mg kg^{-1})	22.8	32.4	14.4	13.2
Cu (mg kg^{-1})	6.1	13.2	4.8	14.4
Fe (mg kg^{-1})	12.2	49.2	15.6	4.6
K (mg kg^{-1})	391	507	96	747
Mg (mg kg^{-1})	254	327	203	529
Na (mg kg^{-1})	158	340	156	290
Ca (mg kg^{-1})	221	925	202	290
Tomato genotype				
Typology	Cherry	Mini plum	Cherry	Mini plum
Genotype	Casarino F1	Dulcemiel F1	Creativo F1	Miele F1
Number of isolates	70	132	85	136

selected isolates were picked off and individually inoculated with a sterile toothpick in 96-microwell cell culture plates (Nunc^{TM} MicroWell^{TM} 96-well, collagen type i–treated, flat-bottom microplate, Thermo Fisher Scientific) containing Luria–Bertani (LB) broth. After overnight incubation, the wells were supplemented with 15% glycerol and stored at −80°C. For routine growth, isolated bacteria were picked off from stock cell cultures using an 8 × 6 replica plater (Sigma).

Phenotypic Characterization of Bacterial Isolates

General and PGPR Traits

Colonies of bacterial isolates were preliminarily characterized in terms of color, shape, opacity, size, and morphology. The Gram reaction was performed using the 3% KOH test (Schaad et al., 2001). The following features were assessed: siderophore production, salt tolerance, and phosphate solubilization. Bacterial isolates from 24 h-old cultures on PDA were plated using the replica-plate device (48 isolates per plate) in the respective media, and results were recorded for up to 3 days of incubation at 28°C. All strains were tested in three independent replicates.

To detect the phosphate solubilizing bacteria, bacterial isolates were streaked onto Pikovskaya's agar medium (Pikovskaya, 1948). Strains that induced a clear zone around the colonies were considered as positive. Siderophore production was determined on chrome-azurol S (CAS) medium (Schwyn and Neilands, 1987). The formation of orange to yellow halos around the colonies confirmed the production of siderophores. The salt tolerance was evaluated by inoculating the isolates on three NA plates containing 0, 2, and 8% NaCl. Bacterial isolates were classified based on their growth at different NaCl concentrations in the medium.

Antimicrobial Activity Against Tomato Pathogens

To phenotype the biocontrol activity potential of the tomato root–associated bacteria, these bacteria were screened for their antimicrobial activity against a set of tomato plant pathogens usually occurring in the area: the Gram-positive bacterium, *Clavibacter michiganensis* subspecies *michiganensis* strain PVCT156.1.1 (*Cmm*), and the Gram-negative bacteria, *Pseudomonas corrugata* strains CFBP5454 (*Pco*), *P. syringae* pv. tomato strains PVCT28.3.1 (*Pto*), *Xanthomonas euvesicatoria* pv. *perforans* strain NCPPB4321 (*Xep*), and *Fusarium oxysporum* f. sp. *lycopersici* strain Saitama ly2 (*Fol*) (**Table 2**).

The antagonistic activity against plant pathogenic bacteria was tested on large PDA plates (Ø 20 cm). Bacterial suspensions in SDW (OD_{600} = 0.01) were obtained from overnight cultures of the plant pathogenic bacteria in nutrient broth. A sterile swab was dipped into the inoculum tube and used to inoculate the plates by streaking the swab three times over the entire agar surface and then rotating the plate approximately 60 degrees each time, as in the Kirby–Bauer antibiotic resistance test (Hudzicki, 2009). After drying, the plates were spot-inoculated with bacterial isolates for testing using sterile toothpicks. Forty-eight bacteria were inoculated on each plate and incubated at 28°C for 1–5 days. The antagonistic activity was expressed as the width (mm) of the growth inhibition area of phytopathogenic bacterium around

TABLE 2 | Tomato pathogens, bacteria and fungi, used in this study.

Species	Strain*	Origin	Disease	References
Pseudomonas corrugata (*Pco*)	CFBP 5454	Italy	Pith necrosis	Trantas et al., 2015
Pseudomonas syringae pv. *tomato* (*Pto*)	PVCT 28.3.1	Italy	Bacterial speck	Bella and Catara, 1998
Clavibacter michiganensis subsp. *michiganensis* (*Cmm*)	PVCT 156.1.1	Italy	Bacterial wilt and canker	Ialacci et al., 2016
Xanthomonas euvesicatoria pv. *perforans* (*Xep*)	NCPPB 4321^T	United States	Bacterial spot	Constantin et al., 2016
Fusarium oxysporum f. sp. *lycopersici* (*Fol*)	Saitama ly2	Japan	*Fusarium* wilt	Hirano and Arie, 2006

**CFBP, International Center for Microbial Resources, French Collection for Plant-associated Bacteria, INRA, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, York, United Kingdom; PVCT, Patologia Vegetale, University of Catania, Catania, Italy.*

the bacterial colonies. The experiments were performed in three independent replicates.

To test the antagonistic activity against *Fol*, bacterial isolates were spot inoculated near the border of small PDA plates (Ø 6 cm, four bacteria per plate). After 24 h of incubation at 28°C, a mycelial plug (0.5 × 0.5 cm) from a 4 day-old culture of *Fol* was placed in the center of each plate. Plates inoculated only with the fungal plug served as the control. All strains were tested in three independent replicates. The antifungal activity was expressed as the percentage of growth inhibition (PGI) according to Vincent (1947): PGI (%) = 100 · (GC − GT)/GC, where GC represents the mean value of the fungus radius in the absence of the bacteria (control), and GT represents the mean value of the fungus radius in the presence of antagonistic bacteria (treatment). Antagonist activity was recorded after incubation at 28°C for up to 5–7 days. The comparison of the antagonistic activity of the bacterial strains was based on two arbitrary 0–3 scales. The antibacterial activity was scored based on the growth inhibition area size as follows: 0, no antagonism; 1, < 3 mm; 2, ≥ 3 and < 10 mm; 3, > 10 mm. Antifungal activity was scored based on the PGI against *Fol* as follows: 0, no inhibition, 1, PGI < 30%; 2, PGI 30–60%; 3, PGI > 60%.

Molecular Identification of the Bacterial Endophytes

The 16S rRNA gene region was amplified and sequenced for taxonomic identification. Bacterial DNA targets for colony PCR were prepared by thermal lysis (10 min at 100°C) of cell suspensions (OD_{600} = 0.01) in 200 μL of SDW. PCR amplicons were generated using the universal 16S rRNA primer pair, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primer set (Edwards et al., 1989; Lane, 1991). Master mixtures included 1 × Taq&Go G2 Hot Start colorless PCR Master Mix (Promega), 0.5 μM of each primer, and 1 μL of template in a total volume of 15 μL. Reactions were performed in a thermal cycler GeneAmp PCR system 9700, with the following thermal protocol: DNA denaturation for 5 min at 95°C, amplification (35 cycles) at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and ended with 10-min extension at 72°C. The 1,400 bp PCR products were analyzed by agarose gel electrophoresis [1.0% (wt/vol) agarose, 90 V, 50 min]. The DNA amplicons were quantified and sequenced by BMR Genomics (Padova, Italy).

Sequence Analysis and Construction of a Phylogenetic Tree

The sequences were searched against the nucleotide collection database at the National Center for Biotechnology Information (NCBI) nucleotide database using Basic Local Alignment Search Tool BLASTN[2]. Taxonomy information was assigned by the NCBI Taxonomy database according to the highest score sequence. Highly homologous sequences were aligned using Clustal-W algorithm within MEGA X; the regions of ambiguous alignment were edited manually and a neighbor-joining tree was generated (Kumar et al., 2018). Sequences were aligned by Clustal W within MEGA X. A phylogenetic tree was built including 16S rRNA gene sequences of the type strains identified by BLAST.

In vivo Biocontrol Activity Assays

Bacterial Pathogens and Antagonists' Inoculum Preparation

Of the 77 endophytes belonging to the genera *Pseudomonas* and *Bacillus*, 10 were selected to evaluate their biocontrol activity *in vivo* on tomato plants against *Cmm* and *Xep*. The endophytes were selected on the basis of their taxonomy, i.e., representativeness of the species and the results of *in vitro* test (**Supplementary Table 1**). The strains selected were *Bacillus velezensis* strains 261, 263, 265, and 306 and *Bacillus megaterium* strain 268; *Pseudomonas citronellolis* strain f1, *Pseudomonas monteilii* strain f53, and *Pseudomonas plecoglossicida* strains 171, 172, and f56. The inoculum of both pathogens and putative biocontrol agents was prepared from bacterial cells grown for 48 h on NDA. Single colonies were transferred into LB broth and incubated at 27°C ± 1°C for 24 h in an orbital shaker at 150 revolutions/min (rpm). The bacterial cultures were centrifuged at 7,500 rpm for 15 min. The pellets were resuspended in sterile tap water, and the density adjusted to 2×10^8 CFU·mL^{-1} (OD_{600} = 0.1).

Plant Material and Inoculation of Bacterial Endophytes

Plantlets of tomato SIR ELYAN F1 3 weeks after germination were obtained from a local nursery and transplanted into square pots (8 cm side) containing nursery peat. In each trial, the pots were arranged in a completely randomized design, with 15 replicates per treatment. Independent trials were set up to assess the effect of the 10 endophytic strains

[2]http://www.ncbi.nlm.nih.gov

on (i) PGP activity, (ii) biocontrol of bacterial canker, and (iii) biocontrol of bacterial spot. Plants were maintained in a growth chamber at 24°C ± 2°C, 68–80% RH, with 16 h of light and 8 h of darkness daily. They were watered as required with the same amount of tap water per pot. All experiments were conducted in duplicate. In all trials, bacterial endophytes were inoculated by soil drenching with 20 mL inoculum. In the PGP trial 30 days after soil treatment, tomato seedlings were harvested. Height, fresh, and dry weight of roots and shoots and shoot-to-root ratio were measured. To determine the dry weight, the samples were dried at 105°C for 24 h. These parameters were compared to mock control plants drenched with tap water.

Plant Challenge With Bacterial Pathogens

Tomato seedlings were inoculated with *Cmm*, bacterial suspension 7 days after treatment with the putative BCAs or water (negative control). Aliquots of 20 mL of *Cmm* were poured into the soil near the stem crown. The roots were then damaged in order to facilitate bacterial penetration by inserting a scalpel at three points located 2 cm from the stem. Bacterial canker symptoms were recorded weekly for 1 month using a 0–5 disease scale developed for root inoculations, where 0 = no symptoms, 1 = chlorosis and loss of turgor, 2 = wilt in 1 or 2 leaves, and/or cankers < 0.5, 3 = wilt in 3 or more leaves, and/or cankers > 0.5, 4 = fully withered plants, and 5 = dead plants (Bella et al., 2012).

The area under disease progress curve (AUDPC) was calculated using weekly recorded data, as described by Madden and Campbell (1990). Using hand-trigger sprayers 3 days after the soil treatment with the putative BCAs or water (negative control), tomato seedlings were spray inoculated with *Xep* onto the abaxial and adaxial leaf surfaces of four replicate tomato plants until runoff. The inoculated plants were preincubated and postincubated for 1 day under transparent polyethylene sheets to increase the RH near 100% to promote bacterial penetration. Ten tomato leaflets per plant were sampled randomly 10 days after pathogen inoculation. Lesions on individual leaflets were counted and leaflet area determined; disease severity was quantified as number of lesions/cm^2 leaflet area (Ji et al., 2006). The leaflet area was obtained by image processing and analysis in Java (ImageJ software). Disease severity data were log transformed and subjected to analysis of variance (ANOVA). Percentage reduction in disease severity compared to the pathogen-only control was calculated according to Ji et al. (2006).

Statistical Analysis

The results of the screening indices were used to perform a principal component analysis (PCA) to detect patterns of similarity among the tomato root–associated bacteria. The PCA was calculated on binary data (0, isolate negative to the test; 1, isolate positive to test) using the "prcomp" function of the "stat" R package (R Core Team, 2013). PCA biplot and loading projections were visualized through the "factoextra" R package (Kassambara and Mundt, 2016). Mosaic plots were drawn using the "stat" R package; the same package was also used to compute ANOVA and the *post hoc* Tukey–Kramer test. Data of biocontrol assays were analyzed by ANOVA using STATGRAPHICS Plus 5. Mean values were compared using the Student–Newman–Keuls test.

RESULTS

Bacterial Population Size in Tomato Root Environment

Cultivable population sizes of total, fluorescent, and spore-forming bacteria in the rhizospheric soil (R) of the four farms ranged from 6.8 to 8.8, from 3.8 to 4.5, and from 3.3 to 6.4 log CFU · g^{-1}, respectively (**Figures 1A–C**). On each farm, the populations were higher in the rhizosphere than in the endorhizosphere (E) (ANOVA; $p < 0.05$) (**Figures 1A–C**). Population sizes in the rhizoplane (RP) and in the endorhizosphere ranged from 6.8 to 8.1 and from 3.7 to 6.4 log CFU · g^{-1} for total bacteria, and from 3.8 to 4.6 and from 2.3 to 3.5 log CFU · g^{-1} for fluorescent bacteria, in the two root compartments, respectively (**Figures 1A,B**). The population sizes of spore-forming bacteria ranged from 3.6 to 6.4

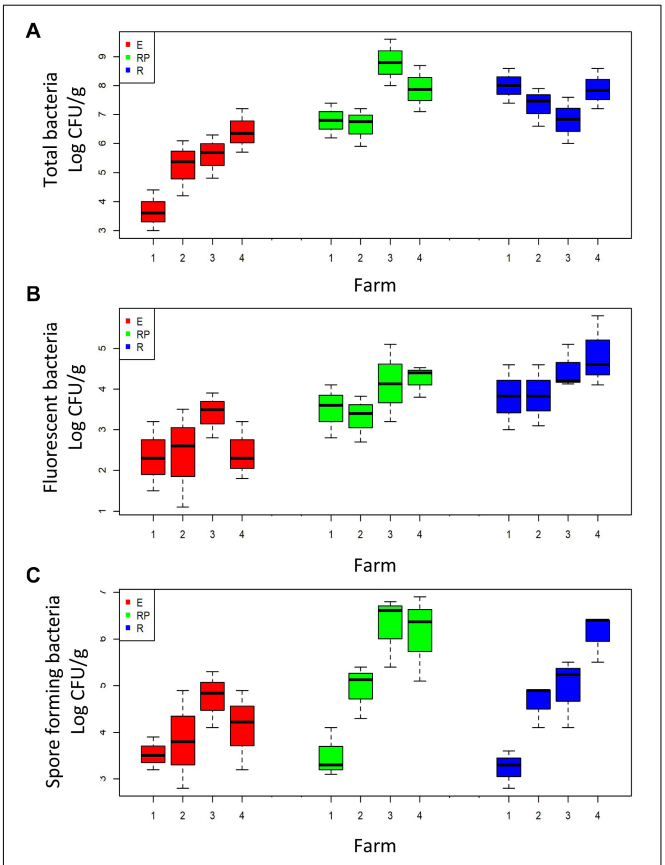

FIGURE 1 | Boxplot of the total **(A)**, fluorescent **(B)**, and spore-forming **(C)** cultivable bacteria in the three root compartments: rhizosphere (R, in red), rhizoplane (RP, in green), and endorhizosphere (E, in blue). Bacteria are grouped according to the farm on which they were collected (*x*-axis).

and from 3.5 to 4.8 log CFU·g^{-1}, for the two root compartments, respectively (**Figure 1C**).

Beneficial Phenotypes of Bacteria From the Root Environment of Tomato Grown in Agricultural Soil

A total of 424 culturable bacterial strains were obtained in pure culture from the isolation plates of the four farms (70, 132, 85, and 136 isolates for farms 1–4, respectively). The percentage of Gram-negative bacteria in the three compartments was 61, 86, and 78% for R, RP, and E, respectively. Among these, fluorescent pseudomonas represented approximately 18.2, 38.6, and 43.2% of the isolates obtained from the R, RP, and E, respectively (**Supplementary Table 1**).

A total of 83.5, 86, and 89% of bacterial isolates from the R, RP, and E, respectively, were able to grow in up to 8% NaCl (**Supplementary Table 1**). The production of siderophores on CAS agar was found in a similar relative frequency in the three rhizoplanes (33, 34, and 30% in R, RP, and E) (**Figure 2A** and **Supplementary Table 1**). A total of 64% of the endophytic isolates showed an ability to solubilize insoluble organic phosphate, whereas the number of isolates showing the same characteristic was 46.5 and 29.5% in R and RP, respectively (**Figure 2B** and **Supplementary Table 1**). All the isolates exhibited at least one of the three PGP traits tested (siderophore production, phosphate solubilization, and tolerance to salinity), and most of the strains tested positive for at least two of the three traits, with 139 of the 424 isolates tested showing the three positive features: tolerance to salinity, siderophore production, and phosphate solubilization (**Supplementary Table 1**).

The tomato root–associated bacterial collection was further screened for the antagonistic ability to inhibit *in vitro* the growth five detrimental tomato phytopathogens (**Table 2** and **Figures 2C–E**). All isolates were therefore tested against the Gram-positive *C. michiganensis* subspecies *michiganensis* strain PVCT156.1.1 (*Cmm*), *P. corrugata* strain CFBP5454 (*Pco*), *Pseudomonas syringae* pv. tomato strain PVTC28.3.1 (*Pto*), *X. euvesicatoria* pv. *perforans* strain NCPPB4321 (*Xep*), and the fungus *F. oxysporum* f. sp. *lycopersici* strain Saitama ly2 (*Fol*). Approximately 30% of the tomato root–associated bacteria (127 of 424 isolates) showed antagonistic activity against all the tested bacterial phytopathogens and *Fol* (**Figure 3** and **Supplementary Table 1**). Of these, 42, 26, and 31% were isolated from R, RP, and E compartments, respectively. The highest activity in terms of the number of antagonistic strains but also effectiveness in terms of inhibition zone was observed against *Cmm* (88% of the isolates) (**Supplementary Figure 1**). Among this group, 98% were ranked within class 3 (inhibition halo > 10 mm). The lowest number of antagonistic bacteria was detected against *Pco* (40%), and the antagonistic activity was ranked with 1 in the scale of activity (< 3 mm). An intermediate behavior was observed against the other two plant pathogenic bacteria (**Supplementary Figure 1**).

The *in vitro* inhibition of Fol was observed, although to different extents, by all but three tomato root–associated bacterial isolates (**Supplementary Figure 1**). The percentage of bacterial isolates with antifungal activity was the highest for RP (33%), followed by R (32%) and E (21%). PGI values of the fungal colonies ranged from 8 to 100% after incubation for 6 days at 24°C (when the colonies on control plates reached the margin). Based on growth inhibition scores (0–3) exhibited toward *Fol*, 214 isolates were ranked in class 2, indicating that their relative percentages of growth inhibition were less than 30% (**Supplementary Figure 1**). Interestingly, 60 isolates led to more than 60% inhibition of pathogen growth and were thus ranked in class 3 (**Supplementary Figure 1**).

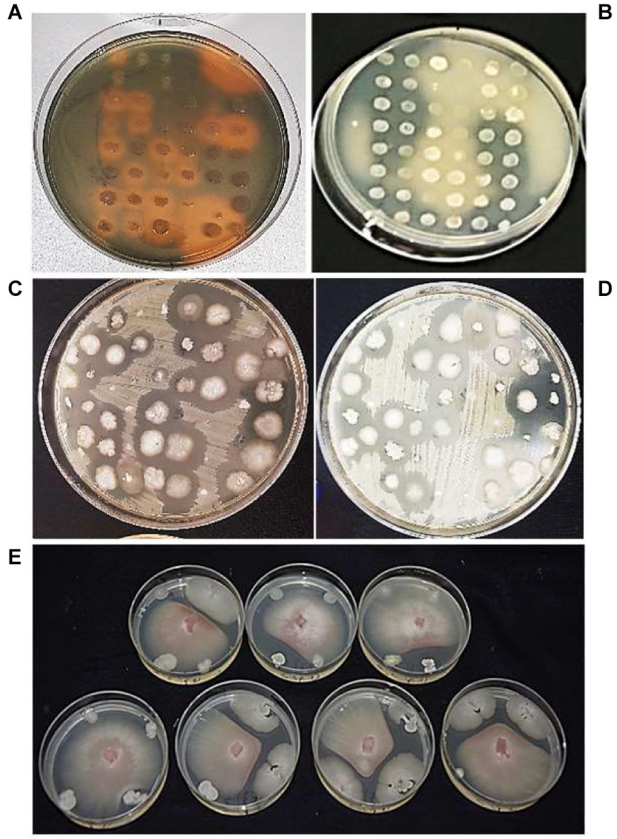

FIGURE 2 | Bacterial isolates, tested for **(A)** siderophore production, orange halos indicate siderophore positive results; **(B)** phosphate solubilization, cleared halos indicate phosphate solubilization positive results; antagonistic activity against **(C)** *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*), **(D)** *Pseudomonas syringae* pv. tomato (*Pto*), and **(E)** *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*).

Source of Isolation Drives Beneficial Traits of Bacterial Isolates

PCA (**Figures 4A,B**) was used to visualize the relationships between the 10 phenotypic traits analyzed (Gram reaction, fluorescence production, siderophore production, phosphate solubilization, salt tolerance, antagonist activity against *Cmm*, *Pco*, *Pto*, *Xep*, and *Fol*) of all bacterial isolates and the source of isolation (farm; root compartment). The first two principal components (PCs) explained 41% of the total phenotypic variability (PC1 = 25.3%, PC2 = 15.7%; **Figures 4A,B**). Results

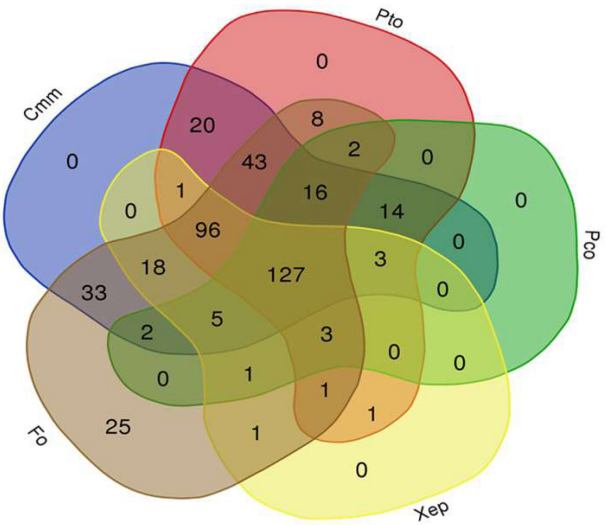

FIGURE 3 | Venn diagram showing the antagonist activity of a collection of 424 bacterial isolates obtained from tomato root environment against the tomato phytopathogenic bacteria *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), *Pseudomonas corrugata* (*Pco*), *P. syringae* pv. *tomato* (*Pto*), and *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*), and the fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) (http://bioinformatics.psb.ugent.be/webtools/Venn/).

enabled the bacteria to be clearly separated according to the farm in which they were isolated (**Figure 4A**), but not to the root compartment (data not shown): bacteria collected on Farm 1 were mainly separated according to PC2, whereas bacteria from Farms 2 and 3 clustered mainly in the upper-right PCA quadrant (PC1 > 0, PC2 > 0); and bacteria collected from Farm 4 were mainly plotted in the lower-right (PC1 < 0, PC2 > 0) and lower-left (PC1 and PC2 < 0) PCA quadrants (**Supplementary Table 2**). The variables greatly influencing the bacteria disposition along the first two PCs were the antagonistic activity against *Pto*, *Pco*, and *Xep* and siderophore production. The antagonistic activity against *Pto* and the siderophore production highlighted opposite directions in the PCA biplot as they were oriented toward the upper-right quadrant and lower-left quadrants, respectively. On the other hand, the antagonist activity against *Xep* and *Pco* was oriented toward the lower-right PCA quadrant.

Overall, the 10 traits employed in the PCA showed a pairwise correlation ranging from -0.45 (p-value < 2.2–16) for siderophore production and antagonist activity against *Pto* to 0.34 (p < 1.1–12) for antagonist activity against *Xep* and *Pto* (**Figure 4C**). An ANOVA test using the collection farm and the 10 traits as categorical variables showed p-values that exceeded the significance threshold ($p < 0.05$) for all traits tested. The traits showing the highest significance ($p < 0.0001$) were the Gram reaction, siderophore production, and the antagonist activity against *Cmm*, *Pto*, *Pco*, and *Fol*.

The bacteria distribution among the four farms was consistent for siderophore production and antagonist activity against *Cmm* and *Pto* (**Figures 5A–J**). Isolates collected from Farm 2 and Farm 3 were characterized by a substantial absence of siderophore production (**Figures 5A,B**) and positive antagonist activity against *Cmm* and *Pto* in all the samples (**Figures 5C–F**), whereas a more admixed configuration was registered for Farm 1 and Farm 4 (**Figures 5C–F**).

The *Pco* antagonistic activity showed statistical differences among all the four farms analyzed (**Figures 5G,H**), with bacteria collected on Farm 3 and Farm 1 showing the highest and lowest number of *Pco* antagonistic activity, respectively (**Figure 5G**). The antagonist activity against *Fol* was detected on all farms (**Figures 5I,J**).

Bioprospecting of Tomato Endophytic Bacteria

Of the 100 total tomato root bacterial endophytes in the working collection, 77 were selected based on their phenotype and representativeness of the PGP and BCA traits, with at least two and/or three PGP traits and antagonistic activity to at least three microorganisms. Partial sequences of the 16S rRNA genes of the 77 isolates obtained from the E were analyzed. According to BLASTN similarity matches, isolates were identified by partial sequencing of their 16S rRNA gene, which enabled the isolates to be classified into three orders, namely, Bacillales, with all the bacterial isolates belonging to the genus *Bacillus*; Pseudomonadales, with bacterial isolates in the genera *Pseudomonas* and *Acinetobacter*; Enterobacteriales with isolates in the genera *Enterobacter*, *Ewingella*, *Pantoea*, *Providencia*, and *Lelliottia*. Putative single-isolate taxon is shown in **Supplementary Table 3**.

Four different *Bacillus* species were identified, three strains with 100% similarity to *Bacillus subtilis* (GenBank accession no CP051860.1, MT081484.1, KU729674.1); two strains with 100% similarity to *Bacillus amyloliquefaciens* (GenBank acc. no MK501609.1); 11 strains with 99–100% similarity to *B. velenzensis* (GenBank acc. no MN559711.1, CP051463.1, KY927398.1, MT365117.1, MN654121.1, and CP024922.1) (all species of the *B. subtilis* clade, Fan et al., 2017); one strain with 99% similarity to *B. megaterium* (GenBank accession no KT883839.1). Two strains were only identified at the genus level as *Bacillus* species (100% similarity to GenBank accession no. CP040881.1).

For isolates among the Enterobacteriales, the best hits were observed with the following species: 10 strains with 97% similarity to *Enterobacter cancerogenus* (GenBank accession no. FJ976582.1); one strain with 97% similarity to *Enterobacter tabaci* (GenBank accession no. MF682952.1); one strain with 97% similarity to *Enterobacter mori* (GenBank accession no. KJ589489.1); 10 strains were only identified at the genus level as *Lelliottia* (96–97% similarity to strain GenBank accession no. JN853247.1); three strains with 98–100% similarity to *Ewingella americana* (GenBank accession no. MT101745.1 and KY126991.1). Three strains with 99% similarity to *Providencia vermicola* (GenBank accession no. KX394623.1 and MK942706.1). Four strains were only identified at the genus level as *Pantoea* species (97% similarity to strains GenBank accession no. MK229045.1 and MH884045.1).

Different species were found in the genus *Pseudomonas* all within the *Pseudomonas putida* group within the *Pseudomonas*

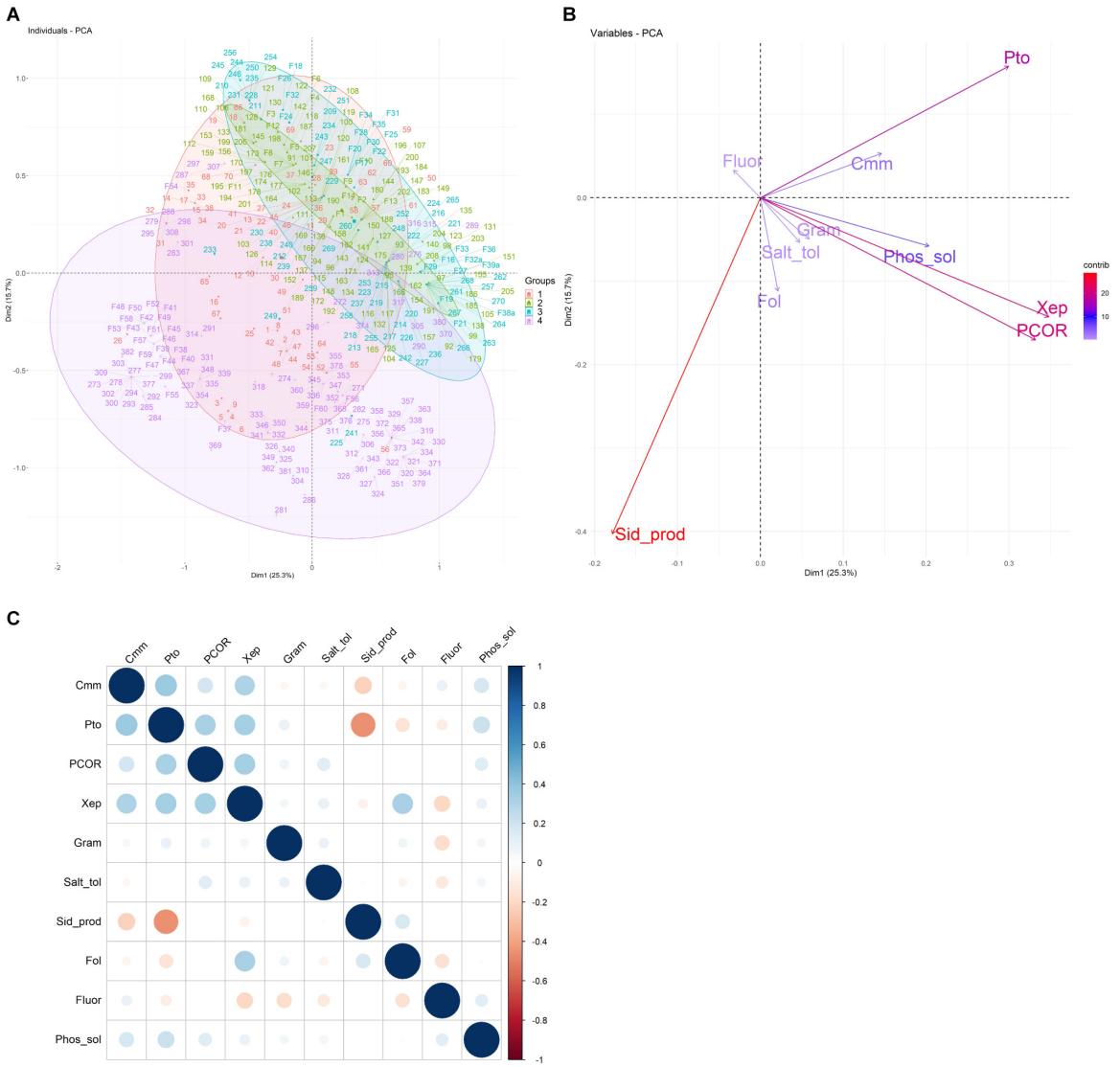

FIGURE 4 | Principal component analysis (PCA) of the characteristics related to the collection of 424 bacterial isolates. **(A)** The first two principal components are shown in a biplot, and bacterial isolates are colored according to the farm on which they were isolated. **(B)** Loading plot of the 10 traits used to compute the PCA, namely, antagonist activity against *Cmm*, *Pco*, *Pto*, *Xep*, and *Fol* (*Cmm*, *Pco*, *Pto*, *Xep*, and *Fol*), fluorescence on King's medium B agar (Fluor), Gram reaction (Gram), phosphate solubilization (Phos_sol), salt tolerance (Salt_tol), and siderophore production (Sid_prod). **(C)** Heatmap of the pairwise correlations between the traits analyzed.

fluorescens lineage (Mulet et al., 2010): 14 strains with 100% similarity to *P. plecoglossicida* (GenBank accession no. MT367715.1); one strain with 100% similarity to *P. citronellolis* (GenkBank accession no. KM210226.1); one strain with 100% similarity to *P. monteilii* (GenkBank accession no. MH603875.1); four strains with 100% similarity to *P. putida* (GenkBank accession no. LN866622.1 and CP026115.2). All the isolates in the genus *Acinetobacter* showed the highest similarity to *Acinetobacter baumannii* (99–100% similarity to GenBank accession no. MT256198.1 and CP050388.1).

In the dendrogram showing the phylogenetic relationships of the endophytic strains in which type strains of the putative bacterial species and some reference species were included, the taxonomic position was confirmed, although some isolates clustered with appropriate the taxonomic clade (e.g., *B. subtilis* or *P. putida* clade) and not with the type strain of the bacterial species resulted from the BLAST similarity analysis. For this reason, sequences of the isolates were deposited at GenBank with the genus and strain name under accession numbers from MW130753 to MW130829 (**Figure 6** and **Supplementary Tables 3**, **4**).

The PCA calculated on the 77 endophytic bacteria showing antagonist activity to at least one pathogen is shown in **Figure 7A**. The first two PCs accounted for 58.8% of the

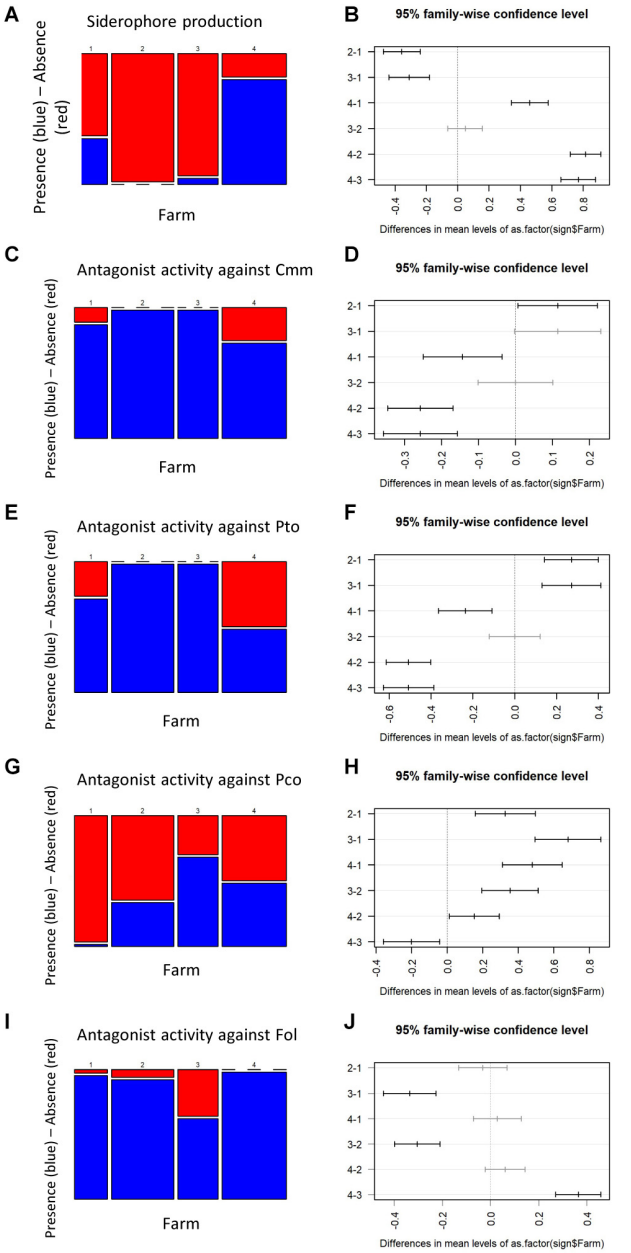

FIGURE 5 | Mosaic plot and Tukey plot of the traits showing the highest differentiation among farms (ANOVA test, $p < 0.0001$), namely, siderophore production **(A)**, antagonistic activity against *Cmm* **(C)**, *Pto* **(E)**, *Pco* **(G)**, and *Fol* **(I)**. Mosaic plots [left of the panels **(B**,**D**,**F**,**H**,**J)**] show the relative frequency of the presence (blue) or absence (red) of a trait (*y*-axis) given the farm (*x*-axis); the width of the columns is proportional to the numerosity of the accessions isolated on each farm. On the right of the panels, the confidence intervals are shown of each pairwise comparison after the Tukey *post hoc* test. Pairwise comparisons that were not statistically different are shown in gray.

total phenotypic variability, with PC1 accounting for 33.1% and PC2 for 25.7%. Bacillales were mainly plotted in the upper-right quadrant of the PCA biplot (PC1 and PC2 > 0), whereas both Enterobacteriales and Pseudomonadales were mainly characterized by PC1 negative values (resulting in a high prevalence of bacteria plotted in the upper-left and lower-left PCA quadrants). The high effectiveness of PC1 in distinguishing between the Bacillales compared to the other two families was confirmed by the ANOVA test, which showed a $p = 0.00003$ (**Figures 7B–D**).

In Planta Bioassays

Tomato seedlings treated by soil drenching with 10 bacterial strains belonging to the genus *Pseudomonas* and *Bacillus,* selected from the endorhizosphere isolated bacteria data set, showed an increase in plant height as compared to water treated control seedlings. Thirty days after the treatment with the bacterial strains, tomato seedlings were from 1.3 to 22% higher than the control plants. *Pseudomonas* strains f56 and f1 and *Bacillus* strain 306 and 261 significantly promoted plant height compared to untreated controls ($p < 0.05$) (**Supplementary Table 5**). Variable results were recorded for the other growth parameters that did not show a clear effect on plant weight (fresh and dry) and dry matter percentage (**Supplementary Table 5**).

Symptoms of bacterial canker, caused by *Cmm*, were first observed in the control plants 14 days postinoculation (dpi). They consisted in the unilateral wilting of one or more leaflets. Generalized wilting symptoms started at 21 dpi. Thirty days postinoculation of *Cmm*, the disease indexes of the plants treated with *P. citronellolis* strain f1 and *B. velenzensis* strain 265 were significantly lower ($p < 0.05$) than that of control plants treated with a water. Both antagonistic isolates also reduced the percentage of dead plants; these were for the *P. citronellolis* strain f1, *B. velenzensis* strain 265, and control plants, 0, 14.30, and 75%, respectively (**Table 3**). The values of the AUDPC, which records the progression of the disease, although were not significant statistically, were also lower (**Table 3**). The effect of the soil treatments with the tomato bacterial endophytes was also evaluated on the tomato leaf pathogen *Xep*. The occurrence of lesions on leaves was observed on positive control plants treated with water starting from 6 dpi. In fact, there were minute chlorotic spots that turned necrotic and expanded by 10 dpi when the data were recorded. Significant differences were observed between the endophyte-treated plants that showed fewer spots than control plants, although differences were observed between bacterial strains. A reduction of the diseases ranging from 30 to 80% was observed (**Table 3**).

DISCUSSION

The main aim of this study was to establish a collection of culturable tomato root–associated bacteria, as well as to bioprospect the natural diversity of root-associated bacterial communities under a real-world environment represented here by an intensive tomato cultivation area characterized by extraseasonal greenhouse production. Although in recent years the advances in next-generation omic technologies have led to the possibility of revealing plant-associated microbiomes, culture-dependent methods are still necessary to bioprospect natural

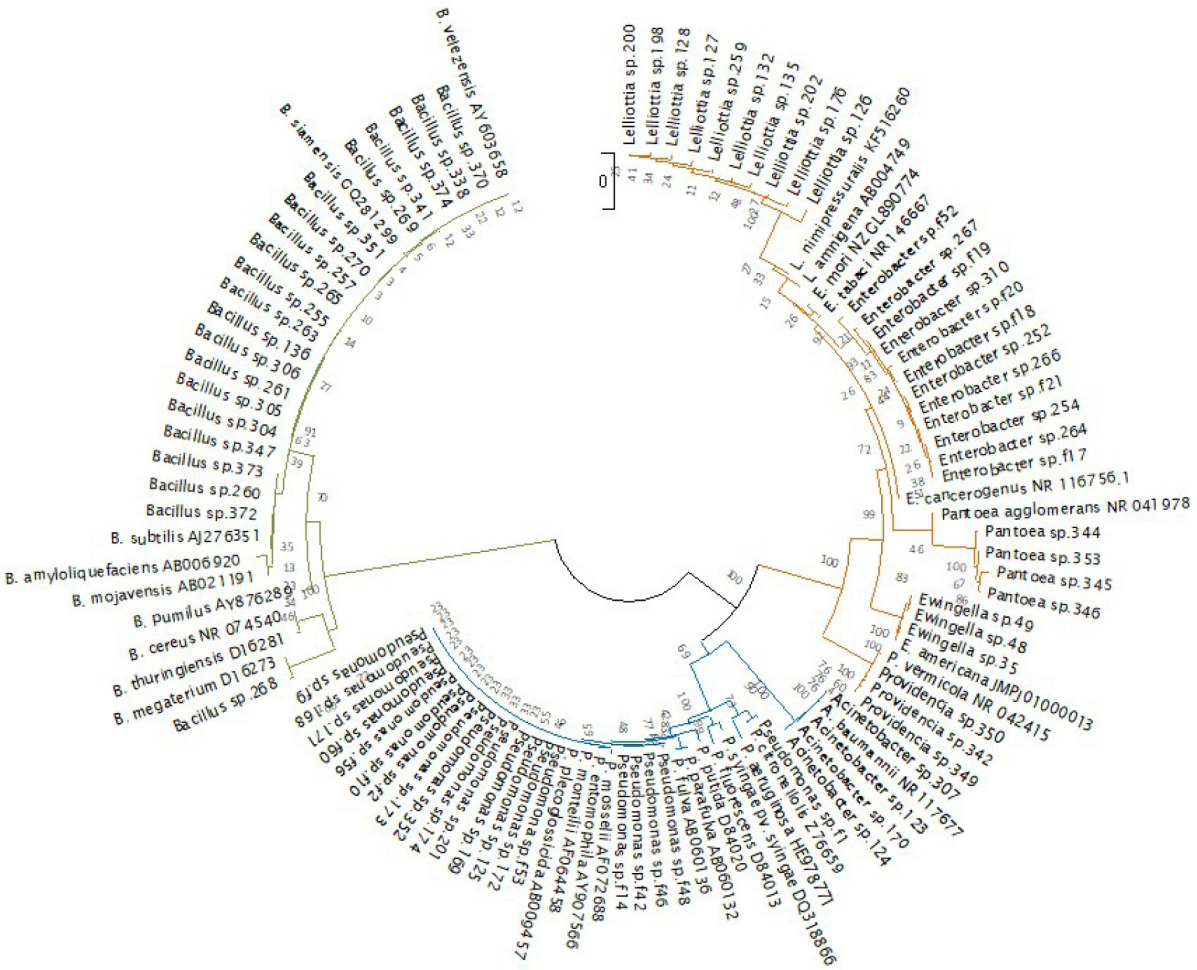

FIGURE 6 | Phylogenetic tree of the 77 endophytic strains isolated in this study and 29 bacterial type strains. (*Bacillales* in green, *Pseudomonadales* in blue, and *Enterobacteriales* in orange). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). There were a total of 824 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

diversity as a source of new tools for sustainable agriculture (Quiza et al., 2015; Lee et al., 2016; Müller et al., 2016).

In this study, bacterial population sizes of the total numbers of fluorescent and spore-forming bacteria associated with the root environment of greenhouse tomato plants grown in agricultural soils from four different farms varied according to the compartment of isolation (i.e., rhizosphere, rhizoplane, endosphere) and, in some cases, the farms. The characterization of a collection of 424 bacterial isolates targeted at phenotypic traits (plant growth promotion and/or biocontrol of detrimental plant pathogens) did not show any clear relationships between the compartments of root isolation. In contrast, the isolates clustered according to the four isolation farms.

The four farms from where the samples were selected shared some common features of the cultivation area. Sicily is the principal tomato greenhouse production area in Italy, and more than half of the tomato production comes from the Province of Ragusa, where the four farms were located. This area is characterized by sandy soil and climatic conditions that facilitate out-of-season production. Tomatoes are grown for one or two cycles within the year in greenhouses covered with a plastic film. In the four greenhouses, four different genotypes of tomato were cultivated, which differed according to the farm management (irrigation, fertilization, and pesticide use and agronomic operations).

Overall, the phenotyping of 424 bacterial isolates from the tomato root environment revealed that this community was more represented by Gram-negative than Gram-positive bacteria and that they possessed interesting PGP bacterial traits. In fact, 139 of the 424 root-associated bacteria isolates were able to produce siderophores, solubilize phosphates, and grow on a saline medium.

These characteristics could be of great interest in developing bioinoculant with also biofertilizer abilities that could also promote plant growth and yield. Phosphate-solubilizing microorganisms play an important role in supplementing

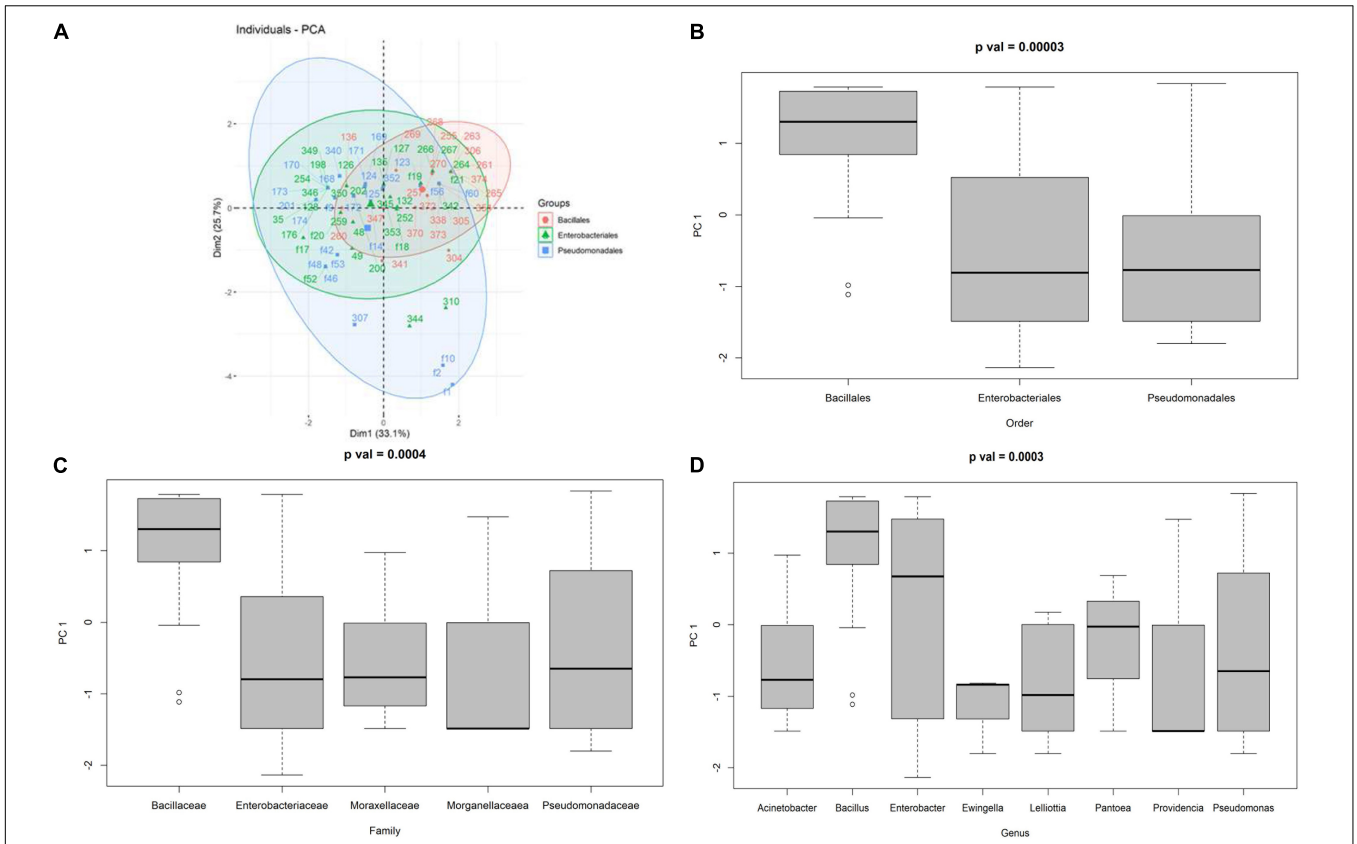

FIGURE 7 | (A) PCA of the antagonist activity of the endophytic bacteria against *Cmm*, *Pco*, *Pto*, *Xep*, and *Fol*, boxplot of the distribution of the first principal component given the order **(B)**, family **(C)**, and genus **(D).**

TABLE 3 | Results of the *in vivo* assays of the biocontrol activity of bacterial endophytes against the tomato bacterial pathogens *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) and *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*).

	Cmm			Xep	
Bacterial strains	**DI 30 dpi[a]**	**AUDPC**	**% of dead plants**	**n. spot/cm^2 leaf area[b]**	**% reduction[c]**
Pseudomonas plecoglossicida _171	4.71cd	51.78d	84.70%	4.02bc	43.50 ± 6.96
P. plecoglossicida _172	4.00abc	35.29abc	57.14%	1.46a	79.50 ± 1.79
*Pseudomonas citronellolis*_f1	2.28a	15.86a	0.00%	4.88c	31.40 ± 14.76
*Pseudomonas monteilii*_f53	4.14abc	40.43abc	57.14%	2.99abc	57.91 ± 1.14
*P. plecoglossicida*_f56	4.71cd	33.00abc	84.70%	4.88c	31.31 ± 12.39
Bacillus velezensis _261	3.28abc	24.86abc	14.30%	2.00ab	71.83 ± 4.40
*B. velezensis*_263	5.00c	47.64bc	100%	2.16ab	69.61 ± 6.53
B. velezensis _265	2.71ab	30.35abc	14.30%	1.30a	81.70 ± 3.20
*Bacillus megaterium*_268	3.00abc	22.71ab	28.57%	2.70ab	61.96 ± 5.67
B. velezensis _306	3.00abc	32.93abc	14.30%	2.06ab	70.97 ± 6.82
Positive control	4.71cd	36.92abc	71.42%	7.11d	/

[a] *DI 30 dpi: disease index based on a 0–5-point disease scale 30 days post-inoculation.*
[b] *Disease severity recorded as number of spot/cm^2 leaf area assessed 10 days post–Xep inoculation.*
[c] *Percentage reduction in lesion numbers per unit leaf area compared to the pathogen-only control ± standard error.*
Means in a column followed by the same letter are not significantly different according to Student–Newman–Keuls test at $p \leq 0.05$.
AUDPC, area under the disease progress curve. http://bioinformatics.psb.ugent.be/webtools/Venn/.

phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. P-solubilizing activity is related to the microbial production of organic acids, which chelate the cation bound to phosphate, thereby converting it to a soluble form (Sagoe et al., 1998; Rashid et al., 2004; Lugtenberg and Kamilova, 2009). The ability to produce secondary metabolites such as siderophore

and antimicrobial peptides has been evaluated in many studies on PGPRs. The ability to produce siderophore and metabolites contributing to antibiosis has been the focus of many studies on PGPR (Sayyed et al., 2004; Maksimov et al., 2011).

The high number of salinity-tolerant bacterial isolates suggests that a selection may have occurred as salinity is one of the typical characteristics of the area (i.e., soils of all the farms showed an EC > 2.0 mmhos cm^{-1} and high Na content). Tomato is moderately sensitive to salinity saline water that is used in greenhouse cultivations; however, high salinity may affect plant physiology (Leonardi and Martorana, 2005). Several reports have shown that halotolerant PGPRs improve the growth of various agricultural crops under salinity stress. Inoculating crops with halotolerant PGPRs isolated from halophytes has been successful in improving crop growth and tolerance under salt stress conditions (Shukla et al., 2012; Khan et al., 2016).

More importantly, approximately 30% (129 strains) of the root-associated bacterial isolates showed antagonistic activity against all the five tested phytopathogens, although to different extents. Their antagonistic activity as assessed *in vitro* suggests the production of secondary metabolites with inhibitory activity against fungi and Gram-positive and Gram-negative plant pathogenic bacteria. Some of these harmful pathogen are seed and/or soil transmitted (Bardin and Gullino, 2020; Catara and Bella, 2020).

A large number of studies have shown that tomato bacterial communities, resolved by metagenomics based on amplicon sequencing, are influenced by different factors. Data, however, refer to the taxonomic operational units, and the PGP and BC activities can only be inferred. Among the biotic and abiotic factors, soil is considered the primary force driving plant–microbiota diversity (Jeanbille et al., 2016). Different studies have demonstrated that the influence of the soil plays a stronger role on plant–microbiota diversity than the plant genotype (Poli et al., 2016; Dong et al., 2019). In addition, transcriptomics and proteomics have demonstrated that the overall characteristics of the substrate contribute more than plant genotype to shaping the molecular responses in tomato roots (Chialva et al., 2018).

Our research also focused on bacterial endophytes, which are good candidates for beneficial inoculants aimed at reducing the chemical inputs in conventional agricultural practices and increasing nutrient uptake and stress resilience in plant species (Ryan et al., 2008; Gupta et al., 2014). In fact, the endophytes interact more closely with their host than rhizospheric bacteria because they are located within the plant tissues (Hallmann et al., 1997; Weyens et al., 2013). In addition, as they live in the apoplast (or in the xylem), endophytes do not need to compete for nutrition and/or niche in the soil as bacteria do in the rhizosphere (Reiter et al., 2002), and they or their metabolites can easily reach the pathogens within the plants (Gupta et al., 2014).

A subset of bacterial endophytes isolated from tomato endorhizosphere (77 isolates), identified by partial sequencing of their 16S rRNA gene, belonged to two phyla (Firmicutes and Proteobacteria) and to three orders, namely, Bacillales (27.3%), with all the isolates in the genus *Bacillus*; Pseudomonadales (31.2%), with isolates in the genera *Pseudomonas* and *Acinetobacter*; and Enterobacteriales (41.6%), with isolates in the genera *Enterobacter*, *Ewingella*, *Pantoea*, *Providencia*, and *Lelliottia*. Similarly, some of these genera (*Bacillus*, *Pseudomonas*, *Acinetobacter*, *Enterobacter*) have been isolated from tomato endorhizosphere in studies on beneficial bacteria (Tian et al., 2017; Singh et al., 2019). Bacterial strains in the genera, *Rhizobium* and *Ralstonia*, have also been isolated from the endorhizosphere of tomato plants (Abbamondi et al., 2016). Bergna et al. (2018) isolated *Ralstonia*, *Stenotrophomonas*, and *Bacillus* strains both from tomato root and seed endosphere. The high-throughput screening and cultivable approach suggested that beneficial bacteria are seed transmitted (Bergna et al., 2018).

Recent studies demonstrated that tomato bacterial communities of the root zone and of the rhizosphere exhibited the highest richness and diversity in comparison to those of the endorhizosphere (Dong et al., 2019; Lee et al., 2019). In general, the richness decreased from the root zone soil to rhizosphere to phyllosphere to endosphere, whereas the diversity decreased in a different order: root zone soil > rhizosphere > endosphere > phyllophere (Ottesen et al., 2013; Dong et al., 2019). Our results, however, suggest that beneficial activities are commonly spread in each root compartment. However, the richness of the bacterial community is the lowest in the endorhizosphere. In this study, when the relationship between the bacterial families and the antagonistic activity of the tomato endophytes was investigated, *Bacillus* isolates were significantly more antagonistic *in vitro* against tomato plant pathogens than bacterial isolates belonging to Pseudomonadales and Enterobacteriales.

Almost 40% of the endophytic bacteria characterized here belong to Enterobacteriales, more specifically to the Enterobacteriaceae family, and as many as five different genera were recorded. Many studies have confirmed that Enterobacteriaceae are indigenous components of the plant microbiome in different species (Brandl, 2006; Teplitski et al., 2011; Erlacher et al., 2014, 2015; Tian et al., 2017). Data on rocket salad suggested that the soil probably provides the largest reservoir from which enterics become established and spread within the whole plant (Cernava et al., 2019). Enterobacteriaceae have been successfully evaluated as biocontrol agents in tomato (Xue et al., 2009); however, there are still some concerns regarding the use of these antagonistic bacteria as some species are human pathogens (Erlacher et al., 2015; Cernava et al., 2019).

Some *Pseudomonas* species show considerable potential for the suppression of plant pathogens, in promoting plant growth, inducing systemic resistance in plants, and are widely used as biocontrol agents (Mercado-Blanco and Bakker, 2007). These bacteria produce several diffusible and/or volatile secondary metabolites with antibiotic properties such as diacetylphloroglucinol, pyrrolnitrin, and cyclic lipopeptides phenazine (Haas and Keel, 2003; Raaijmakers and Mazzola, 2012).

Most endophytic *Bacillus* isolates identified here with the 16S rRNA gene sequence belong to the *B. amyloliquefaciens* and *B. subtilis* group. Members of the *B. subtilis* species complex,

which includes at present more than 20 closely related species such as *B. subtilis*, *B. amyloliquefaciens*, and *Bacillus pumilus* and, to a lesser extent, the genus *Paenibacillus* species, have been proven to be efficient at plant growth promotion and biocontrol against plant pathogens, such as viruses, bacteria, fungi, and nematodes, in the vicinity of plant roots (Vacheron et al., 2013; Fan et al., 2017). To date, bacilli are the most widely used bacteria on the biopesticide market (Borriss, 2011, 2015). This is mainly due to their ability to produce durable endospores, which enable stable bioformulations to be prepared with a long shelf life. These antagonistic strains produce numerous antibiotics including polymyxin, difficidin, subtilin, mycobacillin, and zwittermicin A, which are active against plant pathogenic bacteria and fungi (Borriss, 2015; Caulier et al., 2019).

Biological control of a set of *Bacillus* and *Pseudomonas* isolates from tomato endorhizosphere was tested in a growth chamber in two separate experiments. Two important bacterial pathogens that are common in the area of sampling and that represent important seed-transmitted pathogens were chosen: (i) the vascular pathogenic bacterium *C. michiganensis* pv. *michiganensis*, which causes tomato bacterial canker; and (ii) one of the *Xanthomonas* species that causes bacterial spot of tomato, *X. euvesicatoria* pv. *perforans* (Bella et al., 2012; Aiello et al., 2013; Catara and Bella, 2020).

Biocontrol of bacterial pathogens reduces the impact of copper compounds. Our results were encouraging as in the growth chamber *Cmm* spread very quickly inside the plantlets. In fact, 1 month after inoculation, *Cmm* led to the death of 100% of the plants in the control. Two isolates, *Pseudomonas* species f1 and *Bacillus* species, 265 out of the 10 bacterial isolates tested *in vivo*, significantly reduced bacterial canker by delaying disease progress and reducing the number of dead plants at the end of the trial compared to the control. Several studies have shown that *Pseudomonas* or *Bacillus* strains inoculated in the soil or in the seeds can reduce the incidence and severity of bacterial canker, and in some cases, an induction of systemic resistance has been suggested (Boudyach et al., 2004; Nandi et al., 2018; Abo-Elyousr et al., 2019). In our pathogenicity tests, we used two different pathosystems. In the biocontrol trial on bacterial canker, both the pathogenic bacterium and the biocontrol agent have been inoculated in the soil where the two microorganisms may have interacted by competition and/or antibiotic phenomena. All biocontrol bacteria tested were able to reduce the symptoms of bacterial spot. As the phytopathogenic bacterium in this case was inoculated on the leaves, the spatial distance between the two suggests that the mechanism of action also involves induction of systemic resistance. Phage therapy is currently considered the most effective *Xep* biological control method (Obradovic et al., 2005). However, the effect of foliar biocontrol bacteria and PGPRs and *B. pumilus* in reducing bacterial spot in greenhouse and some field trials has been already demonstrated (Byrne et al., 2005; Ji et al., 2006).

The microbial collection generated in this study could provide the basis for the future development of bioinoculants using single strains or synthetic microbial communities. The bacterial isolates were obtained from the same niche of pathogens; thus, it is conceivable that they could colonize tomato roots, although endophytic colonization is still to be demonstrated. The use of microbial consortia has recently emerged as an approach to combine microorganisms with different traits, effects, or mechanisms of action (Compant et al., 2019). Future *in vivo* studies will demonstrate how successful this bottom-up approach is and whether the isolates could be used to inoculate plantlets in the nursery, thus providing intensive tomato cultivation areas with protected plants.

DATA AVAILABILITY STATEMENT

Sequences of the isolates used in the present study were submitted to the GenBank database. GenBank accession numbers (MW130753–MW130829) are shown in **Supplementary Table 3**.

AUTHOR CONTRIBUTIONS

AA designed and performed all the experiments and wrote the manuscript together with VC. GD and PB contributed to perform 16S rRNA gene amplification, sequencing, and analysis. MD critically contributed to data organization and performed data analysis. RZ planned farm selection and soil analysis and provided sampling. FG, GD, and AA performed the *in vivo* experiments. GC contributed to the critical analysis of the results and together with MD, VC, and PB contributed to the writing and revision of the manuscript. VC conceived, organized, and supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.637582/full#supplementary-material

REFERENCES

Abbamondi, G. R., Tommonaro, G., Weyens, N., Thijs, S., Sillen, W., Gkorezis, P., et al. (2016). Plant growth-promoting effects of rhizospheric and endophytic bacteria associated with different tomato cultivars and new tomato hybrids. *Chem. Biol. Technol. Agric.* 3, 1–10. doi: 10.1186/s40538-015-0051-3

Abdeljalil, N. O. B., Vallance, J., Gerbore, J., Bruez, E., Martins, G., Rey, P., et al. (2016). Characterization of tomato-associated rhizobacteria recovered from various tomato-growing sites in Tunisia. *J. Plant Pathol. Microb*. 7, 1–12. doi: 10.4172/2157-7471.1000351

Abo-Elyousr, K. A., Bagy, H. M. K., Hashem, M., Alamri, S. A., and Mostafa, Y. S. (2019). Biological control of the tomato wilt caused by *Clavibacter michiganensis* subsp. michiganensis using formulated plant growth-promoting bacteria. *Egypt J. Biol. Pest. Control.* 29, 1–8. doi: 10.1186/s41938-019-0152-6

Aiello, D., Scuderi, G., Vitale, A., Firrao, G., Polizzi, G., and Cirvilleri, G. (2013). A pith necrosis caused by *Xanthomonas* perforans on tomato plants. *Eur. J. Plant Pathol.* 137, 29–41. doi: 10.1007/s10658-013-0214-7

Allard, S. M., Walsh, C. S., Wallis, A. E., Ottesen, A. R., Brown, E. W., and Micallef, S. A. (2016). *Solanum lycopersicum* (tomato) hosts robust phyllosphere and rhizosphere bacterial communities when grown in soil amended with various organic and synthetic fertilizers. *Sci. Total Environ.* 573, 555–563. doi: 10.1016/j.scitotenv.2016.08.157

Amaresan, N., Jayakumar, V., Kumar, K., and Thajuddin, N. (2012). Isolation and characterization of plant growth promoting endophytic bacteria and their effect on tomato (*Lycopersicon esculentum*) and chilli (*Capsicum annuum*) seedling growth. *Ann. Microbiol.* 62, 805–810. doi: 10.1007/s13213-011-0321-7

Attia, M. S., El-Sayyad, G. S., Abd Elkodous, M., and El-Batal, A. I. (2020). The effective antagonistic potential of plant growth-promoting rhizobacteria against *Alternaria solani*-causing early blight disease in tomato plant. *Sci. Hortic.* 266:109289. doi: 10.1016/j.scienta.2020.109289

Bardin, M., and Gullino, M. L. (2020). "Fungal diseases," in *Integrated Pest and Disease Management in Greenhouse Crops*, eds M. L. Gullino, R. Albajes, and P. Nicot (Cham: Springer Nature), 55–100.

Bashan, Y. (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnol. Adv.* 16, 729–770. doi: 10.1016/S0734-9750(98)00003-2

Bella, P., and Catara, V. (1998). Macchiettatura batterica su pomodoro in serra fredda in Sicilia. *Colture Protette* 27, 67–70.

Bella, P., Ialacci, G., Licciardello, G., La Rosa, R., and Catara, V. (2012). Characterization of atypical *Clavibacter michiganensis* subsp. michiganensis populations in greenhouse tomatoes in Italy. *J. Plant Pathol* 94, 635–642.

Berg, G., Rybakova, D., Grube, M., and Köberl, M. (2016). The plant microbiome explored: implications for experimental botany. *J. Exp. Bot.* 67, 995–1002. doi: 10.1093/jxb/erv466

Bergna, A., Cernava, T., Rändler, M., Grosch, R., Zachow, C., and Berg, G. (2018). Tomato seeds preferably transmit plant beneficial endophytes. *Phytobiomes J.* 2, 183–193. doi: 10.1094/PBIOMES-06-18-0029-R

Besset-Manzoni, Y., Joly, P., Brutel, A., Gerin, F., Soudière, O., Langin, T., et al. (2019). Does in vitro selection of biocontrol agents guarantee success in planta? A study case of wheat protection against *Fusarium* seedling blight by soil bacteria. *PloS One.* 14:e0225655. doi: 10.1371/journal.pone.0225655

Bloemberg, G. V., and Lugtenberg, B. J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr. Plant Biol.* 4, 343–350. doi: 10.1016/S1369-5266(00)00183-7

Borriss, R. (2011). "Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents," in *Bacteria in Agrobiology: Plant Growth Responses*, ed. D. K. Maheshwari (Heidelberg: Springer), 41–76. doi: 10.1007/978-3-642-20332-9_3

Borriss, R. (2015). "*Bacillus*, a plant beneficial bacterium," in *Principles of Plant-Microbe Interactions. Microbes for Sustainable Agriculture*, ed. B. Lugtenberg (Berlin: Springer), 379–391. doi: 10.1007/978-3-319-08575-3_40

Boudyach, E. H., Fatmi, M., Boubaker, H., Aoumar, A. A. B., and Akhayat, O. (2004). Effectiveness of fluorescent pseudomonads strains HF 22 and HF 142 to control bacterial canker of tomato. *J. Food Agric. Environ.* 2, 115–120.

Brandl, M. T. (2006). Fitness of human enteric pathogens on plants and implications for food safety. *Annu. Rev. Phytopathol.* 44, 367–392. doi: 10.1146/annurev.phyto.44.070505.143359

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Byrne, J. M., Dianese, A. C., Ji, P., Campbell, H. L., Cuppels, D. A., Louws, F. J., et al. (2005). Biological control of bacterial spot of tomato under field conditions at several locations in North America. *Biol. Control* 32, 408–418. doi: 10.1016/j.biocontrol.2004.12.001

Catara, V., and Bella, P. (2020). "Bacterial diseases in integrated pest and disease management," in *Greenhouse Crops*, eds M. L. Gullino, R. Albajes, and P. Nicot (Cham: Springer Nature), 33–54. doi: 10.1007/978-3-030-22304-5_2

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., and Mahillon, J. (2019). Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. *Front. Microbiol.* 10:302. doi: 10.3389/fmicb.2019.00302

Cavazos, B. R., Bohner, T. F., Donald, M. L., Sneck, M. E., Shadow, A., Omacini, M., et al. (2018). Testing the roles of vertical transmission and drought stress in the prevalence of heritable fungal endophytes in annual grass populations. *New Phytol.* 219, 1075–1084. doi: 10.1111/nph.15215

Cernava, T., Erlacher, A., Soh, J., Sensen, C. W., Grube, M., and Berg, G. (2019). *Enterobacteriaceae* dominate the core microbiome and contribute to the resistome of arugula (*Eruca sativa Mill.*). *Microbiome* 7, 1–12. doi: 10.1186/s40168-019-0624-7

Chialva, M., Salvioli di Fossalunga, A., Daghino, S., Ghignone, S., Bagnaresi, P., Chiapello, M., et al. (2018). Native soils with their microbiotas elicit a state of alert in tomato plants. *New Phytol.* 220, 1296–1308. doi: 10.1111/nph.15014

Ciancio, A., Pieterse, C. M., and Mercado-Blanco, J. (2016). Harnessing useful rhizosphere microorganisms for pathogen and pest biocontrol. *Front. Microbiol.* 7:1620. doi: 10.3389/fmicb.2016.01620

Compant, S., Clement, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42, 669–678. doi: 10.1016/j.soilbio.2009.11.024

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004

Constantin, E. C., Cleenwerck, I., Maes, M., Baeyen, S., Van Malderghem, C., De Vos, P., et al. (2016). Genetic characterization of strains named as *Xanthomonas axonopodis* pv. *dieffenbachiae* leads to a taxonomic revision of the *X. axonopodis* species complex. *Plant Pathol.* 65, 792–806. doi: 10.1111/ppa.12461

Dong, C. J., Wang, L. L., Li, Q., and Shang, Q. M. (2019). Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants. *PLoS One* 14:e0223847. doi: 10.1371/journal.pone.0223847

Edwards, U., Rogall, T., Blöcker, H., Emde, M., and Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* 17, 7843–7853. doi: 10.1093/nar/17.19.7843

Enya, J., Shinohara, H., Yoshida, S., Tsukiboshi, T., Negishi, H., Suyama, K., et al. (2007). Culturable leaf-associated bacteria on tomato plants and their potential as biological control agents. *Microb. Ecol.* 53, 524–536. doi: 10.1007/s00248-006-9085-1

Erlacher, A., Cardinale, M., Grosch, R., Grube, M., and Berg, G. (2014). The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome. *Front. Microbiol.* 5:175. doi: 10.3389/fmicb.2014.00175

Erlacher, A., Cardinale, M., Grube, M., and Berg, G. (2015). Biotic stress shifted structure and abundance of *Enterobacteriaceae* in the lettuce microbiome. *PLoS One.* 10:e0118068. doi: 10.1371/journal.pone.0118068

Fan, B., Blom, J., Klenk, H. P., and Borriss, R. (2017). *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Front. Microbiol.* 8:22. doi: 10.3389/fmicb.2017.00022

French, E., Tran, T., and Iyer-Pascuzzi, A. S. (2020). Tomato genotype modulates selection and responses to root microbiota. *Phytobiomes J.* 4, 314–326. doi: 10.1094/PBIOMES-02-20-0020-R

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.* 41, 109–117. doi: 10.1139/m95-015

Gupta, P., Puniya, B., Barun, S., Asthana, M., and Kumar, A. (2014). Isolation and characterization of endophytes from different plants: effects on growth of *Pennisetum typhoides*. *Biosci. Biotechnol. Res. Asia* 11, 223–234. doi: 10.13005/bbra/1259

Haas, D., and Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant

disease. *Annu. Rev. Phytopathol.* 41, 117–153. doi: 10.1146/annurev.phyto.41.052002.095656

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* 43, 895–914. doi: 10.1139/m97-131

Hardoim, P. R., van Overbeek, L. S., and van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.* 16, 463–471. doi: 10.1016/j.tim.2008.07.008

He, F. J., Nowson, C. A., and MacGregor, G. A. (2006). Fruit and vegetable consumption and stroke: meta-analysis of cohort studies. *Lancet* 367, 320–326. doi: 10.1016/S0140-6736(06)68069-0

Heimpel, G. E., and Mills, N. J. (2017). *Biological Control*. Cambridge: Cambridge University Press.

Hirano, Y., and Arie, T. (2006). PCR-based differentiation of *Fusarium oxysporum* ff. sp. *lycopersici* and *radicis-lycopersici* and races of *F. oxysporum* f. sp. *lycopersici*. *J. Gen. Plant Pathol.* 72, 273–283. doi: 10.1007/s10327-006-0287-7

Hudzicki, J. (2009). *Kirby-Bauer Disk Diffusion Susceptibility Test Protocol*. Washington DC: American Society for Microbiology.

Ialacci, G., Bella, P., Licciardello, G., Strano, C. P., Eichenlaub, R., Gartemann, K. H., et al. (2016). Clonal populations of *Clavibacter michiganensis* subsp. *michiganensis* are responsible for the outbreaks of bacterial canker in greenhouse tomatoes in Italy. *Plant Pathol.* 65, 484–495. doi: 10.1111/ppa.12424

Janštová, B., and Lukášová, J. (2001). Heat resistance of *Bacillus* spp. spores isolated from cow's milk and farm environment. *Acta. Vet. Brno*. 70, 179–184. doi: 10.2754/avb200170020179

Jeanbille, M., Buée, M., Bach, C., Cébron, A., Frey-Klett, P., Turpault, M. P., et al. (2016). Soil parameters drive the structure, diversity and metabolic potentials of the bacterial communities across temperate beech forest soil sequences. *Microb. Ecol.* 71, 482–493. doi: 10.1007/s00248-015-0669-5

Ji, P., Campbell, H. L., Kloepper, J. W., Jones, J. B., Suslow, T. V., and Wilson, M. (2006). Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria. *Biol. Control* 36, 358–367. doi: 10.1016/j.biocontrol.2005.09.003

Kassambara, A., and Mundt, F. (2016). *Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R Package Version 1*.

Khan, M. A., Boër, B., Öztur, M., Clüsener-Godt, M., Gul, B., and Breckle, S. W. (2016). *Sabkha Ecosystems*, Vol. V. The Americas: Springer.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120. doi: 10.1007/BF01731581

King, E. O., Ward, M. K., and Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med*. 44, 301–307. doi: 10.5555/uri:pii:002221435490222X

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*. 35, 1547–1549. doi: 10.1093/molbev/msy096

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acid Techniques in Bacterial Systematics*, eds E. Stackebrandt and M. Goodfellow (New York, NY: Wiley), 115–175.

Larousse, M., Rancurel, C., Syska, C., Palero, F., Etienne, C., Nesme, X., et al. (2017). Tomato root microbiota and *Phytophthora parasitica*-associated disease. *Microbiome* 5:56. doi: 10.1186/s40168-017-0273-7

Lee, S. A., Kim, Y., Kim, J. M., Chu, B., Joa, J. H., Sang, M. K., et al. (2019). A preliminary examination of bacterial, archaeal, and fungal communities inhabiting different rhizocompartments of tomato plants under real-world environments. *Sci. Rep.* 9:9300. doi: 10.1038/s41598-019-45660-8

Lee, S. A., Park, J., Chu, B., Kim, J. M., Joa, J. H., Sang, M. K., et al. (2016). Comparative analysis of bacterial diversity in the rhizosphere of tomato by culture-dependent and -independent approaches. *J. Microbiol.* 54, 823–831. doi: 10.1007/s12275-016-6410-3

Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G., Boeufgras, J., et al. (1995). Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum Mill.*), on the diversity of soilborne populations of fluorescent pseudomonads. *Appl. Environ. Microbiol.* 61, 1004–1012. doi: 10.1128/aem.61.3.1004-1012.1995

Leonardi, C., and Martorana, M. (2005). Osmotic stress and tomato under protected cultivation: functional response and management strategies [*Lycopersicon esculentum Mill.*]. *Italus Hortus* 12, 43–56.

Li, J. G., Ren, G. D., Jia, Z. J., and Dong, Y. H. (2014). Composition and activity of rhizosphere microbial communities associated with healthy and diseased greenhouse tomatoes. *Plant Soil* 380, 337–347. doi: 10.1007/s11104-014-2097-6

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Madden, L. V., and Campbell, C. L. (1990). "Nonlinear disease progress curves," in *Epidemics of Plant Diseases*, ed. J. Kranz (Berlin: Springer), 181–229. doi: 10.1007/978-3-642-75398-5_6

Maksimov, I. V., Abizgil'Dina, R. R., and Pusenkova, L. I. (2011). Plant growth promoting rhizobacteria as alternative to chemical crop protectors from pathogens. *Appl. Biochem. Microbiol.* 47, 333–345. doi: 10.1134/S0003683811040090

Manzum, A. A., and Al Mamun, M. A. (2018). Isolation of Bacillus spp. bacteria from soil for production of cellulase. *Nepal J. Biotechnol.* 6, 57–61. doi: 10.3126/njb.v6i1.22338

Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Mercado-Blanco, J., and Bakker, P. A. H. M. (2007). Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Antonie van Leeuwenhoek* 92, 367–389. doi: 10.1007/s10482-007-9167-1

Moriones, E., and Verdin, E. (2020). "Viral diseases," in *Integrated Pest and Disease Management in Greenhouse Crops*, eds M. L. Gullino, R. Albajes, and P. Nicot (Cham: Springer Nature), 3–34. doi: 10.1016/b978-0-12-800964-2.00001-x

Mulet, M., Lalucat, J., and García-Valdés, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environ. Microbiol.* 12, 1513–1530. doi: 10.1111/j.1462-2920.2010.02181.x

Müller, C. A., Obermeier, M. M., and Berg, G. (2016). Bioprospecting plant-associated microbiomes. *J. Biotechnol.* 235, 171–180. doi: 10.1016/j.jbiotec.2016.03.033

Nandi, M., Macdonald, J., Liu, P., Weselowski, B., and Yuan, Z. C. (2018). *Clavibacter michiganensis* ssp. *michiganensis*: bacterial canker of tomato, molecular interactions and disease management. *Molecul. Plant Pathol*. 19, 2036–2050. doi: 10.1111/mpp.12678

Normander, B., and Prosser, J. I. (2000). Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. *Appl. Environ. Microbiol.* 66, 4372–4377. doi: 10.1128/AEM.66.10.4372-4377.2000

Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., et al. (2005). Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Dis.* 89, 712–716. doi: 10.1094/PD-89-0712

Ottesen, A. R., González Peña, A., White, J. R., Pettengill, J. B., Li, C., Allard, S., et al. (2013). Baseline survey of the anatomical microbial ecology of an important food plant: *Solanum lycopersicum* (tomato). *BMC Microbiol.* 13:114. doi: 10.1186/1471-2180-13-114

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Pikovskaya, R. I. (1948). Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiology* 17, 362–370.

Poli, A., Lazzari, A., Prigione, V., Voyron, S., Spadaro, D., and Varese, G. C. (2016). Influence of plant genotype on the cultivable fungi associated to tomato rhizosphere and roots in different soils. *Fungal Biol.* 120, 862–872. doi: 10.1016/j.funbio.2016.03.008

Poudel, R., Jumpponen, A., Kennelly, M. M., Rivard, C. L., Gomez-Montano, L., and Garrett, K. A. (2019). Rootstocks shape the rhizobiome: rhizosphere and endosphere bacterial communities in the grafted tomato system. *Appl. Environ. Microbiol.* 85:2. doi: 10.1128/AEM.01765-18

Quiza, L., St-Arnaud, M., and Yergeau, E. (2015). Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering. *Front. Plant Sci.* 6:507. doi: 10.3389/fpls.2015.00507

Raaijmakers, J. M., and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 50, 403–424. doi: 10.1146/annurev-phyto-081211-172908

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341–361. doi: 10.1007/s11104-008-9568-6

Rashid, M., Khalil, S., Ayub, N., and Alam, S. (2004). Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (Psm) under in vitro conditions. *Pak. J. Biol. Sci.* 7, 187–196. doi: 10.3923/pjbs.2004.187.196

Reiter, B., Pfeifer, U., Schwab, H., and Sessitsch, A. (2002). Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. atroseptica. *Appl. Environ. Microbiol.* 68, 2261–2268. doi: 10.1128/AEM.68.5.2261-2268.2002

Rezki, S., Campion, C., Simoneau, P., Jacques, M. A., Shade, A., and Barret, M. (2018). Assembly of seed-associated microbial communities within and across successive plant generations. *Plant Soil* 422, 67–79. doi: 10.1007/s11104-017-3451-2

Romero, F. M., Marina, M., and Pieckenstain, F. L. (2016). Novel components of leaf bacterial communities of field-grown tomato plants and their potential for plant growth promotion and biocontrol of tomato diseases. *Res. Microbiol.* 167, 222–233. doi: 10.1016/j.resmic.2015.11.001

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiol. Lett.* 278, 1–9. doi: 10.1111/j.1574-6968.2007.00918.x

Sagoe, C. I., Ando, T., Kouno, K., and Nagaoka, T. (1998). Relative importance of protons and solution calcium concentration in phosphate rock dissolution by organic acids. *Soil. Sci. Plant Nutr.* 4, 617–625. doi: 10.1080/00380768.1998.10414485

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425. doi: 10.1093/oxfordjournals.molbev.a040454

Saqib, S., Uddin, S., Zaman, W., Ullah, F., Ayaz, A., Asghar, M., et al. (2020). Characterization and phytostimulatory activity of bacteria isolated from tomato (*Lycopersicon esculentum Mill.*) rhizosphere. *Microb. Pathog.* 140:103966. doi: 10.1016/j.micpath.2020.103966

Sayyed, R. Z., Naphade, B. S., and Chincholkar, S. B. (2005). "Ecologically competent rhizobacteria for plant growth promotion and disease management," in *Recent Trends in Biotechnology,* eds M. K. Rai, N. J. Chikhale, P. V. Thakare, P. A. Wadegaonkar, and A. P. Ramteke (Jodhpur: Scientific Publisher), 1–16.

Schaad, N. W., Jones, J. B., and Chun, W. (2001). *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 3rd Edn. St. Paul, MN: American Phytopathological Society.

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Shukla, P. S., Agarwal, P. K., and Jha, B. (2012). Improved salinity tolerance of *Arachis hypogaea* (L.) by the interaction of halotolerant plant growth promoting rhizobacteria. *J. Plant Growth Regul.* 31, 195–206. doi: 10.1007/s00344-011-9231-y

Singh, M., Kumar, A., and Pandey, K. D. (2019). Biochemical and molecular identification of *Solanum lycopersicum* L. temperature tolerant bacterial endophytes. *Biocatal. Agric. Biotechnol.* 22:101409. doi: 10.1016/j.bcab.2019.101409

Singh, V. K., Singh, A. K., and Kumar, A. (2017). Disease management of tomato through PGPB: current trends and future perspective. *3 Biotech* 7:255. doi: 10.1007/s13205-017-0896-1

Sinno, M., Ranesi, M., Gioia, L., d'Errico, G., and Woo, S. L. (2020). Endophytic fungi of tomato and their potential applications for crop improvement. *Agriculture* 10:587. doi: 10.3390/agriculture10120587

Taffner, J., Bergna, A., Cernava, T., and Berg, G. (2020). Tomato-associated archaea show a cultivar-specific rhizosphere effect but an unspecific transmission by seeds. *Phytobiomes J.* 4, 133–141. doi: 10.1094/PBIOMES-01-20-0017-R

R Core Team (2013). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Teplitski, M., Warriner, K., Bartz, J., and Schneider, K. R. (2011). Untangling metabolic and communication networks: interactions of enterics with phytobacteria and their implications in produce safety. *Trends Microbiol.* 19, 121–127. doi: 10.1016/j.tim.2010.11.007

Tian, B., Zhang, C., Ye, Y., Wen, J., Wu, Y., Wang, H., et al. (2017). Beneficial traits of bacterial endophytes belonging to the core communities of the tomato root microbiome. *Agric. Ecosyst. Environ.* 247, 149–156. doi: 10.1016/j.agee.2017.06.041

Tian, B. Y., Cao, Y., and Zhang, K. Q. (2015). Metagenomic insights into communities, functions of endophytes, and their associates with infection by root-knot nematode, *Meloidogyne incognita*, in tomato roots. *Sci. Rep.* 5:17087. doi: 10.1038/srep17087

Trantas, E., Licciardello, G., Almeida, N. F., Witek, K., Strano, C. P., Duxbury, Z., et al. (2015). Comparative genomic analysis of multiple strains of two unusual plant pathogens: *Pseudomonas corrugata* and *Pseudomonas mediterranea*. *Front. Microbiol.* 6:811. doi: 10.3389/fmicb.2015.00811

Truyens, S., Weyens, N., Cuypers, A., and Vangronsveld, J. (2015). Bacterial seed endophytes: genera, vertical transmission and interaction with plants. *Environ. Microbiol. Rep.* 7, 40–50. doi: 10.1111/1758-2229.12181

Vacheron, J., Desbrosses, G., Bouffaud, M. L., Touraine, B., Moënne-Loccoz, Y., Muller, D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci.* 4:356. doi: 10.3389/fpls.2013.00356

Vacheron, J., Moënne-Loccoz, Y., Dubost, A., Gonçalves-Martins, M., Muller, D., and Prigent-Combaret, C. (2016). Fluorescent *Pseudomonas* strains with only few plant-beneficial properties are favored in the maize rhizosphere. *Front. Plant Sci.* 7:1212. doi: 10.3389/fpls.2016.01212

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Vincent, J. M. (1947). Distortion of fungal hyphae in the presence of certain inhibitors. *Nature* 159, 850–850. doi: 10.1038/159850b0

Weyens, N., Beckers, B., Schellingen, K., Ceulemans, R., Croes, S., Janssen, J., et al. (2013). Plant-associated bacteria and their role in the success or failure of metal phytoextraction projects: first observations of a field-related experiment. *Microb. Biotechnol.* 6, 288–299. doi: 10.1111/1751-7915.12038

Wieland, G., Neumann, R., and Backhaus, H. (2001). Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. *Appl. Environ. Microbiol.* 67, 5849–5854. doi: 10.1128/AEM.67.12.5849-5854.2001

Woo, S. L., and Pepe, O. (2018). Microbial consortia: promising probiotics as plant biostimulants for sustainable agriculture. *Front Plant Sci.* 9:1801. doi: 10.3389/fpls.2018.01801

Xu, M., Sheng, J., Chen, L., Men, Y., Gan, L., Guo, S., et al. (2014). Bacterial community compositions of tomato (*Lycopersicum esculentum* Mill.) seeds and plant growth promoting activity of ACC deaminase producing *Bacillus subtilis* (HYT-12-1) on tomato seedlings. *World J. Microbiol. Biotechnol.* 30, 835–845. doi: 10.1007/s11274-013-1486-y

Xue, Q. Y., Chen, Y., Li, S. M., Chen, L. F., Ding, G. C., Guo, D. W., et al. (2009). Evaluation of the strains of *Acinetobacter* and *Enterobacter* as potential biocontrol agents against *Ralstonia* wilt of tomato. *Biol. Control* 48, 252–258. doi: 10.1016/j.biocontrol.2008.11.004

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REVIEW
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How Mycorrhizal Associations Influence Orchid Distribution and Population Dynamics

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Orchid distribution and population dynamics are influenced by a variety of ecological factors and the formation of holobionts, which play key roles in colonization and ecological community construction. Seed germination, seedling establishment, reproduction, and survival of orchid species are strongly dependent on orchid mycorrhizal fungi (OMF), with mycorrhizal cheating increasingly observed in photosynthetic orchids. Therefore, changes in the composition and abundance of OMF can have profound effects on orchid distribution and fitness. Network analysis is an important tool for the study of interactions between plants, microbes, and the environment, because of the insights that it can provide into the interactions and coexistence patterns among species. Here, we provide a comprehensive overview, systematically describing the current research status of the effects of OMF on orchid distribution and dynamics, phylogenetic signals in orchid–OMF interactions, and OMF networks. We argue that orchid–OMF associations exhibit complementary and specific effects that are highly adapted to their environment. Such specificity of associations may affect the niche breadth of orchid species and act as a stabilizing force in plant–microbe coevolution. We postulate that network analysis is required to elucidate the functions of fungal partners beyond their effects on germination and growth. Such studies may lend insight into the microbial ecology of orchids and provide a scientific basis for the protection of orchids under natural conditions in an efficient and cost-effective manner.

Keywords: orchid mycorrhizal fungi, orchid performance, complementary selection, environmental variables, evolutionary constraints, mycorrhizal networks, keystone taxa

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INTRODUCTION

Mycorrhizal associations play a key role in generating and maintaining plant diversity. Such associations not only enhance the acquisition, transmission, and cycling of nutrients in plants, but also mediate interactions among different plants and between plants and non-mycorrhizal fungi (Tedersoo et al., 2020). A growing body of research suggests that mycorrhizal symbionts are important drivers of biogeographic patterns, distributions, community dynamics, and the health of plants, mediated by their effects on dispersal and coexistence

of species (Delavaux et al., 2019; Trivedi et al., 2020). For example, ectomycorrhizae (EcM) interact with pathogenic fungi to maintain community diversity (Chen et al., 2019a). The family Orchidaceae is extremely diverse (with 28,000+ species), widely distributed across highly heterogeneous microenvironments, and exhibits large spatiotemporal variation in population size (Givnish et al., 2016; Fay, 2018; Djordjević and Tsiftsis, 2020). A common feature of all orchids is their obligatory dependence on orchid mycorrhizal fungi (OMF), which makes the presence of suitable OMF or co-dispersal with partners a prerequisite for the establishment and maintenance of orchid populations (Dearnaley et al., 2012; McCormick and Jacquemyn, 2014). Mycorrhizal symbiosis is especially important for plants associated with OMF and EcM, as they often have high mycorrhizal specificity (Põlme et al., 2018). Most species of EcM fungi exhibit short-distance dispersal and therefore, have limited ranges of distribution (Sato et al., 2012; Tedersoo et al., 2020). Although the major mycorrhizal partners of terrestrial orchids appear to favor a cross-scale distribution with specificity ranging from wide to very narrow (Jacquemyn et al., 2017; Swarts and Dixon, 2017), comparatively little is known about the diversity and biogeography of the associated mycorrhizae of epiphytic orchids. Therefore, the interaction between OMF and orchids is a key factor determining orchid distribution and development, and diversity of OMF has a strong impact on the niche and life cycle of host orchids. For instance, low OMF diversity and high heterogeneity in OMF community composition may lead to weak growth of orchid populations (Kaur et al., 2020).

Orchids exhibit astonishing morphological characteristics, such as labella and modified petals, that indicate their substantial adaptability to the environment (Zhang et al., 2018). On one hand, interactions with specific pollinators promote the reproduction of orchids; on the other hand, symbiotic fungi are required for soil exploitation (Selosse, 2014). Greater environmental heterogeneity and a wider range of resource availability usually contribute to the increase of orchid species diversity (Schödelbauerová et al., 2009; Traxmandlová et al., 2018). Thus, untangling the role of environmental conditions in determining the distribution and abundance of orchids is a prerequisite for effective conservation of these species. Recently, Djordjević and Tsiftsis (2020) systematically sorted out the effects of environmental factors on the distribution, abundance, and richness of orchids. For instance, rainfall and light regime in the habitat are closely related to the flowering patterns and population dynamics of orchids (Wells and Cox, 1991; Jacquemyn et al., 2010a); physical and chemical properties of soil (such as pH, soil moisture, nutrients, etc.) significantly affect the performance of orchid populations (Stuckey, 1967; Tsiftsis et al., 2012). Interestingly, there is growing evidence supports that coexisting orchid species usually exhibit strongly spatially segregated distribution patterns due to strong clustering within individual species and small overlap between species, and that they are often associated with different OMF communities, which are largely explained by differences in soil moisture and pH (e.g., Jacquemyn et al., 2007, 2012, 2014, 2015a; Waud et al., 2017; Chen et al., 2019b; Kaur et al., 2019). These clues strongly suggest that environmental factors may indirectly affect orchid distribution and population dynamics by driving niche partitioning in OMF communities. Similarly, OMF can decompose carbon and nitrogen sources in soil organic matter and transfer them to the associated host orchids (Rasmussen, 1995). In addition, orchids often share EcM with neighboring trees, hence orchid mycorrhiza may mediate the significant effect of vegetation types on orchid niche partitioning (Waterman and Bidartondo, 2008; Jacquemyn et al., 2016a).

Orchid fungi are divided into OMF and orchid non-mycorrhizal fungi (ONF) based on whether or not functional pelotons are present in cortical cells. OMF include at least 17 families of basidiomycetes and five families or genera of ascomycetes (Dearnaley, 2007; Dearnaley et al., 2012). Tulasnellaceae, Ceratobasidiaceae, and Serendipitaceae are most commonly known as rhizoctonia-type Basidiomycetes. Basidiomycetes and Ascomycetes are relatively abundant in the tree roots of forest ecosystems and cultivated species worldwide (Cregger et al., 2018; Wang et al., 2019a; Trivedi et al., 2020), and are commonly associated with orchids as well. Interestingly, a large proportion of ascomycetes associated with orchids are ONF. For example, Helotiales endophytes are the dominant group of ONF associated with host orchids in different habitats as well as major players in plant–fungus associations in a variety of forest ecotypes (Toju et al., 2013; Jacquemyn et al., 2016a, 2017). Previous studies have identified more than 110 genera of ONF, including 76 genera of ascomycetes and 32 genera of basidiomycetes (Ma et al., 2015). Although ONF are often overlooked, recent studies have highlighted their importance in the promotion of orchid seed germination and performance as well as changes in the composition of key chemicals such as sugars. Moreover, they also play a role in mobilizing soil nutrients in the rhizosphere to improve orchid viability and environmental adaptability, and serve as new sources of phytochemicals and bioactive substances to protect the host from soil pathogens. Hence, these ONF provide the plants with promising medicinal and agricultural breeding prospects (Chen et al., 2006; Yuan et al., 2009; Aly et al., 2010; Novotná et al., 2018; Sisti et al., 2019; Hajong and Kapoor, 2020; Wu et al., 2020). Furthermore, ONF may interact with OMF to influence the distribution and population dynamics of orchids. Therefore, the diversity and functionality of ONF should be further explored to gain insight into the associations between orchids and fungi as a whole. In addition, studying how ONF and OMF make rational use of the ecological niches in orchid rhizospheres (i.e., their coexistence mechanism) and the correlation between their distribution and the phylogenetic eigenvectors of orchid species could help us better understand orchid mycorrhizal ecology. We believe that the development of molecular methods and genomics techniques enables the simultaneous consideration of both OMF and ONF in mycorrhizal ecology. This may breed a more complete and informative fungal network that can more accurately predict their association with environmental variables, which may be a key to igniting a new wave of research into orchid mycorrhizae.

The thousands of complex and highly dynamic interactions between plants, microbiomes, and the environment can

be analyzed using ecological networks that play pivotal roles in associations between OMF, EcM, and arbuscular mycorrhizae (AM) fungi, and are often used as black boxes to study the transfer of carbon signals across hosts (Banerjee et al., 2018; Tedersoo et al., 2020). Modular analysis within these networks can be used to identify key microbial groups that are closely related to plant growth and yield. Additionally, the topological roles of the species included in complex networks can be simplified into four categories based on within-module connectivity (*Zi*) and among-module connectivity (*Pi*; **Figure 1A**). Connectors, module hubs, and network hubs are considered ecosystem engineers, which have significant impact on the assembly of communities and can support higher levels of ecosystem function. Network hubs are further defined as keystone taxa due to their high connectivity within these networks, and have more important functional attributes and high interpretation rates on the dynamics of plant microorganisms (Deng et al., 2012; Cavieres et al., 2014; Banerjee et al., 2018; Ma et al., 2020). Thus, they can be used to manipulate the function of the microbiome or predict changes in its community composition. A query of the Web of Science core database using the keyword "orchid mycorrhizal network" retrieved a total of nine articles (as of 15 October 2020), focusing mainly on the interaction between epiphytic orchids in tropical systems or terrestrial orchids in temperate systems and OMF. The methods, ecological premise and revealed network architecture

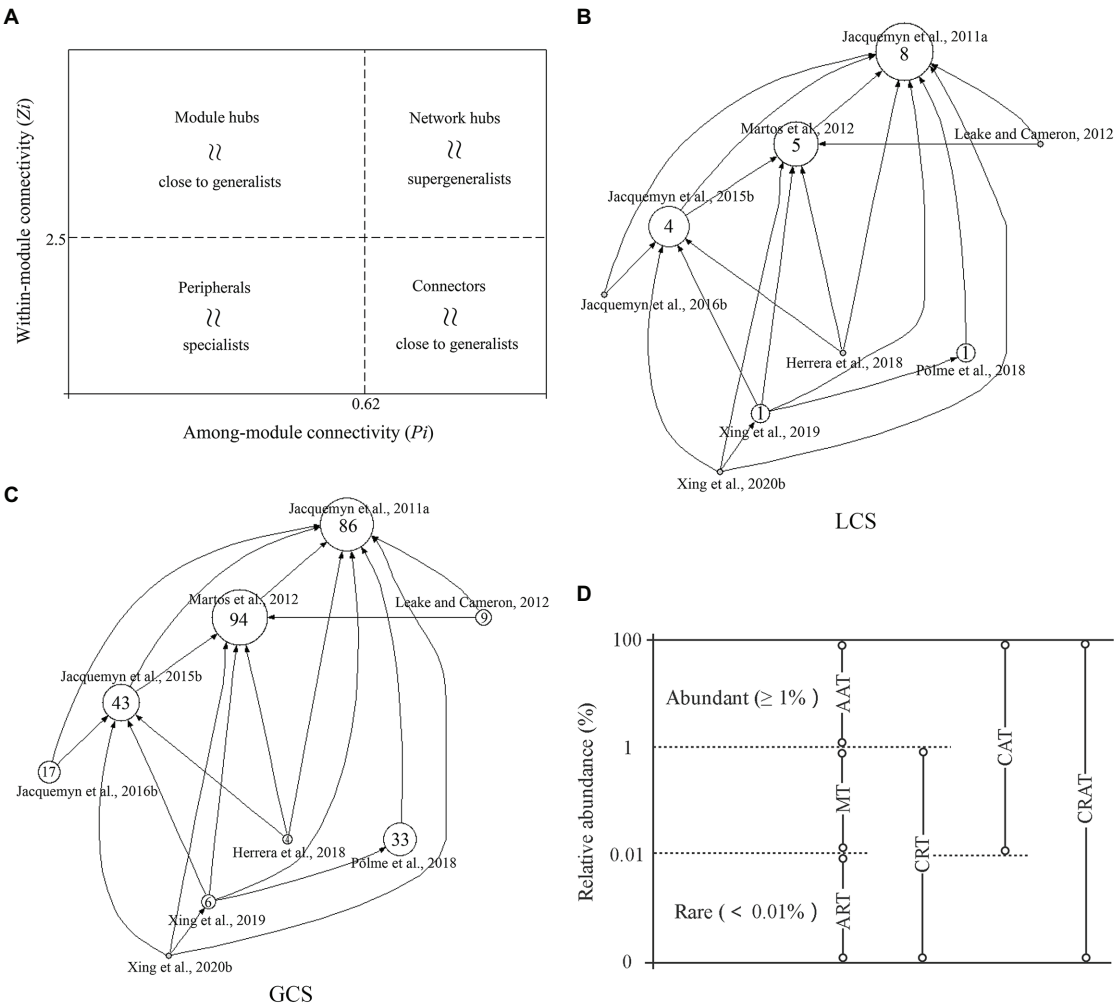

FIGURE 1 | (A) *z–P* plot depicting the role of each species in the interaction network. Peripherals might represent specialists whereas module hubs and connectors are close to generalists and network hubs are supergeneralists. **(B,C)** Local citation score (LCS) and global citation score (GCS) citation diagrams of all orchid mycorrhizal network research papers retrieved from the core Database of Web of Science. Each circle represents a research article. The numbers in the circle are the LCS **(B)** and GCS **(C)** of the corresponding literature, and the size of the circle is proportional to citation scores. Arrows indicate cross-references between the studies. **(D)** Definition and classification of microbial taxa: (i) Always abundant taxa (AAT), operational taxonomic units (OTUs) with abundance always ≥1% in all samples; (ii) Always rare taxa (ART), OTUs with abundance always <0.01% in all samples; (iii) Moderate taxa (MT), OTUs with abundance between 0.01 and 1% in all samples; (iv) Conditionally rare taxa (CRT), OTUs with abundance <0.01% in some samples and below 1% in all samples; (v) Conditionally abundant taxa (CAT), OTUs with abundance greater than 0.01% in all samples and ≥1% in some samples but never rare (<0.01%); and (vi) Conditionally rare and abundant taxa (CRAT), OTUs with abundance varying from rare (≤0.01%) to abundant (≥1%).

in the retrieved articles are described in **Supplementary Table S1**. We used the HistCite software to analyze the citation network and calculate the local citation score (LCS) and global citation score (GCS) from the retrieved articles (**Figures 1B**,**C**). In general, research on the mycorrhizal network of orchids appears to be insufficient, greatly restricting our understanding of the stability and persistence of the species-rich orchid community.

Hence, in this study, we summarize the effects of OMF on orchid distribution and population dynamics, elucidate OMF networks and the phylogenetic relationships in orchid–OMF interactions. More specifically, we would like to discuss the progress in these three aspects to preliminarily clarify the following two issues: (1) the mutual selection mechanism of orchid–OMF and the role of ecology and evolution in this pattern? and (2) the relative importance of nestedness and modularity in orchid mycorrhizal networks? Finally, in the "Prospects" section, we focus on the functional roles of rare groups and discuss several key issues in symbiosis that could benefit from the advancement of OMF network research. Collectively, we summarize the results or conclusions of some case studies related to the target topic, and attempt to focus on the emerging patterns that are more consistent in these summaries. However, it should be noted that the network perpective applies mainly to tropical orchids, and more datasets are needed to expand other biomes. Through this review, we hope to direct attention toward the insufficiently explored field of orchid–fungus mutualism, and encourage the use of beneficial fungal groups for protection of endangered orchids.

EFFECTS OF OMF ON ORCHID DISTRIBUTION AND POPULATION DYNAMICS

The distribution and abundance of orchid populations are curtailed by biotic and abiotic factors. These include latitude, macroclimate, area size, and evolutionary history at the large landscape scale, soil characteristics, light conditions, substrate types, degree of disturbance, pollinating insects, and seed production and dispersal at the local landscape scale, while the effects of altitude, soil moisture, and pH may span both scales (McCormick and Jacquemyn, 2014; Djordjević et al., 2016; Traxmandlová et al., 2018; Tsiftsis et al., 2019; Djordjević and Tsiftsis, 2020). The influence of these factors may depend on the spatiotemporal scale in consideration. Although orchids have dust-like seeds that generally lack nutrients, OMF play a critical role in seed dispersal and germination, establishment of new seedlings, and soil niche partitioning of their lifelong host orchid (McCormick et al., 2006, 2018; Selosse, 2014; Tedersoo et al., 2020). In natural habitats, microhabitats rich in OMF generally facilitate more seed germination and seedling establishment, and more orchids tend to grow in areas with richer distribution of OMF. As a result, changes in OMF composition, abundance, and evenness may greatly influence the fitness of orchids, which in turn affects the distribution and community composition of orchids (McCormick and Jacquemyn, 2014; Waud et al., 2016a; McCormick et al., 2018).

EFFECTS OF OMF ABUNDANCE ON ORCHID DISTRIBUTION

To our knowledge, no reports show that the distribution of orchids at large scales is restricted by OMF. While Hemrová et al. (2019) suggested that fungal symbionts are the main driving forces for the distribution of forest orchids at the landscape scale based on germination experiments and species distribution models integrating multiple habitat characteristics, they did not find molecular evidence for OMF diversity. A possible explanation for this is that OMF has a wide biogeographic distribution, and some major branches have been witnessed on a broad landscape scale (Jacquemyn et al., 2017). Hence, the distribution of OMF assemblages *per se* may not be a limiting factor for the distribution of orchids. However, extensive studies sampling orchids across continents would be required to determine whether the total fungal community enlarges or reduces the distribution of host orchids on the landscape scale. Studies on the local restriction of orchid distribution by OMF abundance include at least nine reports covering 13 specialized orchids (reviewed in McCormick et al., 2018). Most results demonstrate that the germination percentage of seeds is higher near adult orchids where OMF abundance tends to be greater. In addition, some studies show significant positive correlations between mycoheterotrophic orchid abundance and OMF abundance, strongly suggesting that OMF affects the abundance of orchids (McCormick et al., 2009). However, current evidence showing the influence of OMF abundance on the distribution of orchids is mainly based on seed germination and protocorm development (McCormick et al., 2012, 2016, 2018; McCormick and Jacquemyn, 2014). Subsequent studies on the later developmental stages of orchids might unravel more information.

Most orchids experience carbon restrictions during nutrient dormancy. During this phase, orchids require fungi to provide nutrients, and the recovery from dormancy is proposed to be linked to the local abundance of appropriate OMF (Rock-Blake et al., 2017; McCormick et al., 2018; Shefferson et al., 2018). Therefore, OMF abundance may affect the apparent density of orchid populations by affecting the dormancy process, thereby affecting the population characteristics and reproductive success. At the same time, OMF inoculation experiments revealed that the introduction of OMF in areas without orchids but near existing orchid populations can be successful, so that OMF abundance may also increase seed germination and the protocorm formation rate (McCormick et al., 2012, 2016). Interestingly, the relationship between the OMF abundance and the distribution of orchids suggests that the location of adult orchids determines the richness and abundance of OMF to some extent. In addition, several reports demonstrate that fungi obtain nutrients from autotrophic orchids (Cameron et al., 2006; Hynson et al., 2009; Liebel et al., 2015; Yeh et al., 2019). Therefore, we cannot rule out the possibility that the abundance of OMFs may be caused by the density of autotrophic orchids. Furthermore, it is essential to design more tests to explore factors controlling the abundance of OMF and their impact on individuals and populations of orchids.

EFFECTS OF OMF IDENTITY AND SPATIOTEMPORAL VARIABILITY ON ORCHID DISTRIBUTION AND POPULATION DYNAMICS

Most orchids are combined with different OMF in different habitats or climatic conditions, indicating that beneficial OMFs can vary across these conditions (Martos et al., 2012; Jacquemyn et al., 2016a,b; Duffy et al., 2019; Xing et al., 2020a). Germination and growth promotion tests have largely shown that OMF exhibit some specificity (e.g., Zi et al., 2014; Rasmussen et al., 2015; Meng et al., 2019; Zhang et al., 2020), while some studies have suggested that only a few members of OMF assemblages can promote the growth and flowering of orchids under various environmental stresses (McCormick et al., 2009, 2018). Therefore, the identities of OMF and their interactions with the environment may make certain orchid–OMF assemblages more beneficial to the growth of orchids, positively affecting their distribution and population dynamics. In addition, Nurfadilah et al. (2013) found that fungi associated with some widely distributed Australian orchids were more likely to acquire nutritional resources than fungi associated with orchids found only in certain habitats. This has also been confirmed at the genotype level by subsequent multi-omics studies (Kohler et al., 2015; Fochi et al., 2017a,b). Interestingly, *Tulasnella* and some *Serendipita* fungi lack genes for using nitrate and nitrite, though these genes are commonly found in *Ceratobasidium*. Moreover, these three types of rhizoctonias possess different genes that help in the absorption of carbon substrates. Accordingly, recent tests on the sensitivity of orchid seed to nitrate concentration have revealed that nitrates can affect the distribution of orchids by directly inhibiting seed germination (Figura et al., 2020). Hence, nitrates have inhibitory effects on seed germination, growth, and persistence of orchids. With the change of landscape, the rapid increase in nitrate content in intensive pastures or meadows inhabited by a large number of orchid species due to the high soil nitrification rate and the increasing atmospheric nitrogen deposition around the world (Figura et al., 2020; Moore et al., 2020), and eutrophication of habitats (particularly in habitats with severe human disturbance, such as some ancient tea estates in southwest China, the rich orchid resources are faced with the impact of heavy use of chemical fertilizers and human destruction) poses a potential threat to the distribution of orchids. It is worth mentioning that a recent study corroborated that some *Ceratobasidium* symbionts can effectively alleviate the inhibitory effect of nitrate on orchid seed germination (Figura et al., 2021). In addition, recent evidence shows that some cyanobacteria species with nitrogen-fixing activities are present in the velamen of epiphytic orchids (Deepthi and Ray, 2020). Therefore, in the epiphytic niches, *Tulasnella* and some *Serendipita* fungi that cannot use mineral nitrogen may benefit from the velamen roots of epiphytic orchids, at least during adulthood; indicating that the notion that OMF are free-living fungi may need to be reviewed, because epiphytic orchids may indirectly affect OMF performance by recruiting cyanobacteria species.

In addition to the distribution, abundance, and identity of OMF, spatiotemporal variation in OMF may more important factor in determining the distribution and population dynamics of orchids. The high spatiotemporal turnover rate of OMF may also reduce competition for resources, *via* niche separation, by promoting the coexistence of more orchid species in the natural environment. This is corroborated by several studies on the coexistence of terrestrial orchids that use a combination of spatial point pattern analysis and OMF phylogenetic analysis. These studies have revealed that coexisting orchid species have different OMF communities with little overlap over fine spatial scales, and that individual orchid species commonly occur as high-density clusters, displaying high local dominance (Jacquemyn et al., 2012, 2014, 2015a; Waud et al., 2016b). Therefore, the co-occurrence of terrestrial orchids observed in nature may be mediated by spatial distribution and interactions of the associated OMF. However, minimal research has been conducted on the relationship between the spatial distribution of epiphytic orchids on different phorophytes and OMF. Since epiphytic orchids account for about 70% of orchids (Dearnaley et al., 2012; Chase et al., 2015), the lack of such research limits our understanding of how OMF affect the distribution and population dynamics of orchids.

ROLES OF *TULASNELLA* IN ORCHID COMMUNITY CONSTRUCTION

The fungi *Tulasnella* mostly reside in various orchid tissues (mainly roots and stems) and tend to be extremely sensitive to environmental variables, indicating a strong dependence on host orchids or surrounding plants. Therefore, recovery from disruption of the multi-nutrient balance among *Tulasnella*, orchids, and accompanying plants (the plant species that often occur around a certain orchid species and do not belong to the Orchidaceae in natural habitats and are expected to have a specific association with the orchid species) that is established by long-term coevolution could be difficult. This can result in orchid breeders or orchid enthusiasts facing problems, while reestablishing conditions for growing orchids. Studies have shown that the use of peat-based, a mixture of coconut shells, bark shavings, soil from the original habitat, or a mixture of pinecone scales, mosses, and humus, can induce symbiosis between transplanted orchid individuals or asymbiotically cultured orchid individuals grown *ex situ* and Tulasnellaceae fungi (Han et al., 2016; Kaur et al., 2018; Qin et al., 2019). However, no *Tulasnella* symbiotic with orchid individuals were obtained when transplanted in original habitat soil or sawdust (May et al., 2020; Li et al., unpublished data). Preliminary research suggests that the dominant OMF and ONF in fungal communities reconstructed by cultured orchid individuals are different from those seen in wild populations. Overall, fungi belonging to the groups Atractiellales, Auriculariales, Ceratobasidiaceae, and *Fusarium* tended to increase, while the abundance of Tulasnellaceae and Pyronemataceae tended to decrease (Downing et al., 2017; Qin et al., 2019; May et al., 2020), as a consequence of transplantation, and the extent of such changes may depend on the time scales of culture or transplantation. This indicates low survival rates of cultured orchids, which may be caused by the loss of some key

OMF (e.g., Tulasnellaceae fungi) or the restricted construction of new mycorrhizal communities. Hence, selecting substrates with high OMF diversity, using molecular identification of OMF composition of substrates, is critical for improving culturing practices. However, further research is required to determine the extent of similarity in *Tulasnella* taxa between cultivated and wild orchids across different time scales.

A FRAMEWORK FOR HOW OMF AFFECT THE DISTRIBUTION AND POPULATION DYNAMICS OF ORCHIDS

Orchid mycorrhizal fungi exhibit widespread biogeographical distributions with major clades found all over the world, suggesting that the widespread distribution of orchids is driven by OMF (Jacquemyn et al., 2017). However, the mechanisms by which OMF affect the distribution and population dynamics of orchids are still poorly understood since most current knowledge is based on molecular data from adult plant symbionts, while complex mycorrhizal associations with orchids occur at different stages of their life cycle. In this paper, we postulate a simple framework for the effects of OMF on orchid distribution and population dynamics, though this conception may be biased. We argue that orchid–OMF associations exhibit complementary and specific effects of selection that are highly adapted to the environment, and promote the niche breadth of orchid species, which may act as a stabilizing force. More specifically, orchid species with specific mycorrhizae are usually symbiotic with generalist OMF, while OMF associated with host orchids with generalist mycorrhizae are often limited in their distribution. In other words, the distribution of orchids is shaped by coupled influences of environmental variables and efficient complementary selection between OMF and orchids. The most well-known example of association between generalist OMF and orchid species with mycorrhizal specificity is that of Serendipitaceae, which are symbiotic partners specifically associated with many host orchids. Serendipitaceae are also widely distributed, shared by orchids and their accompanying plants in several habitats, or serving as a beneficial growth-promoting fungus for a wide range of agricultural crops (e.g., Davis et al., 2015; Jacquemyn et al., 2015a; Fritsche et al., 2020; Reiter et al., 2020). While *Platanthera leucophaea*, which is protected by the United States federal government, is highly dependent on *Ceratobasidium* in the tallgrass prairie ecosystems of North America, *Ceratobasidium* species has also been isolated from various orchid species found in other locations (Thixton et al., 2020). Similarly, two rare *Orchis* sister species have high specificity for the dominant fungal symbiont *Tulasnella helicospora*, even though this fungus is found across the world (Calevo et al., 2020). Contrarily, generalist mycorrhizal orchids recruit a large number of symbiont partners, but these compatible OMF are rarely found in other areas (Jacquemyn et al., 2011a). While biogeography is the main factor affecting the microbial communities (including fungi and bacteria) associated with *Gymnadenia conopsea* (i.e., composition varies greatly with location), the species still exhibits certain specificities (Lin et al., 2020; Xing et al., 2020a). Similarly, Gao et al. (2020) found that OMF isolated from orchid species that coexist with *G. conopsea* in the wild do not promote its seed germination and protocorm formation *in vitro*, since they require specific OMF.

Environmental filtering largely accounts for the narrow distribution of OMF associated with these generalist mycorrhizal orchids. Recent evidence indicates that phosphorus content is higher in the roots of larger populations of *Platanthera cooperi* and the surrounding bulk soil, which are mainly colonized by the Tulasnellaceae. In contrast, higher zinc content and a higher relative abundance of Ceratobasidiaceae are observed in smaller *P. cooperi* populations (Kaur et al., 2020). Interestingly, Ceratobasidiaceae are more abundant in phosphorus-rich restored grasslands, while Serendipitaceae are more common in semi-natural grasslands with higher organic matter content (Vogt-Schilb et al., 2020). This may be due to differences in how variables in a particular habitat are weighed. In addition, it has been widely reported that several environmental variables (such as soil water content, pH, and soil nitrogen content) can affect the composition and abundance of OMF communities (Jacquemyn et al., 2015a; Waud et al., 2017; Duffy et al., 2019; Kaur et al., 2019; Mujica et al., 2020). Hence, the structure of OMF communities is significantly related to microenvironmental changes. Thus, these factors exert a joint effect on the formation and structure of orchid populations.

It must be noted that this framework applies mainly to terrestrial orchids. Since orchid–OMF interactions are of a higher order due to the presence of abundant phorophytes in epiphytic orchids, equilibrium dynamics underlying their mutual selection are complicated. More scenarios need to be considered to address this problem though a recent study has shown that phorophytes and epiphytic orchids harbor different fungal communities (Eskov et al., 2020). Moreover, there should be greater focus on the fungal taxa associated with epiphytic orchids, epiphytic niches, and accompanying plants as well as their mutual selection mechanisms. Lastly, the orchid–OMF complementary selection mechanisms may be related to evolutionary constraints, which will be discussed in detail in the following section.

In summary, the patchy distribution, heterogeneous abundance, identities, and spatiotemporal variability of OMF have crucial effects on the local distribution and population dynamics of orchids. The local distribution of orchids may in turn promote the formation and diversification of orchid species by curtailing the population size and gene flow among populations, which may be responsible for the huge species diversity of Orchidaceae. Since the distribution of orchids is affected by various factors, the relationship between OMF and the distribution of orchids should be further explored. Future studies must particularly focus on the influence of availability of OMF, flow of nutrient resources between orchids and OMF, and abiotic factors on the distribution of tropical orchids.

PHYLOGENETIC SIGNALS IN ORCHID–OMF INTERACTIONS

The composition and distribution of biological assemblages are strongly influenced by a series of ecological and evolutionary

processes (Heilmann-Clausen et al., 2016; Boeraeve et al., 2018; Beng and Corlett, 2019; Wang et al., 2019a). The genetic relationship between hosts in antagonistic or mutualistic interactions and its influence on the assembly of fungal communities has been unraveled by recent studies (e.g., Põlme et al., 2013; van der Linde et al., 2018). For example, different degrees of evolutionary constraints have been observed in AM, EcM, plant pathogens, and fungi in general (Erlandson et al., 2018; Wang et al., 2019a,b; Yang et al., 2019). In addition to these biotrophic fungal guilds, the dissimilarities among free-living soil fungal communities significantly increase over large spatial scales and with increasing plant phylogenetic distance, however, the explained variation is relatively lower than that of pathogens and EcM fungi (Yang et al., 2019). Controlled experiments also suggest that soil microorganisms that are obligately symbiotic with some trees usually promote growth on these trees or closely related species, but do not affect growth as much on distantly related species (Liang et al., 2019). These results suggest that closely related hosts usually have certain fungal specificities. Considering the dependence and specificity of orchids on OMF, the evolution of host orchids may be a key factor affecting the composition of the OMF community.

In recent years, some studies have indicated the existence of phylogenetic conservatism in the interplay between orchids and OMF. Jacquemyn et al. (2011b) revealed that the phylogenetic structure of 16 species of the genus *Orchis* distributed across 11 different regions in Europe can explain the community differences in associated Tulasnellaceae. Many orchid species that are closely related within genera host similar rhizoctonias or Tulasnellaceae operational taxonomic units (OTUs), such as *Cypripedium* (Shefferson et al., 2007), *Goodyera* (Shefferson et al., 2010), *Neottia* (Těšitelová et al., 2015), *Teagueia* (Suárez and Kottke, 2016), *Caladenia* (Phillips et al., 2016), *Dendrobium* (Xing et al., 2017), *Pleione* (Qin et al., 2019), and Cypripedioideae (lady's slipper subfamily; Shefferson et al., 2019). Therefore, orchid–OMF specific associations during their evolutionary history may result in strong influences of orchid phylogeny on OMF communities. However, rhizoctonias display little or no coevolution with host orchids. This indicates asymmetric interactions during coevolution process and phylogenetic conservatism of functional traits of orchids (De Deyn and van der Putten, 2005; Heilmann-Clausen et al., 2016; Wang et al., 2019a).

Nevertheless, studies on the interactions between orchids of multiple genera and rhizoctonias have produced inconsistent conclusions. Martos et al. (2012) performed a phylogenetic analysis of tropical orchids and symbiotic rhizoctonias distributed on Reunion Island, utilizing a narrow resolution (34 angraecoid species) and a broader resolution (25 orchid genera), and showed that the overall phylogenetic signal was weak. At a narrow resolution, the evolutionary constraints between orchids and rhizoctonias tended to be asymmetric, with phylogenetic signals only observed in orchids. Interestingly, at the broader resolution, both orchids and rhizoctonias in the epiphytic sub-networks displayed significant phylogenetic signals. Similarly, a recent analysis of the mycorrhizal association of 44 tropical orchids covering three life forms (terrestrial, epiphytic, and lithophytic) with rhizoctonias or *Tulasnella*, revealed low phylogenetic signals in both orchids and fungi (Xing et al., 2019). Moreover, no significant differences were observed in phylogenetic signals between the three types of orchids and the sub-networks formed by rhizoctonias or *Tulasnella* separately, which were close to zero. Differences in the results of these two studies could be related to the phylogenetic spectra of the orchids involved (Tedersoo et al., 2014; Xing et al., 2019). The number of terrestrial, epiphytic, and lithophytic orchid species in the latter study was less than 20, and the phylogenetic diversity focused on fewer orchid genera. No significant phylogenetic signals were consistently detected on either side of the interactions in the binary network formed by seven species of orchids and rhizoctonias belonging to different genera distributed in Song Mountain, Beijing (Chen et al., 2019b). Thus, further research could sample greater numbers of different types of orchids focusing on the broader orchid phylogenetic spectra. Furthermore, the associations of phylogenetically related host orchids with similar fungal communities in rhizosphere soil and orchid-occupied bulk soil may be worth investigating.

The phylogenetic niche conservatism theory proposes that host species with close genetic relationships tend to possess highly similar morphologies and functions (Losos, 2008). There is substantial evidence for the effects of phylogenetic eigenvectors and species-specific functional traits on fungal communities often overlap significantly. The phylogenetic effects of hosts can be explained by the conservatism of plant functional traits. In addition, the phylogenetic relatedness of hosts could explain the similarity of functional traits to a large extent, which would allow a rough prediction of either of these features based on the other (Wardle et al., 2004; Legay et al., 2014; López-García et al., 2017; Wang et al., 2019a; Yang et al., 2019). This is probably one of the major reasons for the greater likelihood of observing phylogenetic signals from the same orchid genus during interactions with rhizoctonias. However, since data regarding root traits that are important in the construction of underground communities is lacking, further investigations are required to examine the extent to which evolutionary constraints of orchid genera are caused by their own functional traits. In addition, little to no phylogenetic signals were observed in the narrow phylogenetic spectra of orchids. This could be because the associated rhizoctonias were mostly saprotrophic and endophytic fungi (Smith and Read, 2008; Jacquemyn et al., 2017) with relatively high functional redundancy (particularly saprotrophic fungi) and sensitivity of local species pools to abiotic environmental filtering, which would substantially obscure the influence of orchid phylogeny (Setälä and McLean, 2004; Erlandson et al., 2018).

When a plant invades or is transplanted into a new environment, existing microorganisms in the environment may adapt or be redistributed. Over time, the outcomes of these adaptations may not be very beneficial to hosts of other genotypes (Batstone et al., 2020), which indicates that shared evolutionary history is an important factor in the mutual selection between hosts and microorganisms. Consistent with this view, recent studies have shown that "invasive orchids were capable of associating with a broader range of mycorrhizal fungi than co-occurring native congeners (a generalist strategy)" but they were also less likely to harbor pathogenic fungal groups (Downing et al., 2020).

However, continual monitoring on longer time scales is required to verify whether beneficial associations developed by invasive orchids are driven by evolutionary history.

Hence, to summarize these two sections, the composition of OMF communities are determined jointly by ecological and evolutionary constraints, the relative importance of which depends on the specific time, space, and orchid species studied. Subsequent case studies could determine the contributions of these constraints through variation partitioning analysis (VPA) and help us understand the relationship between orchid ecology and evolution.

ORCHID MYCORRHIZAL NETWORK

The architecture of plant–fungus interactions varies according to the mycorrhizal type. The association network of AM and plants is usually characterized by a nested assembly such that host plants that are symbiotic with fewer AM prefer to form symbiotic associations with AM that are symbiotic with most host plants (Chagnon et al., 2012; Montesinos-Navarro et al., 2012). Orchid mycorrhizae and ericoid mycorrhizae (ErM) interaction networks form a modular structure (Martos et al., 2012; Jacquemyn et al., 2015b; Toju et al., 2016; Xing et al., 2019) with high specificity between host plants and partners, while EcM network architectures tend to assume an intermediate structure (Bahram et al., 2014; van der Heijden et al., 2015). Recently, Põlme et al. (2018) performed a meta-analysis of 111 datasets of plant–fungus interactions, which showed that the OMF community responded most strongly to orchid host identity, with significantly higher levels of specificity than other types and higher modularity than EcM and AM. In general, the orchid mycorrhizal network has significant characteristics of modules as a whole. However, the contributions of modularity and nestedness in the local network often change, and the orchid–OMF interaction in different ecosystems shows inconsistency with the whole in network eigenvalues. Temperate and Mediterranean ecosystems exhibit slightly different architectures: the former tends to be significantly nested (Jacquemyn et al., 2011a, 2015b, 2016b), whereas tropical orchids and OMF symbiosis are more diversified (Martos et al., 2012; Kottke et al., 2013; Herrera et al., 2018; Xing et al., 2019, 2020b).

The distribution of orchid species and OMF and their selective effects constitute a complex orchid mycorrhizal network, and the tight junctions present in the network are particularly important for the coexistence and population dynamics of orchid species. Jacquemyn et al. (2011a) applied network analysis for studying symbiotic relationships between orchids and OMF for the first time, and analyzed the architecture of the interaction between 16 *Orchis* species distributed in 11 regions of Europe and OMF. From this study, they confirmed that the interaction between orchids and OMF at the community level showed a nested structure similar to a mutualistic relationship network seen in pollination and seed dispersal, as well as networks of predation. This study was identified as a pioneering work in the field of orchid mycorrhizal networks based on our LCS analysis (**Figure 1B**). To our knowledge, this is also one of the first studies to apply network analysis methods to provide insight into complex mycorrhizal symbiotic relationships. Immediately afterward, Martos et al. (2012) built a binary network of nearly half of the tropical orchid species and 95 rhizoctonia fungi associated with them on Reunion Island, and found that the overall orchid mycorrhizal network showed high modularity due to the ecological barrier between epiphytic and terrestrial orchids. However, the epiphytic subnetwork formed a highly nested pattern. This study constructed the largest orchid mycorrhizal network to date, which was another important milestone in the progress of the orchid mycorrhizal network and continues to influence fields other than orchid mycorrhizal networks (**Figure 1C**). Subsequently, three studies further supported the highly modular structure of the orchid mycorrhizal network. The structure of the mycorrhizal network, formed by species of the genus *Dactylorhiza* with different levels of ploidy and inhabiting a wide range of habitats (including acid peat bogs, wet alkaline grasslands, dry meadows, and forests), is characterized by modularity that is significantly dependent on local environmental conditions (Jacquemyn et al., 2016b). Although orchid species of different life forms are all simultaneously symbiotic with multiple OMF, the overall interconnected network remains highly modular due to the enhanced specificity of Tulasnellaceae from terrestrial to epiphytic or lithophytic orchids (Xing et al., 2019). Interestingly, overall OMF diversity in a narrow transect of 10 × 1,000 m with relatively similar habitats could be partitioned into a subset of 20 terrestrial orchids with mycorrhizal diversity belonging to five coexisting genera, low overlap among the subsets, and multiple isolated groups present in the interconnected network (Jacquemyn et al., 2015b).

In contrast, four studies supported significantly nested network features. The nested network formed between the highly diverse epiphytic orchids and rhizoctonia distributed in tropical montane rainforests of southern Ecuador may be influenced by climate, as climate is the main driving force for OMF community turnover among sites (Kottke et al., 2013). Herrera et al. (2018) further confirmed that terrestrial and epiphytic orchids shared abundant Tulasnellaceae mycobionts in different habitats within tropical forests of southern Ecuador, and the network showed a nested structure with generalists forming the core. Due to the large degree of overlap seen in the mycorrhizal communities of epiphytic and lithophytic orchids, the network structure formed by these two types of orchids and sympatrically distributed terrestrial orchids is highly modular but also shows significant nestedness (Xing et al., 2019; Qin et al., 2020). Notably, the OMF network of *Dendrobium* species inhabiting the same phorophyte escaped strong selection by the host and showed significant asymmetric specialization (Xing et al., 2020b). In addition, consistent with pollination networks, robustness analysis revealed that generalist OMF and orchid species play an important role in the stability of interrelated networks, and their loss may drive the cascading loss of biodiversity (Memmott et al., 2004; Burgos et al., 2007; Herrera et al., 2018). Moreover, the robustness of the symbiotic network formed by terrestrial and epiphytic orchids was only slightly different, implying that OMF is equally important for the fate of both life forms of orchids (Herrera et al., 2018).

FORMATION OF SIGNIFICANTLY NESTED STRUCTURES

Although there was no significant difference in community nestedness among different mycorrhizal types, it was significantly negatively correlated with annual average rainfall. Moreover, the nestedness values of orchid mycorrhizal networks showed large variation, implying that the significant nested structures formed by orchids and OMF may be more sensitive to fluctuations in environmental conditions (Põlme et al., 2018). This is consistent with the hypothesis proposed by Kottke et al. (2013) that climate may be the cause of nested networks. More specifically, the similarity of OMF communities resulting from habitat variation may explain the observed nested structure, such as the significant nested structure seen in *Orchis* species due to low variation in habitats. Contrarily, *Dactylorhiza* species exhibit greater habitat differentiation, resulting in rare overlap among OMF communities between different populations and a highly modular structure (Jacquemyn et al., 2011a, 2016b). From an evolutionary point of view, mycorrhizal associations of some species of *Orchis* and *Cypripedium* may be undergoing expansion of phylogenetic breadth, presenting broad specificity, and gradually driving network attributes to have generalists at the core order to maximize adaptation to the environment and absorb nutrients (Shefferson et al., 2007; Jacquemyn et al., 2011b).

In addition, the research scale and the threshold for species delimitation may be two important factors affecting network nestedness. The smaller the scale of the network constructed, the tighter interaction within the network, resulting in increased nestedness of the network (Caruso et al., 2012; Öpik and Moora, 2012; Chagnon et al., 2016). Thus, sampling should be performed at the similar scales to compare the mycorrhizal network of coexisting orchid species in different habitats. As the OTU sequence similarity threshold increases, the number of OTUs and rare associations increase, while the strength of nestedness decreases (Toju et al., 2014; Põlme et al., 2018). However, the pattern of nestedness observed in the orchid mycorrhizal network did not vary according to the OTU delimitation threshold, probably because most of the OMF considered in these studies were highly abundant species (Jacquemyn et al., 2011a; Herrera et al., 2018). Considering the importance of rare species in subsurface ecosystem services, the impact of OTU classification on network nestedness should be fully considered, while performing in-depth analysis of orchid–fungal (including ONF) networks.

FORMATION OF HIGHLY MODULAR FEATURES

Since coexisting orchid species commonly exhibit highly spatially clustered and strongly spatially segregated distribution patterns and are often associated with different OMF communities, and these OMF communities with patchy distributions rarely overlap (Jacquemyn et al., 2007, 2014; Waterman et al., 2011; Waud et al., 2016b), the interaction network between orchids and OMF often shows highly modular characteristics. On the one hand, this may be due to the specific selection of OMF taxa by host orchids (Jacquemyn et al., 2015b; Xing et al., 2019). In order to maximize mutual symbiosis in a complex environment, hosts usually allocate more carbohydrates to better quality partners, resulting in increased levels of specificity for the association between orchids and OMF, which in turn form a symmetrical and modular network structure of interactions (Kiers et al., 2011). Notably, the host selection effect of ErM associations is low but still presents a high degree of modularity, which may be explained by the high sensitivity of the modularity metric to total links in the dataset (Bahram et al., 2014). Moreover, the modularity of networks may be due to environmental variables that enhance specific selection of OMF by hosts (Jacquemyn et al., 2010b; Shefferson et al., 2019). Specific environmental gradients or distinct niche differentiation may allow host orchids to be specifically associated with OMF characterized by greater taxonomic richness or functional diversity. In addition, the presence of forbidden links may explain the strong modular structure (Olesen et al., 2011). For example, the inconsistency of spatiotemporal dynamic changes in OMF limits certain pairwise interactions that may occur throughout the network.

A FRAMEWORK FOR WEIGHING THE RELATIVE IMPORTANCE OF NESTEDNESS AND MODULARITY

Based on these factors that affect the characteristics of the orchid mycorrhizal network, we initially proposed a framework to weigh the relative importance of nestedness and modularity of the orchid mycorrhizal network (**Figure 2**). The characteristics of complex mycorrhizal networks formed by orchids and widely distributed OMF mainly depend on the relative strength between specific selection of host orchids and generalist selection of OMF. When the coupled influences of phylogenetic spectra, root traits, and environmental differences of orchids increases the intensity of specific selection of host orchids beyond that by OMF, the network structure is highly modular. Contrarily, when the similarity of environmental conditions drives coexisting orchid species to share similar OMF communities, resulting in greater intensity of generalist selection of OMF, the network structure shows significant nested assembly.

FUTURE DIRECTIONS

Co-occurrence network analysis is a promising method to gain insight into ecological communities and may serve as a potential approach to more efficiently and conveniently study the distributions of microorganisms, the pattern of symbiotic relationships, and their impact on plant distribution and population dynamics at the community level. However, this method has not been fully applied to multiple mutualisms of orchids, fungi, and their accompanying plants or phorophytes. Therefore, more attention should be paid to the in-depth analysis of the total fungal co-occurrence network among different habitats (populations) of the same species, different orchid species coexisting in the same habitat, and orchid species of different life forms

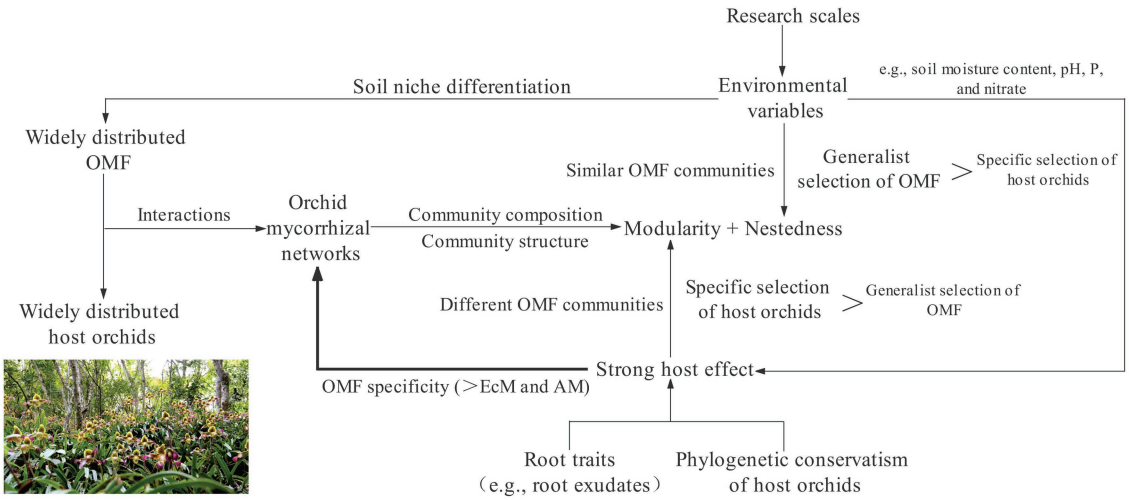

FIGURE 2 | A framework of the architecture of the orchid mycorrhizal network. The intensity between the specific selection of host orchids and the generalist selection of orchid mycorrhizal fungi (OMF) indirectly determines the relative importance of modularity and nestedness by affecting the degree of similarity of OMF communities. The thick line implies that the phylogenetic conservatism and root traits of host orchids as well as environmental variables jointly drive OMF to be significantly more specific than ectomycorrhizae (EcM) and arbuscular mycorrhizae (AM) fungi.

or at different developmental stages in further studies. In order to increase the accuracy of orchid fungal networks, spatial autocorrelations should be avoided as far as possible when collecting samples and, combinations of analytical methods should be used. Moreover, analytical methods such as IDEN should be developed for the investigation of orchid traits and examining bipartite networks of orchid–fungus interaction, which can help understand cross-kingdom associations between vegetation data and microorganisms (Feng et al., 2019). Alternatively, interactions among orchid-associated fungi can be jointly analyzed by MENA and SparCC, using CoNet, which comprehensively considers multiple correlations, or SPIEC-EASI, which uses a more inferential function (Deng et al., 2012; Faust et al., 2012; Friedman and Alm, 2012; Kurtz et al., 2015).

The abundance of microorganisms in a local community is extremely uneven, as shown by a few dominant groups playing major roles in active growth along with a large number of rare groups (Jia et al., 2018). To distinguish abundant and rare microbes in a community in terms of their roles and contributions, all OTUs detected within a community are usually divided into six exclusive categories based on relative abundance (**Figure 1D**; Dai et al., 2016; Chen et al., 2019c). Recently, an increasing number of studies have emphasized the importance of rare biosphere microbes, which includes more metabolically active microorganisms than abundant groups, plays a key role in co-occurrence networks, ecosystem versatility, and plant performance. These microbes are not only highly resistant to environmental stresses, but also enhance the function of abundant microbes to some extent (Jousset et al., 2017; Ziegler et al., 2018; Liang et al., 2020; Xiong et al., 2020). Similarly, some rare OMF affiliated with Serendipitaceae (such as *Serendipita indica* and *Serendipita restingae*) have been demonstrated to promote the germination of orchid seeds and the growth and adaptation of plantlets (Schäfer and Kogel, 2009; Oliveira et al., 2014; Shah et al., 2019; Fritsche et al., 2020). Interestingly, these rare OMF coexist with a wide range of plants and increase the reproduction and fitness of symbiotic hosts. Moreover, *S. indica* has value in agricultural applications due to its effect on increasing yield of tomatoes by 65%, while also inducing their resistance to salt stress (Abdelaziz et al., 2019).

Increasing attention to the mycorrhizal network of orchids, rare taxa in the network, and adaptive evolution between orchids and fungi helps identify functions of key orchid fungi and provides clues about orchid distribution, population dynamics, and the mechanisms underlying orchid–fungal mutualisms. Thus, such studies may lend insight into the following aspects with significant implications:

1. Since the protective effect of mycorrhizal symbiosis on plants is a redundant feature, some key taxa revealed by network analysis can be used as targets. The isolation and culture of these target strains can be achieved as far as possible using medium prediction techniques in combination with some characteristics of target strains (such as the increase in the proportion of rare microorganisms in acidic environments) or simulating the growth conditions of orchids in the field. In addition, the effects of their combinations on orchid germination and various growth stages can be examined to identify simplified microbial groups that dominate the community and can meet the demand for host nutrients. Tentative exploration of orchid SynCom is would be a major advancement in orchid microbiome research and a key step for the application of scientific research achievements into greenhouse and natural environments.
2. Metagenomic analysis can assist in the functional study of key species, and binning assembly with the help of contigs obtained by metagenomic splicing can help in the annotation of genes and their functions. Moreover, comparative genome analysis

and evolutionary analysis of inseparable key species at the strain level may benefit from such approaches and advance our understanding of the mechanisms of ecological adaptation, nutrient mutualism, and metabolic functions of the strains.

3. Since plant roots continuously secrete carbon and other nutrients to the rhizosphere environment, the rhizosphere is known as one of the most dynamic interfaces on Earth. This significantly affects the arms race within complex microbial communities as well as the growth and health of hosts (Jogaiah et al., 2013; Raaijmakers, 2015; Lee et al., 2020). However, reports on fungal communities in the rhizosphere of orchids are currently limited. Therefore, future studies should focus on the composition, dynamics, and function of orchid fungal communities in this microdomain and the correlation of their biogeographic patterns with the distribution and population dynamics of orchids.
4. Attempts should be made to correlate the underground fungal diversity with the genetic characteristics of aboveground orchid populations to gain insight into the genetic diversity and dynamic history of orchid populations. This can be done through SSR molecular markers and ABC models or SNP markers and DADI models, to indirectly predict the dynamics of fungal diversity with the aim of protecting orchid gene pools (including orchid provenances and fungal sources).
5. Increasing evidence suggests that nitrates significantly affect the composition and abundance of OMF and inhibit the germination of orchid seeds in natural habitats (Duffy et al., 2019; Figura et al., 2020). Further work should focus on the effects of specific environmental variables, such as soil moisture content, nitrate content, and pH, which are frequently reported to affect OMF communities in network analyses, to gain insight into how these metrics affect sub-modules and the entire network. At the same time due to the lack of reports on orchid seed-associated microbial communities, we know little to nothing about the heritability of microorganisms associated with orchid species and the vertical transmission ability of the microbiome at the plant level. These are important aspects of orchid–fungus mutualism that require urgent attention.

Finally, in order to better point out the research direction of orchid fungal networks, while emphasizing the importance of considering ONF and making full use of network analysis methods in orchid–fungal interaction, we also provide a conceptual framework illustrating that orchid fungal networks are critical for insight into the complex and dynamic linkages between the orchid, fungi, and the environment, as well as the exploration of orchid conservation practices (**Figure 3**). Furthermore, several forward-looking studies have recently confirmed that certain

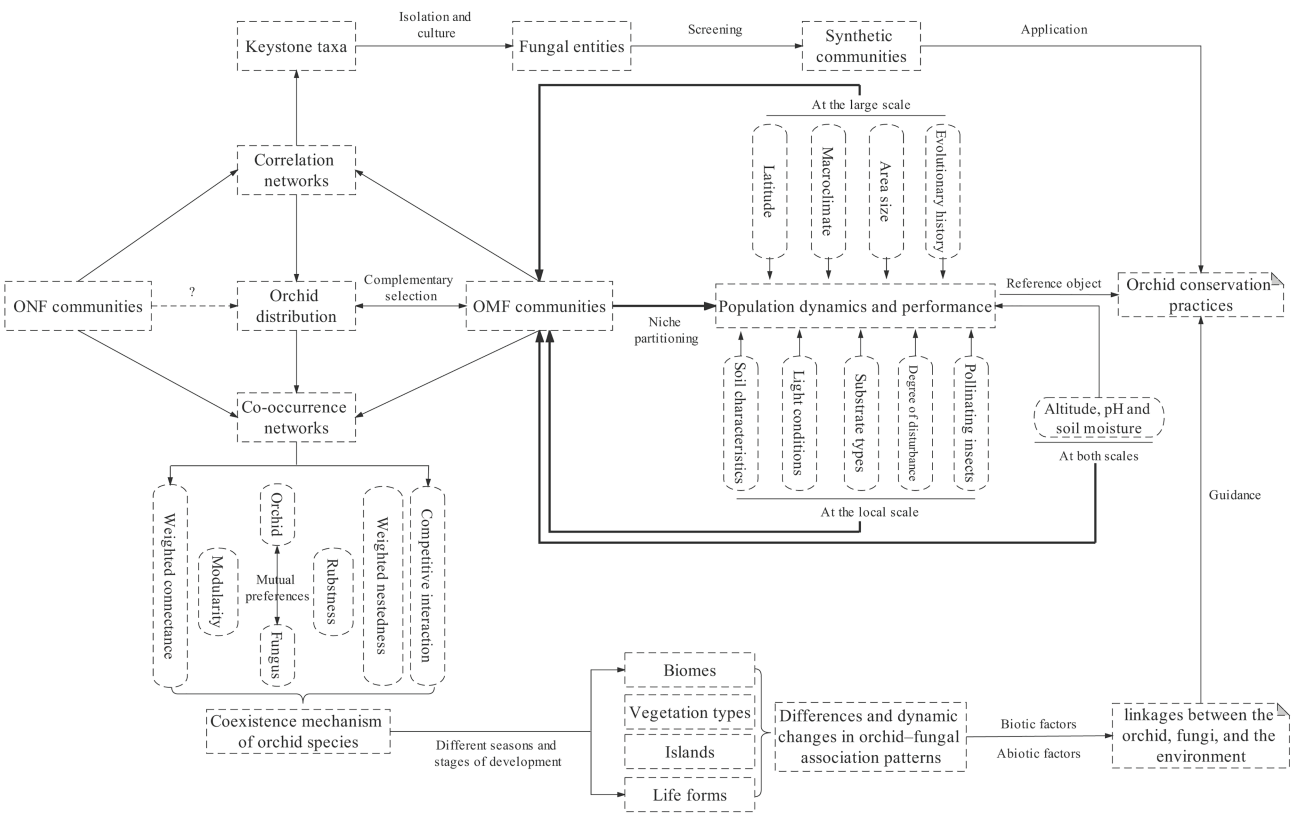

FIGURE 3 | A framework illustrating that orchid fungal networks are critical for insight into the complex and dynamic linkages between the orchid, fungi, and the environment, as well as the exploration of orchid conservation practices. The thick lines imply increasing evidence that environmental factors may indirectly affect orchid distribution and population dynamics by driving niche partitioning in OMF communities (see the second paragraph in the Introduction); dashed line with question marks indicate hypothetical relationships that have rarely been studied.

bacterial taxa as well as microbial interkingdom interactions are essential for plant growth (Durán et al., 2018; Finkel et al., 2020). Observations under the microscope suggest that bacterial taxa mainly reside within the root caps of orchids, while fungal taxa are found in various subdivisions of roots. The spatial distribution pattern of such cross-kingdom microorganisms in roots seems to be consistent with their characteristics because fungal hyphae can act as highways for bacterial movement and also as transport systems for microorganisms belonging to other kingdoms. However, it is unclear whether microorganisms in different kingdoms engage in mutualism within the roots, and whether such mutualism affects the distribution and population dynamics of orchids. These issues should be focus areas of research on orchids and other plants in the future.

AUTHOR CONTRIBUTIONS

JG and M-AS designed the outline of the manuscript. TL, JG, SW, and WY collected the data and wrote the manuscript. M-AS and JG polished the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this review can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.647114/full#supplementary-material

REFERENCES

Abdelaziz, M. E., Abdelsattar, M., Abdeldaym, E. A., Atia, M. A. M., Mahmoud, A. W. M., Saad, M. M., et al. (2019). Piriformospora indica alters Na+/K+ homeostasis, antioxidant enzymes and LeNHX1 expression of greenhouse tomato grown under salt stress. *Sci. Hortic.* 256:108532. doi: 10.1016/j.scienta.2019.05.059

Aly, A. H., Debbab, A., Kjer, J., and Proksch, P. (2010). Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Divers.* 41, 1–16. doi: 10.1007/s13225-010-0034-4

Bahram, M., Harend, H., and Tedersoo, L. (2014). Network perspectives of ectomycorrhizal associations. *Fungal Ecol.* 7, 70–77. doi: 10.1016/j.funeco.2013.10.003

Banerjee, S., Schlaeppi, K., and van der Heijden, M. G. A. (2018). Keystone taxa as drivers of microbiome structure and functioning. *Nat. Rev. Microbiol.* 16, 567–576. doi: 10.1038/s41579-018-0024-1

Batstone, R. T., O'Brien, A. M., Harrison, T. L., and Frederickson, M. E. (2020). Experimental evolution makes microbes more cooperative with their local host genotype. *Science* 370, 476–478. doi: 10.1126/science.abb7222

Beng, K. C., and Corlett, R. T. (2019). Identifying the mechanisms that shape fungal community and metacommunity patterns in Yunnan. *Fungal Ecol.* 42;100862. doi: 10.1016/j.funeco.2019.08.006

Boeraeve, M., Honnay, O., and Jacquemyn, H. (2018). Effects of host species, environmental filtering and forest age on community assembly of ectomycorrhizal fungi in fragmented forests. *Fungal Ecol.* 36, 89–98. doi: 10.1016/j.funeco.2018.08.003

Burgos, E., Ceva, H., Perazzo, R. P. J., Devoto, M., Medan, D., Zimmermann, M., et al. (2007). Why nestedness in mutualistic networks? *J. Theor. Biol.* 249, 307–313. doi: 10.1016/j.jtbi.2007.07.030

Calevo, J., Voyron, S., Ercole, E., and Girlanda, M. (2020). Is the distribution of two rare *Orchis* sister species limited by their main mycobiont? *Diversity* 12:262. doi: 10.3390/d12070262

Cameron, D. D., Leake, J. R., and Read, D. J. (2006). Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. *New Phytol.* 171, 405–416. doi: 10.1111/j.1469-8137.2006.01767.x

Caruso, T., Rillig, M. C., and Garlaschelli, D. (2012). On the application of network theory to arbuscular mycorrhizal fungi-plant interactions: the importance of basic assumptions. *New Phytol.* 194, 891–894. doi: 10.1111/j.1469-8137.2012.04163.x

Cavieres, L. A., Brooker, R. W., Butterfield, B. J., Cook, B. J., Kikvidze, Z., Lortie, C. J., et al. (2014). Facilitative plant interactions and climate simultaneously drive alpine plant diversity. *Ecol. Lett.* 17, 193–202. doi: 10.1111/ele.12217

Chagnon, P., Bradley, R. L., and Klironomos, J. N. (2012). Using ecological network theory to evaluate the causes and consequences of arbuscular mycorrhizal community structure. *New Phytol.* 194, 307–312. doi: 10.1111/j.1469-8137.2011.04044.x

Chagnon, P., U'Ren, J. M., Miadlikowska, J., Lutzoni, F., and Arnold, A. E. (2016). Interaction type influences ecological network structure more than local abiotic conditions: evidence from endophytic and endolichenic fungi at a continental scale. *Oecologia* 180, 181–191. doi: 10.1007/s00442-015-3457-5

Chase, M. W., Cameron, K. M., Freudenstein, J. V., Pridgeon, A. M., Salazar, G., and van den Berg, C. (2015). An updated classification of Orchidaceae. *Bot. J. Linn. Soc.* 177, 151–174. doi: 10.1111/boj.12234

Chen, Y. H., Gao, Y., Song, L. L., Zhao, Z. Y., Guo, S. X., and Xing, X. K. (2019b). Mycorrhizal fungal community composition in seven orchid species inhabiting song mountain, Beijing, China. *Sci. China Life Sci.* 62, 838–847. doi: 10.1007/s11427-018-9471-x

Chen, X. M., Guo, S. X., and Meng, Z. X. (2006). Effects of fungal elicitor on the protocorms of *Dendrobium candidum*. *J. Chin. Pharm. Sci.* 41, 1692–1694.

Chen, W., Ren, K., Isabwe, A., Chen, H., Liu, M., and Yang, J. (2019c). Stochastic processes shape microeukaryotic community assembly in a subtropical river across wet and dry seasons. *Microbiome* 7:138. doi: 10.1186/s40168-019-0749-8

Chen, L., Swenson, N. G., Ji, N. N., Mi, X. C., Ren, H. B., Guo, L. D., et al. (2019a). Differential soil fungus accumulation and density dependence of trees in a subtropical forest. *Science* 366, 124–128. doi: 10.1126/science.aau1361

Cregger, M. A., Veach, A. M., Yang, Z. K., Crouch, M. J., Vilgalys, R., Tuskan, G. A., et al. (2018). The *Populus* holobiont: dissecting the effects of plant niches and genotype on the microbiome. *Microbiome* 6:31. doi: 10.1186/s40168-018-0413-8

Dai, T., Zhang, Y., Tang, Y., Bai, Y., Tao, Y., Huang, B., et al. (2016). Identifying the key taxonomic categories that characterize microbial community diversity using full-scale classification: a case study of microbial communities in the sediments of Hangzhou Bay. *FEMS Microbiol. Ecol.* 92:fiw150. doi: 10.1093/femsec/fiw150

Davis, B. J., Phillips, R. D., Wright, M., Linde, C. C., and Dixon, K. W. (2015). Continent-wide distribution in mycorrhizal fungi: implications for the

biogeography of specialized orchids. *Ann. Bot.* 116, 413–421. doi: 10.1093/aob/mcv084

De Deyn, G. B., and Van der Putten, W. H. (2005). Linking aboveground and belowground diversity. *Trends Ecol. Evol.* 20, 625–633. doi: 10.1016/j.tree.2005.08.009

Dearnaley, J. D. W. (2007). Further advances in orchid mycorrhizal research. *Mycorrhiza* 17, 475–486. doi: 10.1007/s00572-007-0138-1

Dearnaley, J. D. W., Martos, F., and Selosse, M.-A. (2012). "Orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects," in *Fungal Associations*. *2nd Edn.* ed. B. Hock (Berlin, Heidelberg: Springer), 207–230.

Deepthi, A. S., and Ray, J. G. (2020). Algal associates and the evidence of cyanobacterial nitrogen fixation in the velamen roots of epiphytic orchids. *Glob. Ecol. Conserv.* 22:e00946. doi: 10.1016/j.gecco.2020.e00946

Delavaux, C. S., Weigelt, P., Dawson, W., Duchicela, J., Essl, F., van Kleunen, M., et al. (2019). Mycorrhizal fungi influence global plant biogeography. *Nat. Ecol. Evol.* 3, 424–429. doi: 10.1038/s41559-019-0823-4

Deng, Y., Jiang, Y.-H., Yang, Y. F., He, Z. L., Luo, F., and Zhou, J. Z. (2012). Molecular ecological network analyses. *BMC Bioinformatics* 13:113. doi: 10.1186/1471-2105-13-113

Djordjević, V., and Tsiftsis, S. (2020). "The role of ecological factors in distribution and abundance of terrestrial orchids," in *Orchids Phytochemistry, Biology and Horticulture: Reference Series in Phytochemistry*. eds. J. M. Mérillon and H. Kodja (Switzerland, Cham: Springer), 1–77.

Djordjević, V., Tsiftsis, S., Lakušić, D., Jovanović, S., and Stevanović, V. (2016). Factors affecting the distribution and abundance of orchids in grasslands and herbaceous wetlands. *Syst. Biodivers.* 14, 355–370. doi: 10.1080/14772000.2016.1151468

Downing, J. L., Liu, H., McCormick, M. K., Arce, J., Alonso, D., and Lopez-Perez, J. (2020). Generalized mycorrhizal interactions and fungal enemy release drive range expansion of orchids in southern Florida. *Ecosphere* 11:e3228. doi: 10.1002/ecs2.3228

Downing, J. L., Liu, H., Shao, S. C., Wang, X. L., McCormick, M., Deng, R. Y., et al. (2017). Contrasting changes in biotic interactions of orchid populations subject to conservation introduction vs. conventional translocation in tropical China. *Biol. Conserv.* 212, 29–38. doi: 10.1016/j.biocon.2017.05.021

Duffy, K. J., Waud, M., Schatz, B., Petanidou, T., and Jacquemyn, H. (2019). Latitudinal variation in mycorrhizal diversity associated with a European orchid. *J. Biogeogr.* 46, 968–980. doi: 10.1111/jbi.13548

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis* survival. *Cell* 175, 973–983. doi: 10.1016/j.cell.2018.10.020

Erlandson, S., Wei, X. J., Savage, J., Cavender-Bares, J., and Peay, K. (2018). Soil abiotic variables are more important than Salicaceae phylogeny or habitat specialization in determining soil microbial community structure. *Mol. Ecol.* 27, 2007–2024. doi: 10.1111/mec.14576

Eskov, A. K., Voronina, E. Y., Tedersoo, L., Tiunov, A. V., Manh, V., Prilepsky, N. G., et al. (2020). Orchid epiphytes do not receive organic substances from living trees through fungi. *Mycorrhiza* 30, 697–704. doi: 10.1007/s00572-020-00980-w

Faust, K., Sathirapongsasuti, J. F., Izard, J., Segata, N., Gevers, D., Raes, J., et al. (2012). Microbial co-occurrence relationships in the human microbiome. *PLoS Comput. Biol.* 8:e1002606. doi: 10.1371/journal.pcbi.1002606

Fay, M. F. (2018). Orchid conservation: how can we meet the challenges in the twenty-first century? *Bot. Stud.* 59:16. doi: 10.1186/s40529-018-0232-z

Feng, K., Zhang, Y., He, Z., Ning, D., and Deng, Y. (2019). Interdomain ecological networks between plants and microbes. *Mol. Ecol. Resour.* 19, 1565–1577. doi: 10.1111/1755-0998.13081

Figura, T., Tylová, E., Jersáková, J., Vohník, M., and Ponert, J. (2021). Fungal symbionts may modulate nitrate inhibitory effect on orchid seed germination. *Mycorrhiza* 31, 231–241. doi: 10.1007/s00572-021-01021-w

Figura, T., Weiser, M., and Ponert, J. (2020). Orchid seed sensitivity to nitrate reflects habitat preferences and soil nitrate content. *Plant Biol.* 22, 21–29. doi: 10.1111/plb.13044

Finkel, O. M., Salas-González, I., Castrillo, G., Conway, J. M., Law, T. F., Teixeira, P. J. P. L., et al. (2020). A single bacterial genus maintains root growth in a complex microbiome. *Nature* 587, 103–108. doi: 10.1038/s41586-020-2778-7

Fochi, V., Chitarra, W., Kohler, A., Voyron, S., Singan, V. R., Lindquist, E. A., et al. (2017a). Fungal and plant gene expression in the *Tulasnella calospora-Serapias vomeracea* symbiosis provides clues about nitrogen pathways in orchid mycorrhizas. *New Phytol.* 213, 365–379. doi: 10.1111/nph.14279

Fochi, V., Falla, N., Girlanda, M., Perotto, S., and Balestrini, R. (2017b). Cell-specific expression of plant nutrient transporter genes in orchid mycorrhizae. *Plant Sci.* 263, 39–45. doi: 10.1016/j.plantsci.2017.06.015

Friedman, J., and Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLoS Comput. Biol.* 8:e1002687. doi: 10.1371/journal.pcbi.1002687

Fritsche, Y., Lopes, M. E., Selosse, M., Stefenon, V. M., and Guerra, M. P. (2020). *Serendipita restingae* sp. nov. (Sebacinales): an orchid mycorrhizal agaricomycete with wide host range. *Mycorrhiza* 31, 1–15. doi: 10.1007/s00572-020-01000-7

Gao, Y., Zhao, Z. Y., Li, J. Y., Liu, N., Jacquemyn, H., Guo, S. X., et al. (2020). Do fungal associates of co-occurring orchids promote seed germination of the widespread orchid species *Gymnadenia conopsea*? *Mycorrhiza* 30, 221–228. doi: 10.1007/s00572-020-00943-1

Givnish, T. J., Spalink, D., Ames, M., Lyon, S. P., Hunter, S. J., Zuluaga, A., et al. (2016). Orchid historical biogeography, diversification, Antarctica and the paradox of orchid dispersal. *J. Biogeogr.* 43, 1905–1916. doi: 10.1111/jbi.12854

Hajong, S., and Kapoor, R. (2020). An amalgam of pathogenic and beneficial endophytic fungi colonizing four *Dendrobium* species from Meghalaya. *J. Basic Microbiol.* 60, 415–423. doi: 10.1002/jobm.201900631

Han, J. Y., Xiao, H. F., and Gao, J. Y. (2016). Seasonal dynamics of mycorrhizal fungi in Paphiopedilum spicerianum (Rchb.F) Pfitzer—a critically endangered orchid from China. *Glob. Ecol. Conserv.* 6, 327–338. doi: 10.1016/j.gecco.2016.03.011

Heilmann-Clausen, J., Maruyama, P. K., Bruun, H. H., Dimitrov, D., Læssøe, T., Frøslev, T. G., et al. (2016). Citizen science data reveal ecological, historical and evolutionary factors shaping interactions between woody hosts and wood-inhabiting fungi. *New Phytol.* 212, 1072–1082. doi: 10.1111/nph.14194

Hemrová, L., Kotilínek, M., Konečná, M., Paulič, R., Jersáková, J., Těšitelová, T., et al. (2019). Identification of drivers of landscape distribution of forest orchids using germination experiment and species distribution models. *Oecologia* 190, 411–423. doi: 10.1007/s00442-019-04427-8

Herrera, P., Kottke, I., Molina, M. C., Méndez, M., and Suárez, J. P. (2018). Generalism in the interaction of Tulasnellaceae mycobionts with orchids characterizes a biodiversity hotspot in the tropical Andes of southern Ecuador. *Mycoscience* 59, 38–48. doi: 10.1016/j.myc.2017.08.003

Hynson, N. A., Preiss, K., and Gebauer, G. (2009). Is it better to give than to receive? A stable isotope perspective on orchid–fungal carbon transport in the green orchid species *Goodyera repens* and *Goodyera oblongifolia*. *New Phytol.* 182, 8–11. doi: 10.1111/j.1469-8137.2009.02778.x

Jacquemyn, H., Brys, R., Cammue, B. P. A., Honnay, O., and Lievens, B. (2011b). Mycorrhizal associations and reproductive isolation in three closely related *Orchis* species. *Ann. Bot.* 107, 347–356. doi: 10.1093/aob/mcq248

Jacquemyn, H., Brys, R., and Jongejans, E. (2010a). Size-dependent flowering and costs of reproduction affect population dynamics in a tuberous perennial woodland orchid. *J. Ecol.* 98, 1204–1215. doi: 10.1111/j.1365-2745.2010.01697.x

Jacquemyn, H., Brys, R., Lievens, B., and Wiegand, T. (2012). Spatial variation in below-ground seed germination and divergent mycorrhizal associations correlate with spatial segregation of three co-occurring orchid species. *J. Ecol.* 100, 1328–1337. doi: 10.1111/j.1365-2745.2012.01998.x

Jacquemyn, H., Brys, R., Merckx, V. S. F. T., Waud, M., Lievens, B., and Wiegand, T. (2014). Coexisting orchid species have distinct mycorrhizal communities and display strong spatial segregation. *New Phytol.* 202, 616–627. doi: 10.1111/nph.12640

Jacquemyn, H., Brys, R., Vandepitte, K., Honnay, O., Roldán-Ruiz, I., and Wiegand, T. (2007). A spatially explicit analysis of seedling recruitment in the terrestrial orchid *Orchis purpurea*. *New Phytol.* 176, 448–459. doi: 10.1111/j.1469-8137.2007.02179.x

Jacquemyn, H., Brys, R., Waud, M., Busschaert, P., and Lievens, B. (2015b). Mycorrhizal networks and coexistence in species-rich orchid communities. *New Phytol.* 206, 1127–1134. doi: 10.1111/nph.13281

Jacquemyn, H., Duffy, K. J., and Selosse, M.-A. (2017). "Biogeography of orchid mycorrhizas," in *Biogeography of Mycorrhizal Symbiosis: Ecological Studies*. ed. L. Tedersoo (Switzerland, Cham: Springer), 159–177.

Jacquemyn, H., Honnay, O., Cammue, B. P. A., Brys, R., and Lievens, B. (2010b). Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. *Mol. Ecol.* 19, 4086–4095. doi: 10.1111/j.1365-294X.2010.04785.x

Jacquemyn, H., Merckx, V., Brys, R., Tyteca, D., Cammue, B. P. A., Honnay, O., et al. (2011a). Analysis of network architecture reveals phylogenetic constraints on mycorrhizal specificity in the genus *Orchis* (Orchidaceae). *New Phytol.* 192, 518–528. doi: 10.1111/j.1469-8137.2011.03796.x

Jacquemyn, H., Waud, M., Lievens, B., and Brys, R. (2016a). Differences in mycorrhizal communities between *Epipactis palustris*, *E. helleborine* and its presumed sister species *E. neerlandica*. *Ann. Bot.* 118, 105–114. doi: 10.1093/aob/mcw015

Jacquemyn, H., Waud, M., Merckx, V. S. F. T., Brys, R., Tyteca, D., Hedrén, M., et al. (2016b). Habitat-driven variation in mycorrhizal communities in the terrestrial orchid genus *Dactylorhiza*. *Sci. Rep.* 6:37182. doi: 10.1038/srep37182

Jacquemyn, H., Waud, M., Merckx, V. S. F. T., Lievens, B., and Brys, R. (2015a). Mycorrhizal diversity, seed germination and long-term changes in population size across nine populations of the terrestrial orchid *Neottia ovata*. *Mol. Ecol.* 24, 3269–3280. doi: 10.1111/mec.13236

Jia, X., Dini-Andreote, F., and Falcão Salles, J. (2018). Community assembly processes of the microbial rare biosphere. *Trends Microbiol.* 26, 738–747. doi: 10.1016/j.tim.2018.02.011

Jogaiah, S., Abdelrahman, M., Tran, L. P., and Shin-ichi, I. (2013). Characterization of rhizosphere fungi that mediate resistance in tomato against bacterial wilt disease. *J. Exp. Bot.* 64, 3829–3842. doi: 10.1093/jxb/ert212

Jousset, A., Bienhold, C., Chatzinotas, A., Gallien, L., Gobet, A., Kurm, V., et al. (2017). Where less may be more: how the rare biosphere pulls ecosystems strings. *ISME J.* 11, 853–862. doi: 10.1038/ismej.2016.174

Kaur, J., Andrews, L., and Sharma, J. (2019). High specificity of a rare terrestrial orchid toward a rare fungus within the north American tallgrass prairie. *Fungal Biol.* 123, 895–904. doi: 10.1016/j.funbio.2019.09.010

Kaur, J., Phillips, C., and Sharma, J. (2020). Host population size is linked to orchid mycorrhizal fungal communities in roots and soil, which are shaped by microenvironment. *Mycorrhiza* 31, 17–30. doi: 10.1007/s00572-020-00993-5

Kaur, J., Poff, K. E., and Sharma, J. (2018). A rare temperate terrestrial orchid selects similar *Tulasnella* taxa in ex situ and in situ environments. *Plant Ecol.* 219, 45–55. doi: 10.1007/s11258-017-0776-0

Kiers, E. T., Duhamel, M., Beesetty, Y., Mensah, J. A., Franken, O., Verbruggen, E., et al. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* 333, 880–882. doi: 10.1126/science.1208473

Kohler, A., Kuo, A., Nagy, L. G., Morin, E., Barry, K. W., Buscot, F., et al. (2015). Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nat. Genet.* 47, 410–415. doi: 10.1038/ng.3223

Kottke, I., Setaro, S., Haug, I., Herrera, P., Cruz, D., Fries, A., et al. (2013). "Mycorrhiza networks promote biodiversity and stabilize the tropical mountain rain forest ecosystem: perspectives for understanding complex communities," in *Ecosystem Services, Biodiversity and Environmental Change in a Tropical Mountain Ecosystem of South Ecuador: Ecological Studies*. eds. J. Bendix, E. Beck, A. Bräuning, F. Makeschin, R. Mosandl, S. Scheu et al. (Berlin, Heidelberg: Springer), 187–203.

Kurtz, Z. D., Müller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., and Bonneau, R. A. (2015). Sparse and compositionally robust inference of microbial ecological networks. *PLoS Comput. Biol.* 11:e1004226. doi: 10.1371/journal.pcbi.1004226

Lee, S., Kong, H. G., Song, G. C., and Ryu, C. (2020). Disruption of firmicutes and actinobacteria abundance in tomato rhizosphere causes the incidence of bacterial wilt disease. *ISME J.* 15, 330–347. doi: 10.1038/s41396-020-00785-x

Legay, N., Baxendale, C., Grigulis, K., Krainer, U., Kastl, E., Schloter, M., et al. (2014). Contribution of above-and below-ground plant traits to the structure and function of grassland soil microbial communities. *Ann. Bot.* 114, 1011–1021. doi: 10.1093/aob/mcu169

Liang, M. X., Liu, X. B., Parker, I. M., Johnson, D., Zheng, Y., Luo, S., et al. (2019). Soil microbes drive phylogenetic diversity-productivity relationships in a subtropical forest. *Sci. Adv.* 5:eaax5088. doi: 10.1126/sciadv.aax5088

Liang, Y., Xiao, X., Nuccio, E. E., Yuan, M., Zhang, N., Xue, K., et al. (2020). Differentiation strategies of soil rare and abundant microbial taxa in response to changing climatic regimes. *Environ. Microbiol.* 22, 1327–1340. doi: 10.1111/1462-2920.14945

Liebel, H. T., Bidartondo, M. I., and Gebauer, G. (2015). Are carbon and nitrogen exchange between fungi and the orchid *Goodyera repens* affected by irradiance? *Ann. Bot.* 115, 251–261. doi: 10.1093/aob/mcu240

Lin, M., Xiong, H., Xiang, X., Zhou, Z., Liang, L., and Mei, Z. (2020). The effect of plant geographical location and developmental stage on root-associated microbiomes of *Gymnadenia conopsea*. *Front. Microbiol.* 11:1257. doi: 10.3389/fmicb.2020.01257

López-García, Á., Varela-Cervero, S., Vasar, M., Öpik, M., Barea, J. M., and Azcón-Aguilar, C. (2017). Plant traits determine the phylogenetic structure of arbuscular mycorrhizal fungal communities. *Mol. Ecol.* 26, 6948–6959. doi: 10.1111/mec.14403

Losos, J. B. (2008). Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity among species. *Ecol. Lett.* 11, 995–1003. doi: 10.1111/j.1461-0248.2008.01229.x

Ma, X. Y., Kang, J. C., Nontachaiyapoom, S., Wen, T. C., and Hyde, K. D. (2015). Non-mycorrhizal endophytic fungi from orchids. *Curr. Sci.* 109, 72–87.

Ma, B., Wang, Y., Ye, S., Liu, S., Stirling, E., Gilbert, J. A., et al. (2020). Earth microbial co-occurrence network reveals interconnection pattern across microbiomes. *Microbiome* 8:82. doi: 10.1186/s40168-020-00857-2

Martos, F., Munoz, F., Pailler, T., Kottke, I., Gonneau, C., and Selosse, M.-A. (2012). The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. *Mol. Ecol.* 21, 5098–5109. doi: 10.1111/j.1365-294X.2012.05692.x

May, M., Jąkalski, M., Novotná, A., Dietel, J., Ayasse, M., Lallemand, F., et al. (2020). Three-year pot culture of *Epipactis helleborine* reveals autotrophic survival, without mycorrhizal networks, in a mixotrophic species. *Mycorrhiza* 30, 51–61. doi: 10.1007/s00572-020-00932-4

McCormick, M. K., and Jacquemyn, H. (2014). What constrains the distribution of orchid populations? *New Phytol.* 202, 392–400. doi: 10.1111/nph.12639

McCormick, M. K., Taylor, D. L., Juhaszova, K., Burnett, R. K., Whigham, D. F., and O'Neill, J. P. (2012). Limitations on orchid recruitment: not a simple picture. *Mol. Ecol.* 21, 1511–1523. doi: 10.1111/j.1365-294X.2012.05468.x

McCormick, M. K., Taylor, D. L., Whigham, D. F., and Burnett, R. K. (2016). Germination patterns in three terrestrial orchids relate to abundance of mycorrhizal fungi. *J. Ecol.* 104, 744–754. doi: 10.1111/1365-2745.12556

McCormick, M. K., Whigham, D. F., and Canchani-Viruet, A. (2018). Mycorrhizal fungi affect orchid distribution and population dynamics. *New Phytol.* 219, 1207–1215. doi: 10.1111/nph.15223

McCormick, M. K., Whigham, D. F., O'Neill, J. P., Becker, J. J., Werner, S., Rasmussen, H. N., et al. (2009). Abundance and distribution of *Corallorhiza odontorhiza* reflect variations in climate and ectomycorrhizae. *Ecol. Monogr.* 79, 619–635. doi: 10.1890/08-0729.1

McCormick, M. K., Whigham, D. F., Sloan, D., O'Malley, K., and Hodkinson, B. (2006). Orchid–fungus fidelity: a marriage meant to last? *Ecology* 87, 903–911. doi: 10.1890/0012-9658(2006)87[903:OFAMMT]2.0.CO;2

Memmott, J., Waser, N. M., and Price, M. V. (2004). Tolerance of pollination networks to species extinctions. *Proc. Biol. Sci.* 271, 2605–2611. doi: 10.1098/rspb.2004.2909

Meng, Y. Y., Zhang, W. L., Selosse, M.-A., and Gao, J. Y. (2019). Are fungi from adult orchid roots the best symbionts at germination? A case study. *Mycorrhiza* 29, 541–547. doi: 10.1007/s00572-019-00907-0

Montesinos-Navarro, A., Segarra-Moragues, J. G., Valiente-Banuet, A., and Verdú, M. (2012). The network structure of plant-arbuscular mycorrhizal fungi. *New Phytol.* 194, 536–547. doi: 10.1111/j.1469-8137.2011.04045.x

Moore, J. A. M., Anthony, M. A., Pec, G. J., Trocha, L. K., Trzebny, A., Geyer, K. M., et al. (2020). Fungal community structure and function shifts with atmospheric nitrogen deposition. *Glob. Chang. Biol.* 27, 1–16. doi: 10.1111/gcb.15444

Mujica, M. I., Pérez, M. F., Jakalski, M., Martos, F., and Selosse, M.-A. (2020). Soil P reduces mycorrhizal colonization while favors fungal pathogens: observational and experimental evidence in *Bipinnula* (Orchidaceae). *FEMS Microbiol. Ecol.* 96:fiaa178. doi: 10.1093/femsec/fiaa178

Novotná, A., Benítez, Á., Herrera, P., Cruz, D., Filipczyková, E., and Suárez, J. P. (2018). High diversity of root-associated fungi isolated from three epiphytic orchids in southern Ecuador. *Mycoscience* 59, 24–32. doi: 10.1016/j.myc.2017.07.007

Nurfadilah, S., Swarts, N. D., Dixon, K. W., Lambers, H., and Merritt, D. J. (2013). Variation in nutrient-acquisition patterns by mycorrhizal fungi of rare and common orchids explains diversification in a global biodiversity hotspot. *Ann. Bot.* 111, 1233–1241. doi: 10.1093/aob/mct064

Olesen, J. M., Bascompte, J., Dupont, Y. L., Elberling, H., Rasmussen, C., and Jordano, P. (2011). Missing and forbidden links in mutualistic networks. *Proc. Biol. Sci.* 278, 725–732. doi: 10.1098/rspb.2010.1371

Oliveira, S. F., Bocayuva, M. F., Veloso, T. G. R., Bazzolli, D. M. S., Da Silva, C. C., Pereira, O. L., et al. (2014). Endophytic and mycorrhizal fungi associated with roots of endangered native orchids from the Atlantic forest, Brazil. *Mycorrhiza* 24, 55–64. doi: 10.1007/s00572-013-0512-0

Öpik, M., and Moora, M. (2012). Missing nodes and links in mycorrhizal networks. *New Phytol.* 194, 304–306. doi: 10.1111/j.1469-8137.2012.04121.x

Phillips, R. D., Barrett, M. D., Dalziell, E. L., Dixon, K. W., and Swarts, N. D. (2016). Geographical range and host breadth of *Sebacina* orchid mycorrhizal fungi associating with *Caladenia* in South-Western Australia. *Bot. J. Linn. Soc.* 182, 140–151. doi: 10.1111/boj.12453

Põlme, S., Bahram, M., Jacquemyn, H., Kennedy, P., Kohout, P., Moora, M., et al. (2018). Host preference and network properties in biotrophic plant-fungal associations. *New Phytol.* 217, 1230–1239. doi: 10.1111/nph.14895

Põlme, S., Bahram, M., Yamanaka, T., Nara, K., Dai, Y. C., Grebenc, T., et al. (2013). Biogeography of ectomycorrhizal fungi associated with alders (*Alnus* spp.) in relation to biotic and abiotic variables at the global scale. *New Phytol.* 198, 1239–1249. doi: 10.1111/nph.12170

Qin, J., Zhang, W., Ge, Z. W., and Zhang, S. B. (2019). Molecular identifications uncover diverse fungal symbionts of *Pleione* (Orchidaceae). *Fungal Ecol.* 37, 19–29. doi: 10.1016/j.funeco.2018.10.003

Qin, J., Zhang, W., Zhang, S., and Wang, J. (2020). Similar mycorrhizal fungal communities associated with epiphytic and lithophytic orchids of *Coelogyne corymbosa*. *Plant Divers.* 42, 362–369. doi: 10.1016/j.pld.2020.07.005

Raaijmakers, J. (2015). "The minimal rhizosphere microbiome," in *Principles of Plant-Microbe Interactions*. ed. B. Lugtenberg (Cham: Springer International Publishing), 411–417.

Rasmussen, H. N. (1995). *Terrestrial Orchids: From Seed to Mycotrophic Plant*. New York, NY: Cambridge University Press.

Rasmussen, H. N., Dixon, K. W., Jersáková, J., and Těšitelová, T. (2015). Germination and seedling establishment in orchids: a complex of requirements. *Ann. Bot.* 116, 391–402. doi: 10.1093/aob/mcv087

Reiter, N., Phillips, R. D., Swarts, N. D., Wright, M., Holmes, G., Sussmilch, F. C., et al. (2020). Specific mycorrhizal associations involving the same fungal taxa in common and threatened *Caladenia* (Orchidaceae): implications for conservation. *Ann. Bot.* 126, 943–955. doi: 10.1093/aob/mcaa116

Rock-Blake, R., McCormick, M. K., Brooks, H. E. A., Jones, C. S., and Whigham, D. F. (2017). Symbiont abundance can affect host plant population dynamics. *Am. J. Bot.* 104, 72–82. doi: 10.3732/ajb.1600334

Sato, H., Tsujino, R., Kurita, K., Yokoyama, K., and Agata, K. (2012). Modelling the global distribution of fungal species: new insights into microbial cosmopolitanism. *Mol. Ecol.* 21, 5599–5612. doi: 10.1111/mec.12053

Schäfer, P., and Kogel, K. H. (2009). "The sebacinoid fungus Piriformospora indica: an orchid mycorrhiza which may increase host plant reproduction and fitness," in *Plant Relationships*. ed. H. B. Deising (Berlin, Heidelberg: Springer), 99–112.

Schödelbauerová, I., Roberts, D. L., and Kindlmann, P. (2009). Size of protected areas is the main determinant of species diversity in orchids. *Biol. Conserv.* 142, 2329–2334. doi: 10.1016/j.biocon.2009.05.015

Selosse, M.-A. (2014). The latest news from biological interactions in orchids: in love, head to toe. *New Phytol.* 202, 337–340. doi: 10.1111/nph.12769

Setälä, H., and McLean, M. A. (2004). Decomposition rate of organic substrates in relation to the species diversity of soil saprophytic fungi. *Oecologia* 139, 98–107. doi: 10.1007/s00442-003-1478-y

Shah, S., Thapa, B. B., Chand, K., Pradhan, S., Singh, A., Varma, A., et al. (2019). *Piriformospora indica* promotes the growth of the in-vitro-raised *Cymbidium aloifolium* plantlet and their acclimatization. *Plant Signal. Behav.* 14:1596716. doi: 10.1080/15592324.2019.1596716

Shefferson, R. P., Bunch, W., Cowden, C. C., Lee, Y. I., Kartzinel, T. R., Yukawa, T., et al. (2019). Does evolutionary history determine specificity in broad ecological interactions? *J. Ecol.* 107, 1582–1593. doi: 10.1111/1365-2745.13170

Shefferson, R. P., Cowden, C. C., McCormick, M. K., Yukawa, T., Ogura-Tsujita, Y., and Hashimoto, T. (2010). Evolution of host breadth in broad interactions: mycorrhizal specificity in east Asian and north American rattlesnake plantains (*Goodyera* spp.) and their fungal hosts. *Mol. Ecol.* 19, 3008–3017. doi: 10.1111/j.1365-294X.2010.04693.x

Shefferson, R. P., Kull, T., Hutchings, M. J., Selosse, M.-A., Jacquemyn, H., Kellett, K. M., et al. (2018). Drivers of vegetative dormancy across herbaceous perennial plant species. *Ecol. Lett.* 21, 724–733. doi: 10.1111/ele.12940

Shefferson, R. P., Taylor, D. L., Weiß, M., Garnica, S., McCormick, M. K., Adams, S., et al. (2007). The evolutionary history of mycorrhizal specificity among lady's slipper orchids. *Evolution* 61, 1380–1390. doi: 10.1111/j.1558-5646.2007.00112.x

Sisti, L. S., Flores-Borges, D. N. A., Andrade, S. A. L., Koehler, S., Bonatelli, M. L., and Mayer, J. L. S. (2019). The role of non-mycorrhizal fungi in germination of the mycoheterotrophic orchid *Pogoniopsis schenckii* Cogn. *Front. Plant Sci.* 10:1589. doi: 10.3389/fpls.2019.01589

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*. Cambridge, UK: Academic Press.

Stuckey, I. H. (1967). Environmental factors and the growth of native orchids. *Am. J. Bot.* 54, 232–241. doi: 10.1002/j.1537-2197.1967.tb06914.x

Suárez, J. P., and Kottke, I. (2016). Main fungal partners and different levels of specificity of orchid mycorrhizae in the tropical mountain forests of Ecuador. *Lankesteriana* 16, 299–305. doi: 10.15517/lank.v16i2.26014

Swarts, N. D., and Dixon, K. W. (2017). *Conservation Methods for Terrestrial Orchids*. Plantation, FL: J. Ross Publishing.

Tedersoo, L., Bahram, M., and Dickie, I. A. (2014). Does host plant richness explain diversity of ectomycorrhizal fungi? Re-evaluation of Gao et al. (2013) data sets reveals sampling effects. *Mol. Ecol.* 23, 992–995. doi: 10.1111/mec.12660

Tedersoo, L., Bahram, M., and Zobel, M. (2020). How mycorrhizal associations drive plant population and community biology. *Science* 367:eaba1223. doi: 10.1126/science.aba1223

Těšitelová, T., Kotilínek, M., Jersáková, J., Joly, F. X., Košnar, J., Tatarenko, I., et al. (2015). Two widespread green *Neottia* species (Orchidaceae) show mycorrhizal preference for Sebacinales in various habitats and ontogenetic stages. *Mol. Ecol.* 24, 1122–1134. doi: 10.1111/mec.13088

Thixton, H. L., Esselman, E. J., Corey, L. L., and Zettler, L. W. (2020). Further evidence of *Ceratobasidium* D.P. Rogers (Basidiomycota) serving as the ubiquitous fungal associate of *Platanthera leucophaea* (Orchidaceae) in the north American tallgrass prairie. *Bot. Stud.* 61:12. doi: 10.1186/s40529-020-00289-z

Toju, H., Guimarães, P. R., Olesen, J. M., and Thompson, J. N. (2014). Assembly of complex plant-fungus networks. *Nat. Commun.* 5:5273. doi: 10.1038/ncomms6273

Toju, H., Tanabe, A. S., and Ishii, H. S. (2016). Ericaceous plant-fungus network in a harsh alpine-subalpine environment. *Mol. Ecol.* 25, 3242–3257. doi: 10.1111/mec.13680

Toju, H., Yamamoto, S., Sato, H., and Tanabe, A. S. (2013). Sharing of diverse mycorrhizal and root-endophytic fungi among plant species in an oak-dominated cool-temperate forest. *PLoS One* 8:e78248. doi: 10.1371/journal.pone.0078248

Traxmandlová, I., Ackerman, J. D., Tremblay, R. L., Roberts, D. L., Štípková, Z., and Kindlmann, P. (2018). Determinants of orchid species diversity in world islands. *New Phytol.* 217, 12–15. doi: 10.1111/nph.14862

Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T., and Singh, B. K. (2020). Plant-microbiome interactions: from community assembly to plant health. *Nat. Rev. Microbiol.* 18, 607–621. doi: 10.1038/s41579-020-0412-1

Tsiftsis, S., Štípková, Z., and Kindlmann, P. (2019). Role of way of life, latitude, elevation and climate on the richness and distribution of orchid species. *Biodivers. Conserv.* 28, 75–96. doi: 10.1007/s10531-018-1637-4

Tsiftsis, S., Tsiripidis, I., and Papaioannou, A. (2012). Ecology of orchid *Goodyera repens* in its southern distribution limits. *Plant Biosyst.* 146, 857–866. doi: 10.1080/11263504.2011.642416

van der Heijden, M. G. A., Martin, F. M., Selosse, M.-A., and Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytol.* 205, 1406–1423. doi: 10.1111/nph.13288

van der Linde, S., Suz, L. M., Orme, C. D. L., Cox, F., Andreae, H., Asi, E., et al. (2018). Environment and host as large-scale controls of ectomycorrhizal fungi. *Nature* 558, 243–248. doi: 10.1038/s41586-018-0189-9

Vogt-Schilb, H., Těšitelová, T., Kotilínek, M., Sucháček, P., Kohout, P., and Jersáková, J. (2020). Altered rhizoctonia assemblages in grasslands on ex-arable land support germination of mycorrhizal generalist, not specialist orchids. *New Phytol.* 227, 1200–1212. doi: 10.1111/nph.16604

Wang, Y. L., Gao, C., Chen, L., Ji, N. N., Wu, B. W., Li, X. C., et al. (2019b). Host plant phylogeny and geographic distance strongly structure Betulaceae-associated ectomycorrhizal fungal communities in Chinese secondary forest ecosystems. *FEMS Microbiol. Ecol.* 95:fiz037. doi: 10.1093/femsec/fiz037

Wang, Z. H., Jiang, Y., Deane, D. C., He, F. L., Shu, W. S., and Liu, Y. (2019a). Effects of host phylogeny, habitat and spatial proximity on host specificity and diversity of pathogenic and mycorrhizal fungi in a subtropical forest. *New Phytol.* 223, 462–474. doi: 10.1111/nph.15786

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setälä, H., van der Putten, W. H., and Wall, D. H. (2004). Ecological linkages between aboveground and belowground biota. *Science* 304, 1629–1633. doi: 10.1126/science.1094875

Waterman, R. J., and Bidartondo, M. I. (2008). Deception above, deception below: linking pollination and mycorrhizal biology of orchids. *J. Exp. Bot.* 59, 1085–1096. doi: 10.1093/jxb/erm366

Waterman, R. J., Bidartondo, M. I., Stofberg, J., Combs, J. K., Gebauer, G., Savolainen, V., et al. (2011). The effects of above-and belowground mutualisms on orchid speciation and coexistence. *Am. Nat.* 177, E54–E68. doi: 10.1086/657955

Waud, M., Brys, R., Van Landuyt, W., Lievens, B., and Jacquemyn, H. (2017). Mycorrhizal specificity does not limit the distribution of an endangered orchid species. *Mol. Ecol.* 26, 1687–1701. doi: 10.1111/mec.14014

Waud, M., Busschaert, P., Lievens, B., and Jacquemyn, H. (2016a). Specificity and localised distribution of mycorrhizal fungi in the soil may contribute to co-existence of orchid species. *Fungal Ecol.* 20, 155–165. doi: 10.1016/j.funeco.2015.12.008

Waud, M., Wiegand, T., Brys, R., Lievens, B., and Jacquemyn, H. (2016b). Nonrandom seedling establishment corresponds with distance-dependent decline in mycorrhizal abundance in two terrestrial orchids. *New Phytol.* 211, 255–264. doi: 10.1111/nph.13894

Wells, T. C. E., and Cox, R. (1991). "Demographic and biological studies on *Ophrys apifera*: some results from a 10 year study," in *Population Ecology of Terrestrial Orchids*. eds. T. C. E. Wells and J. H. Willems (Netherlands, Hague: SPB Academic Publishing), 47–61.

Wu, L., Dong, W., Si, J., Liu, J., and Zhu, Y. (2020). Endophytic fungi, host genotype, and their interaction influence the growth and production of key chemical components of *Dendrobium catenatum*. *Fungal Biol.* 124, 864–876. doi: 10.1016/j.funbio.2020.07.002

Xing, X. K., Gao, Y., Zhao, Z. Y., Waud, M., Duffy, K. J., Selosse, M.-A., et al. (2020a). Similarity in mycorrhizal communities associating with two widespread terrestrial orchids decays with distance. *J. Biogeogr.* 47, 421–433. doi: 10.1111/jbi.13728

Xing, X. K., Jacquemyn, H., Gai, X. G., Gao, Y., Liu, Q., Zhao, Z. Y., et al. (2019). The impact of life form on the architecture of orchid mycorrhizal networks in tropical forest. *Oikos* 128, 1254–1264. doi: 10.1111/oik.06363

Xing, X., Liu, Q., Gao, Y., Shao, S., Guo, L., Jacquemyn, H., et al. (2020b). The architecture of the network of orchid–fungus interactions in nine co-occurring *Dendrobium* species. *Front. Ecol. Evol.* 8:130. doi: 10.3389/fevo.2020.00130

Xing, X. K., Ma, X. T., Men, J. X., Chen, Y. H., and Guo, S. X. (2017). Phylogenetic constrains on mycorrhizal specificity in eight *Dendrobium* (Orchidaceae) species. *Sci. China Life Sci.* 60, 536–544. doi: 10.1007/s11427-017-9020-1

Xiong, C., He, J., Singh, B. K., Zhu, Y., Wang, J., Li, P., et al. (2020). Rare taxa maintain the stability of crop mycobiomes and ecosystem functions. Environ. Microbiol. doi: 10.1111/1462-2920.15262 [Epub ahead of print]

Yang, T., Tedersoo, L., Soltis, P. S., Soltis, D. E., Gilbert, J. A., Sun, M., et al. (2019). Phylogenetic imprint of woody plants on the soil mycobiome in natural mountain forests of eastern China. *ISME J.* 13, 686–697. doi: 10.1038/s41396-018-0303-x

Yeh, C. M., Chung, K. M., Liang, C. K., and Tsai, W. C. (2019). New insights into the symbiotic relationship between orchids and fungi. *Appl. Sci.* 9:585. doi: 10.3390/app9030585

Yuan, Z. L., Chen, Y. C., and Yang, Y. (2009). Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid (*Dendrobium nobile*): estimation and characterization. *World J. Microbiol. Biotechnol.* 25, 295–303. doi: 10.1007/s11274-008-9893-1

Zhang, Y., Li, Y. Y., Chen, X. M., Guo, S. X., and Lee, Y. I. (2020). Effect of different mycobionts on symbiotic germination and seedling growth of *Dendrobium officinale*, an important medicinal orchid. *Bot. Stud.* 61:2. doi: 10.1186/s40529-019-0278-6

Zhang, S. B., Yang, Y. J., Li, J. W., Qin, J., Zhang, W., Huang, W., et al. (2018). Physiological diversity of orchids. *Plant Divers.* 40, 196–208. doi: 10.1016/j.pld.2018.06.003

Zi, X. M., Sheng, C. L., Goodale, U. M., Shao, S. C., and Gao, J. Y. (2014). In situ seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, *Dendrobium aphyllum* (Orchidaceae). *Mycorrhiza* 24, 487–499. doi: 10.1007/s00572-014-0565-8

Ziegler, M., Eguíluz, V. M., Duarte, C. M., and Voolstra, C. R. (2018). Rare symbionts may contribute to the resilience of coral-algal assemblages. *ISME J.* 12, 161–172. doi: 10.1038/ismej.2017.151

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Temperature Differentially Influences the Capacity of *Trichoderma* Species to Induce Plant Defense Responses in Tomato Against Insect Pests

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Species of the ecological opportunistic, avirulent fungus, *Trichoderma* are widely used in agriculture for their ability to protect crops from the attack of pathogenic fungi and for plant growth promotion activity. Recently, it has been shown that they may also have complementary properties that enhance plant defense barriers against insects. However, the use of these fungi is somewhat undermined by their variable level of biocontrol activity, which is influenced by environmental conditions. Understanding the source of this variability is essential for its profitable and wide use in plant protection. Here, we focus on the impact of temperature on *Trichoderma afroharzianum* T22, *Trichoderma atroviride* P1, and the defense response induced in tomato by insects. The *in vitro* development of these two strains was differentially influenced by temperature, and the observed pattern was consistent with temperature-dependent levels of resistance induced by them in tomato plants against the aphid, *Macrosiphum euphorbiae,* and the noctuid moth, *Spodoptera littoralis*. Tomato plants treated with *T. afroharzianum* T22 exhibited enhanced resistance toward both insect pests at 25°C, while *T. atroviride* P1 proved to be more effective at 20°C. The comparison of plant transcriptomic profiles generated by the two *Trichoderma* species allowed the identification of specific defense genes involved in the observed response, and a selected group was used to assess, by real-time quantitative reverse transcription PCR (qRT-PCR), the differential gene expression in *Trichoderma*-treated tomato plants subjected to the two temperature regimens that significantly affected fungal biological performance. These results will help pave the way toward a rational selection of the most suitable *Trichoderma* isolates for field applications, in order to best face the challenges imposed by local environmental conditions and by extreme climatic shifts due to global warming.

Keywords: induced systemic resistance, defense genes, gene expression analysis, *Macrosiphum euphorbiae*, *Spodoptera littoralis*, biological control

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INTRODUCTION

The need to reduce the use of chemical pesticides in agriculture has promoted the development of bio-based strategies of plant protection, exploiting beneficial organisms, and the ecological services they provide. There are various biological products available on the market used for disease and pest control, as well as plant biostimulants and fertilizer/soil enhancers that are based on plant beneficial microbes, such as bacteria [*Bacillus, Pseudomonas* (Ferreira et al., 1991; Walsh et al., 2001)] or fungi [*Trichoderma,* mycorrhizae*, Beauveria* (Castillo Lopez and Sword, 2015; Rouphael et al., 2015; Russo et al., 2015; Sinno et al., 2020)]. Numerous *Trichoderma* strains are the key components of commercially available microbial biofungicides (Harman et al., 2004; Woo et al., 2014; Alfiky and Weisskopf, 2021; Ferreira and Musumeci, 2021; Poveda, 2021). Among the 377 *Trichoderma* species identified, only 20–30 species are found in soils as ecological opportunists saprophytes, associated with the rhizosphere, avirulent to plants, that may be useful in agriculture (Druzhinina et al., 2011; Cai and Druzhinina, 2021). It cannot be generalized that all *Trichoderma* are extensive root colonizers or that they are endophytes (Howell, 2003; Harman et al., 2004). For example, some isolates exhibit localized penetration of plant tissues, whereas others are rhizosphere competent, and are able to colonize the roots by growing in the epidermis and outer cortex of the tissues (Harman, 2000; Harman et al., 2004; Hermosa et al., 2012; Lace et al., 2015). Many *Trichoderma* are indeed free-living microorganisms in the soil rhizosphere and have limited contact with the plant; however, by releasing microbial compounds recognized by the plant, they are still able to interact in a molecular cross-talk that influences the plant defense response (Lorito et al., 2010). Some of these antagonists of phytopathogens, not only contribute to biocontrol, but also provide well-recognized positive effects to the plant, such as improved vegetative-root growth, development, and yield; they also enhance nutrient availability and uptake by the plant (Vinale et al., 2008). For example, T. *afroharzianum* (ex-T. *harzianum*; Cai and Druzhinina, 2021) strain T22, the active component of a commercial biofungicide product, was also found to improve the plant growth of important horticultural crops, such as lettuce, tomatoes, peppers, ornamentals, and woody crops, and to prevent diseases, both under greenhouse and field conditions (Harman et al., 2004; Lorito et al., 2010; Woo et al., 2014).

Different *Trichoderma* species or strains can have a diverse impact on different crop species, or even genotypes of the same crop, as observed in tomato in which *T. afroharzianum* T22 and *Trichoderma atroviride* P1, diversely affected plant growth and resistance against *Botrytis cinerea,* depending upon the tomato genotype (Tucci et al., 2011). Furthermore, the crop genotype was found to significantly influence the colonization by different *Trichoderma* strains in the rhizosphere of lentils (Bazghaleh et al., 2020). *Trichoderma* may promote plant endogenous defenses against biotic (phytopathogenic fungi) and abiotic stress factors by induced local or systemic resistance (ISR), similar to those activated by plant growth promoting rhizobacteria (PGPR) that result in the priming of the plant to subsequent attacks by pathogens or other parasites (Harman et al., 2004; Lorito et al., 2010; Hermosa et al., 2013; Conrath et al., 2015; Martínez-Medina et al., 2017; Adnan et al., 2019). *Trichoderma* is also capable of stimulating an ISR plant defense response against nematodes, modulated by a cross-talk between salicylic acid (SA) and jasmonic acid (JA) signaling pathways (Martínez-Medina et al., 2017). Furthermore, it was observed that plants exposed to *Trichoderma* root colonization were more resistant to pest insects, such as aphids (Coppola et al., 2019a), thrips (Muvea et al., 2014), caterpillars (Contreras-Cornejo et al., 2018; Coppola et al., 2019b), and nematodes (Poveda et al., 2020). Indirect defense barriers against insects are also induced in plants colonized by *Trichoderma*, whereby the plant releases compounds that attract the parasitoids (Coppola et al., 2017, 2019a) and/or predators (Battaglia et al., 2013) of the pest insects that attack the plants.

One of the major problems associated with the use of beneficial microbes in agriculture is the variability of their effects on target organisms, which can be affected by environmental conditions. Indeed, the selection of appropriate *Trichoderma* for applications in agriculture depends not only on the targeted use, that is, the biological control of pathogens or pests or the use as biostimulants, but also on the ecological adaptability of the strains to diverse environments (i.e., soils of different properties—structure, pH, and organic matter), availability of water and nutrients, climatic conditions and to crops (i.e., species or genotypes) that may influence their efficacy in the field (Hjeljord and Tronsmo, 1998). Many authors reported that temperature has an effect on spore germination, hyphal growth, and colonization of the biocontrol agent that consequently influences the competitive and antagonistic capabilities of the *Trichoderma* strain, due to its differential interaction with the target fungal pathogen (Tronsmo and Dennis, 1978; Mukherjee and Raghu, 1997; Kredics et al., 2003). Furthermore, the disparity in the growth and development of this plant beneficial microbe at diverse temperatures can have a key role in the *Trichoderma*-host plant interaction, the establishment of the molecular cross-talk that underpins the activation of the plant defense response involving induced resistance to pathogen infection or parasite infestation. The study of the variability in direct and indirect biocontrol efficacy that is dependent on temperature is essential to enhance the action and the application of these microbial agents.

To fill this research gap, the objective of this study is to investigate how temperature impacts the biological performance of two different *Trichoderma* strains (*T. afroharzianum* T22 and *T. atroviride* P1) and the consequential effects they have on transcriptional reprogramming in treated tomato plants to activate defense barriers against insects. Our results will help pave the way toward a rational selection of the most suitable strains of *Trichoderma* for field applications, in order to better face the challenges posed by local environmental settings and by conditions resulting from sudden extreme changes due to global warming.

MATERIALS AND METHODS

Fungal Isolates

Trichoderma afroharzianum strain T22, hereafter indicated as T22, was isolated from the commercial biofungicide product, Trianum (kindly provided by Koppert Biological Systems, Rotterdam, the Netherlands). *Trichoderma atroviride* strain P1, hereafter indicated as P1 (ATCC 74058) was obtained from the microbial collection of the Department of Agricultural Sciences, University of Naples at Portici, Italy. It is a strain that was isolated from wood chips and selected as an effective biological control agent against foliar and post-harvest pathogens, such as *B. cinerea*, and for use in cold storage (Tronsmo, 1991).

Both fungi were maintained on potato dextrose agar (PDA; HiMedia) at room temperature (25°C) and sub-cultured regularly. Conidia were collected from the surface of sporulating fungal cultures (5–7 days), in sterile distilled water and adjusted to a concentration of 10^7 sp ml^{-1} by using a hemocytometer (Coppola et al., 2019b).

Plant Material and *Trichoderma* Treatment

A seed-coating treatment was conducted on surface-sterilized seeds of *Solanum lycopersicum* cv "Dwarf San Marzano" following the protocol of Coppola et al. (2019a). Treated seeds were planted in 60-well planting trays containing sterilized soil (Floragard, Universal Potting Soil, Oldenburg, Germany), then 7–10 days after emergence, plant plugs were transplanted to 10 cm diameter vases and grown at different temperature conditions of 20 ± 1°C or 25 ± 1°C, photoperiod 16: 8 h light/dark. Under these two temperatures, experimental plants attained the size for their use in the insect bioassays after 7 weeks at 20°C and 5 weeks at 25°C.

Insect Rearing

Spodoptera littoralis is permanently lab-reared at the Department of Agricultural Sciences and derives from a population collected on flower crops in Agro-Pontino (Latina, Italy). The larvae were reared on an artificial diet (41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methyl-4-hydroxybenzoate, and 29.6 g/l agar), as previously described (Di Lelio et al., 2014), at 25 ± 1°C, 70 ± 5% RH, and photoperiod of 16:8 h light/dark.

The aphid *Macrosiphum euphorbiae* was collected on tomato crops (Battipaglia, Salerno, Italy) and is permanently reared on tomato plants (*S. lycopersicum*, cultivar Dwarf San Marzano) placed in anti-aphid netcages, in a greenhouse at 20 ± 2°C, 70 ± 10% RH, and photoperiod of 16:8 h light/dark.

Fungal Development at Different Temperatures

The two *Trichoderma* (strains T22 and P1) were cultured in 90 mm Petri plates containing PDA, incubated at 25°C, for 3 days, in the dark. A fungal plug (5 mm) was transferred to the center of new PDA plates and both *Trichoderma* inoculated cultures were incubated in the same controlled growth conditions as the plants reared for the insect bioassays, at temperatures of 20 and 25°C. Four plates were inoculated for each fungal strain, for each of the two incubation temperatures ($4 \times 2 = 8$ cultures per strain); and the experiment was repeated two times. The radial growth of the fungal mycelia was measured at 24, 48, and 72 h.

Fungal Colony-Forming Units in Soil

At the end of insect bioassays (30 days at 20°C and 32 days at 25°C), the samples were collected from the soil in the vases of potted tomato plants at the first flowering stage, to quantify the number of fungal colony-forming units (CFUs) and confirm the presence of living *Trichoderma* after the seed treatments. A mixture of 10 g of soil containing plant roots was added to 90 ml Ringer solution (Sigma-Aldrich, Milan, Italy), containing 0.162 g of sodium pyrophosphate, then placed in agitation for 30 min on a "tilting top" mixer (SSL1, Stuart, Staffordshire, United Kingdom), and this soil suspension was used to prepare a serial dilution from 10^{-3} to 10^{-7} with sterile water. A 100 µl aliquot of the soil suspension from each dilution series was transferred to plates containing *Trichoderma* selective media agar (TSM; HiMedia Laboratories LLC, PA, United States) augmented with Igepal, then distributed uniformly on the substrate surface with an L-spreader, and incubated at 25°C. After 5 days, the number of *Trichoderma* colonies was counted to determine the abundance of fungi present in the soil rhizosphere.

Spodoptera littoralis Bioassay

The larval feeding bioassay was performed at 25 ± 1°C and 70 ± 5% RH, and photoperiod of 16:8 h light/dark, in 4-wells plastic rearing trays (RT32W, Frontier Agricultural Sciences, Pitman, NJ, United States). In each well, 3 ml of 1.5% agar-agar (w/v) were dispensed, in order to keep the tomato leaves turgid in a moist environment, and the rearing wells were closed by perforated plastic lids (RTCV4, Frontier Agricultural Sciences, Pitman, NJ, United States). Groups of 25 newly hatched larvae were isolated into a single well (for a total of 400 larvae/treatment) and allowed to feed on sub-apical tomato leaf disks of 6 week-old plants. Then, newly molted third instar larvae were singly transferred into the wells of a new tray, prepared as above, and were offered fresh leaf disks daily, obtained from sub-apical leaves of tomato plants reared at 20°C or at 25°C. For each treatment, 32 larvae and 60 tomato plants were used.

On a daily basis, the following parameters were recorded: survival of the larvae and their weight, the number of days to attain the pupal stage, the weight of the pupae, the rate of adult emergence, and longevity. To assess the fertility of the emerged adults and the viability of their eggs, each female moth was fed soon after emergence with water/honey solution (50%) and allowed to mate with two males for 24 h, then isolated in a box ($40 \times 30 \times 20$) to assess the number of eggs laid daily and their rate of hatching.

Macrosiphum euphorbiae Bioassay

To assess the effects of plant defense barriers induced by *Trichoderma* on sucking insects, a bioassay on aphids was carried out. Briefly, five apterous adult aphids were gently transferred onto a single plant using a paintbrush. After 24 h, the adult aphids were removed and only five nymphs of the newly laid progeny were left on the plant. Aphid survival was recorded daily, until

the survival of the last aphid. The laid nymphs were counted and removed every day until the end of reproductive activity. The bioassay was conducted in a glasshouse under controlled climatic conditions, at the temperature of 20 or 25°C in separate experimental settings, kept at 70 ± 10% RH and photoperiod of 16:8 h light/dark. A total of 11 replicates (a single tomato plant constituted a replicate) were carried out for each experimental treatment (T22, P1, control).

Gene Expression Analysis

Expression levels of defense-related genes were quantified by real-time PCR (RT-PCR). Fully expanded leaves from plants grown at 20 and 25°C were collected at seven and 5 weeks after sowing, respectively, and immediately frozen in liquid nitrogen.

The isolation of total RNA and the synthesis of the first strand of cDNA were performed according to standard procedures, as already described (Corrado et al., 2012). Expression analysis was carried out using two technical replicates for each of the three biological replicates per sample. Relative quantification of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Student's *t*-test was used to compare the relative quantification of transcripts in treated samples compared to the untreated controls, used as calibrator. The housekeeping gene EF-1α was used as an endogenous reference gene for the normalization of the expression levels of the target genes. Primers and their main features are reported in **Supplementary Table 1**.

The differentially expressed genes (DEGs) originating from two public datasets reporting the transcriptomic profiles of tomato plants modulated by *T. atroviride* P1 (Coppola et al., 2019a) or by *T. afroharzianum* T22 treatments (Coppola et al., 2019b), generated from tomato plants grown at 20°C, were compared by Venny (Oliveros, 2007). DEGs were mapped to MapMan bins for data visualization and pathway analysis (version 3.6.0). To this end, the tomato MapMan ontologies (http://www.gomapman.org/export/ current/mapman/sly_SL2.40_ITAG2.3_2015-01-09_mapping.txt.tgz) were retrieved from the GO MapMan web resource and imported in the MapMan tool.

Statistical Analysis

Survival curves of *S. littoralis* and *M. euphorbiae* were compared by using Kaplan–Meier and Log-rank analysis. Unpaired Student's *t*-test was used for pairwise comparisons of the means, and one-way ANOVA test was used when more than two groups were involved. Normality of data was checked with Shapiro–Wilk test and Kolmogorov–Smirnov test, while homoscedasticity was tested with Levene's test and Barlett's test. When significant effects were observed ($P < 0.05$), Bonferroni's *post-hoc* test was used. If one of the one-way ANOVA assumptions was not met, even after the transformation of the data, Kruskal–Wallis (non-parametric ANOVA) test was employed. Fisher's test was performed at $P \leq 0.05$ to compare the mean mycelial growth of *Trichoderma* strains (MiniTab, Windows). Data were analyzed using Prism (GraphPad Software Inc. version 6.0b, San Diego, CA, United States).

TABLE 1 | Effect of temperature (20°C and 25°C) on the mycelial growth (colony diameter in mm) of *Trichoderma* strains T22 and P1 cultured on PDA, measured at 24 h intervals.

		Mycelial growth (mm) over time		
Temperature	***Trichoderma***	**24 h**	**48 h**	**72 h**
20°C	T22	21.25 ± 1.26 c	41.50 ± 1.73 c	64.00 ± 2.58 c
20°C	P1	22.50 ± 1.92 c	47.50 ± 2.06 b	90.00 ± 0.00 a
25°C	T22	42.75 ± 1.71 a	75.00 ± 2.45 a	90.00 ± 0.00 a
25 °C	P1	27.00 ± 2.16 b	49.25 ± 2.99 b	74.00 ± 3.65 b

Values are the means of four replicates ± SD. Different letters in the same column indicate significant differences within each sampling time, according to Fisher's test (P = 0.05).

RESULTS

Fungal Development

Trichoderma strains, T22 and P1 showed differential growth at 20°C and 25°C in the *in vitro* plate cultures (**Table 1**, **Figure 1**). At 20°C, the mycelial growth of P1 was more rapid than that of T22 (**Table 1**), already significantly higher at the second day [one-way ANOVA: $F_{(1, 6)} = 21.55$; $P = 0.004$], and by day three, the difference was even more pronounced [one-way ANOVA: $F_{(1, 6)} = 405.60$; $P < 0.0001$]. At 72 h, the P1 mycelium was dense and completely covered the surface of the Petri dish (90 mm diameter), whereas the T22 culture was thin and required an additional day to cover the entire substrate (**Figure 1** left, **Table 1**). At 25°C, the growth of the two strains was inversed, with T22 demonstrating a significantly faster growth than P1 at all sampling times [one-way ANOVA: 24 h $F_{(1, 6)} = 130.85$; $P < 0.0001$; 48 h $F_{(1, 6)} = 170.97$; $P < 0.0001$; 72 h $F_{(1, 6)} = 76.80$; $P < 0.0001$], and its dense mycelium completely covered the plate by day three, whereas P1 required an additional day to attain the same dimension (**Figure 1** right, **Table 1**). It can be noted that T22 had a faster mycelial growth rate than P1, in particular, when the fungal development was compared at the optimal temperature for each strain [one-way ANOVA: 24 h $F_{(3, 12)} = 122.10$; $P < 0.0001$; 48 h $F_{(3, 12)} = 157.20$; $P < 0.0001$; 72 h $F_{(3, 12)} = 130.93$; $P < 0.0001$) (**Table 1**).

Fungi in Soil

At 20°C, a significantly higher number of fungal colonies was obtained from soils of plants receiving the P1 seed treatment (Student's *t*-test: t = 9.88, $P < 0.001$, dF = 4), whereas at 25°C, a significantly higher number of CFUs was found in the soils from the T22 treated plants (Student's *t*- test: t = 14.632, $P < 0.0001$, dF = 4) (**Figure 2**). The CFU abundance in the soil of the *Trichoderma* strains at the two temperatures of incubation was indicative of the corresponding trend observed for the differential mycelial growth of P1 at 20°C and T22 at 25°C demonstrated in the *in vitro* tests.

Spodoptera littoralis Bioassay

Since the two *Trichoderma* strains tested showed highly different performances at the two experimental temperatures considered, it was assessed whether the resulting different interaction with colonized plants at different temperatures can affect the

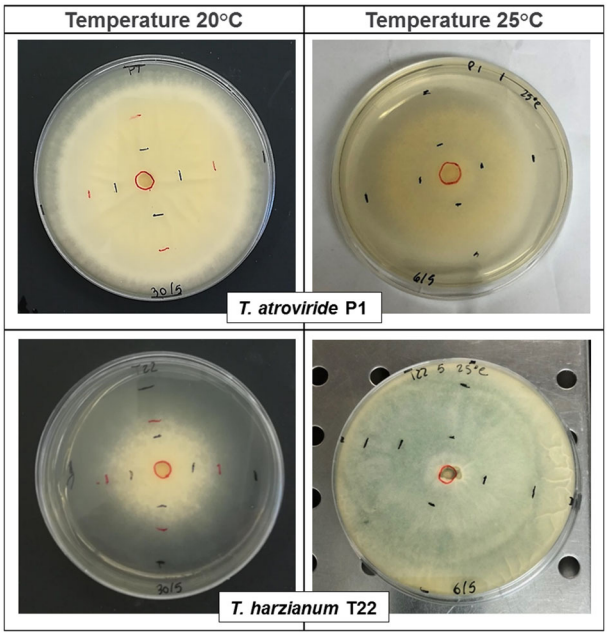

FIGURE 1 | Effect of temperature, at 20°C (left) and 25°C (right), on the mycelial growth of *Trichoderma atroviride* P1 and *T. afroharzianum* T22 after 72 h. Measurements were conducted every 24 h, as indicated by the black/red markings delineating the periphery of the fungal colony. The fungi were cultured on potato dextrose agar (PDA) and maintained at a constant temperature of 20 ± 1°C or 25 ± 1°C and photoperiod of 16: 8 h light/dark.

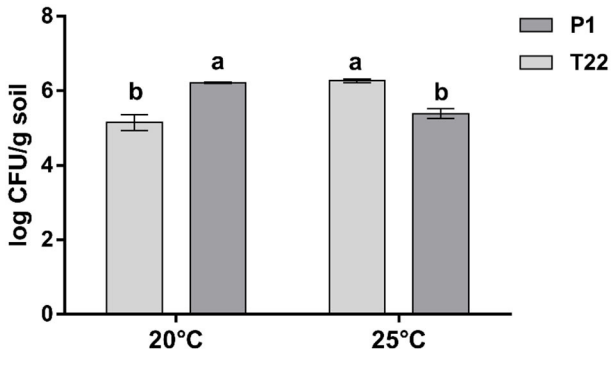

FIGURE 2 | *Trichoderma* colonies in soil. Effect of temperature (20 and 25°C) on the number (means ± SD) of fungal colony-forming units (CFU) in soil samples obtained from the rhizosphere of tomato plants inoculated with *Trichoderma* strain T22 or P1, 5 days after inoculation on *Trichoderma* selective media (TSM). Values are the means of three replicates; bars with different letters indicate significant differences according to Fisher's test at $P \leq 0.05$.

activation of defense barriers against insects. At 20°C, *S. littoralis* showed the lowest fitness when reared on plants inoculated with P1 (**Figure 3A**). T22 and P1 treatments to tomato plants grown at 20°C significantly affected larval survival until pupation (Log-rank test: $\chi^2 = 16.80$; $P = 0.0002$; dF = 2). However, the survival rate of larvae fed on P1-tomato leaves was significantly lower, compared both to controls and T22-tomato plants (Log-rank test: P1 vs. control $\chi^2 = 13.58$; $P = 0.0002$; df = 1; P1 vs. T22 $\chi^2 = 5.676$; $P = 0.0172$; dF = 1), while no difference was registered between the latter two (Log-rank test: $\chi^2 = 3.098$; $P = 0.0784$; dF = 1) (**Figure 3A**).

Conversely, at 25°C, the strain most negatively affecting *S. littoralis* fitness was T22 (**Figure 3B**). Under these experimental conditions, both *Trichoderma* strains negatively affected larval survival (Log-rank test: $\chi^2 = 31.55$; $P < 0.0001$; dF = 2), but, unlike what observed at the lower temperature, T22 had a significantly higher negative impact on larval survival compared both to the control (Log-rank test: $\chi^2 = 23.12$; $P < 0.0001$; dF = 1), and to the P1 treatment (Log-rank test: $\chi^2 = 11.90$; $P = 0.0006$; dF = 1) (**Figure 3B**).

The pattern of reduced survival across larval-pupal development, as affected by temperature, was observed also for the weight of larvae and pupae and for the time required to attain the pupal stage (**Supplementary Figures 1**, **2**, and **Supplementary Table 2**).

Tomato plants colonized by *Trichoderma* had a negative impact on *Spodoptera* adult longevity, which was significantly higher when they were fed as larvae on P1-tomato at 20°C [one-way ANOVA: $F_{(2,65)} = 79.84$; $P < 0.0001$] (**Figure 4A**), while, at 25°C, T22-tomato resulted as the food source most detrimental for adult longevity [one-way ANOVA: $F_{(2, 59)} = 126.1$; $P < 0.0001$] (**Figure 4B**).

Adult fertility was similarly affected, with P1 resulting most detrimental at 20°C [one-way ANOVA: $F_{(2, 24)} = 13.59$; $P < 0.0001$] (**Figure 5A**), while at 25°C, the reverse occurred, with females deriving from larvae fed on T22-tomato leaves producing less eggs [one-way ANOVA: $F_{(2, 26)} = 61.86$; $P < 0.0001$] (**Figure 5B**).

Macrosiphum euphorbiae Bioassay

M. euphorbiae showed a lower survival on tomato plants inoculated with *T. atroviride* P1 and incubated at 20°C, while at 25°C, the reverse was observed, with *T. afroharzianum* T22 showing a higher negative impact on the insect.

At 20°C, aphid survival was affected by feeding on plants receiving the *Trichoderma* treatment (Log-rank test: $\chi^2 = 6.740$; $P = 0.0344$; dF = 2), showing a significant reduction on P1-tomato plants compared to control (Log-rank test: $\chi^2 = 4.898$; $P = 0.0269$; dF =1), or to T22-tomato (Log-rank test: $\chi^2 = 3.666$; $P = 0.0555$; dF = 1), which did not significantly differ between them (Log-rank test: $\chi^2 = 0.1031$; $P = 0.7481$; dF = 1) (**Figure 6**).

At 25°C, aphid survival was affected by *Trichoderma*-treated plants (Log-rank test: $\chi^2 = 15.68$; $P = 0.0004$; dF = 2), but, conversely to what observed at 20°C, survival rates registered on T22-tomato plants were significantly lower than those induced by both P1-plants (Log-rank test: $\chi^2 = 11.92$; $P = 0.0006$; dF = 1) and controls (Log-rank test: $\chi^2 = 11.45$; $P = 0.0007$; dF = 1), which did not differ between them (Log-rank test: $\chi^2 = 0.1273$; $P = 0.72$; dF = 1) (**Figure 7**).

Aphid fertility was not influenced by *Trichoderma* treatments when plants were reared at 20°C [one-way ANOVA: $F_{(2, 23)} = 2.023$; $P < 0.1551$; data not shown], while a significant reduction was induced at 25°C by the T22-tomato treatment [one-way ANOVA: $F_{(2, 29)} = 3.447$; $P < 0.0454$] (**Figure 8**).

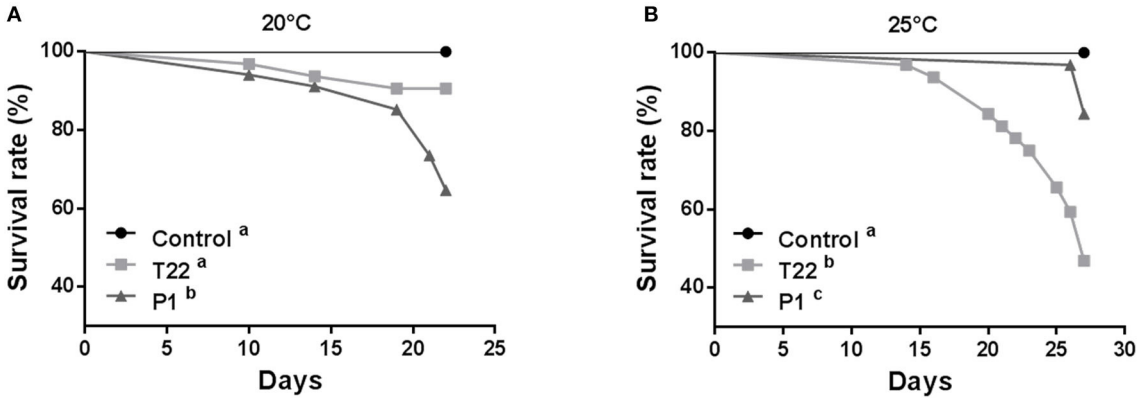

FIGURE 3 | *Spodoptera littoralis* larvae survival when fed leaves from tomato plants inoculated with *Trichoderma strain* T22 or P1 then grown at 20°C **(A)** or 25°C **(B)**. At 20°C, **(A)** survival was significantly lower on T22-tomato with respect to P1- and Control- tomato, whereas, when plants were grown at 25°C, **(B)** larvae fed on T22- or P1-tomato showed higher mortality compared to Control. Different letters denote significant differences in the survival curves (Log-Rank test, $P < 0.0001$).

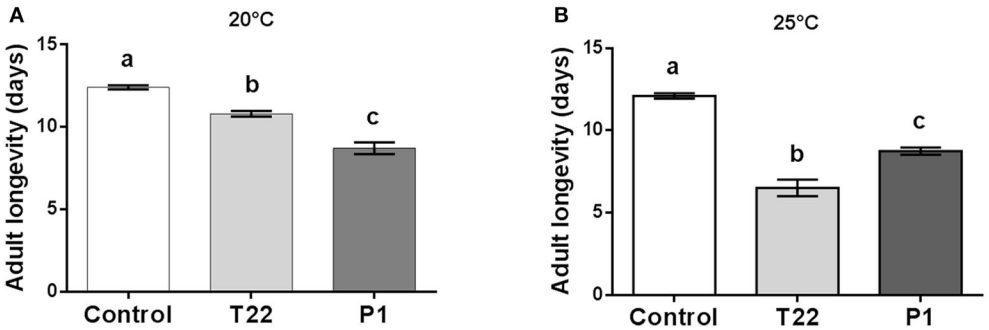

FIGURE 4 | *Spodoptera littoralis* adult longevity. Adults emerging from larvae fed on T22- and P1-tomato leaf disks showed a significantly lower longevity compared to Control adults. At 20°C, **(A)** P1-tomato had the highest negative impact on longevity, whereas at 25°C, the reverse was observed, with T22 showing a significantly higher negative impact on longevity. The values are means ± SD. Mean values denoted with different letters are significantly different (one-way ANOVA **(A)** or Kruskal–Wallis test **(B)** ($P < 0.05$).

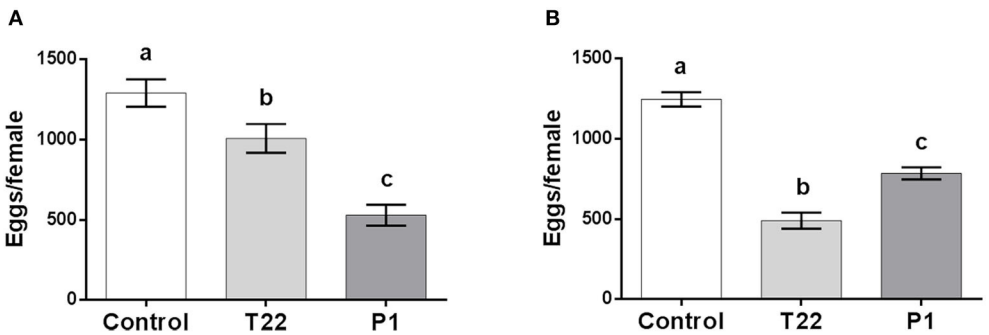

FIGURE 5 | *Spodoptera littoralis* fertility. Adults emerging from larvae fed on T22- and P1-tomato leaf showed lower fertility compared to adults. At 20°C, **(A)** the lower performance was observed on P1-tomato, whereas at 25°C, **(B)** fertility was significantly poorer on T22-tomato. The values are means ± SD. Mean values denoted with different letters are significantly different (one-way ANOVA test, $P < 0.0001$).

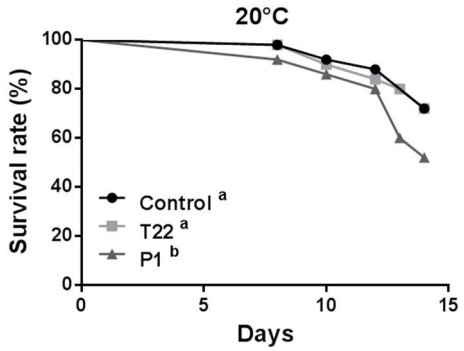

FIGURE 6 | Aphid survival rate at 20°C. *Macrosiphum euphorbiae* reared on P1-tomato showed higher mortality compared to aphids reared on T22- and Control-tomato plants. Different letters indicate a significant difference (Log-rank test, $P < 0.001$).

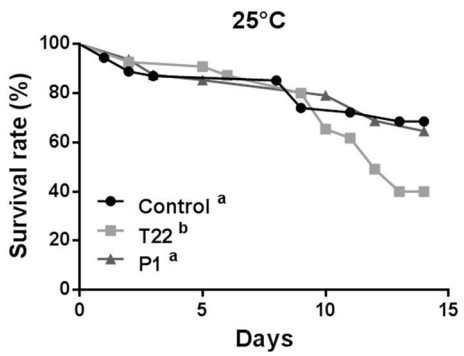

FIGURE 7 | Aphid survival rate at 25°C. *Macrosiphum euphorbiae* reared on T22-tomato showed higher mortality compared to aphids on P1- and Control-tomato plants. Different letters indicate a significant difference (Log-rank test, $P < 0.001$).

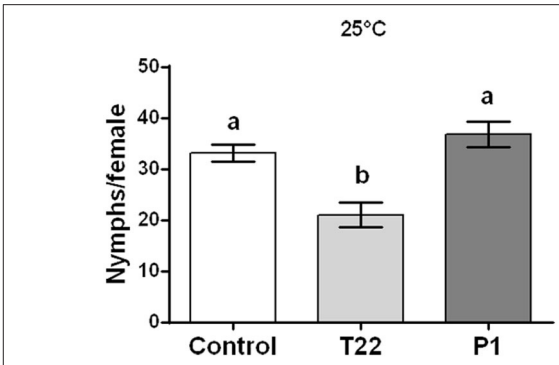

FIGURE 8 | Aphid fertility on tomato plants grown at 25°C. Offspring of *Macrosiphum euphorbiae* reared for their whole lifespan on T22-, P1-, and Control-tomato plants. Data represent mean ± SE. Different letters indicate a significant difference (one-way ANOVA test, $P < 0.05$).

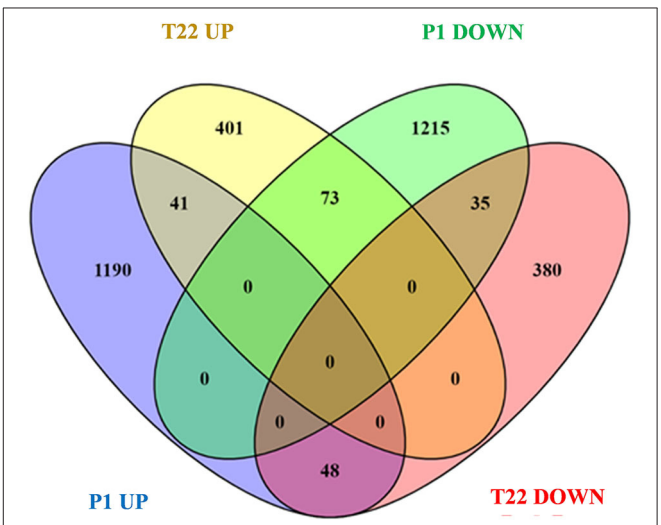

FIGURE 9 | Venn diagram visualization of differentially regulated genes (DEGs) in P1 and T22 treated plants at 20°C. DEGs of tomato plants inoculated with *T. atroviride P1* (P1) and tomato plants inoculated with *T. afroharzianum* T22 (T22).

In silico Comparison of Plant Transcriptomic Profiles Affected by *T. atroviride* P1 and *T. afroharzianum* T22

To assess the impact of the two *Trichoderma* species on the main defense-related pathways, we compared the transcriptomic profiles of tomato plants, as previously analyzed in precedent studies: tomato as affected by treatments with *T. atroviride* P1 (Coppola et al., 2019a) and *T. afroharzianum* T22 grown at 20°C (Coppola et al., 2019b).

A Venn diagram representation of the *in silico* comparison of the two datasets (**Figure 9**) showed a common effect of the two fungi on gene expression, which shared 41 upregulated and 35 downregulated genes (**Supplementary Tables 3A,B**). Common genes induced by the tomato-*Trichoderma* interaction included genes coding for several classes of transcription factors, most of them associated with ethylene signaling (**Table 2**): ethylene-responsive transcription factor, TINY, ethylene-responsive transcription factor, MYB transcription factor 31, and AP2/EREBP transcription factor. In addition, several genes involved in tomato defense were induced by P1 or T22 seed treatments, such as polyphenol oxidase precursor, late embryogenesis abundant protein, heat-shock protein, purple acid phosphatase 17, metallocarboxypeptidase inhibitor (**Supplementary Table 3A**).

TABLE 2 | Common DEGs of datasets coding for transcription factors in tomato treated with *Trichoderma* strains P1 or T22 and grown at 20°C.

Gene ID	P1 FC	T22 FC	Description
Solyc06g066540.1	2.93	1.47	Ethylene-responsive transcription factor TINY
Solyc01g090340.2	2.77	1.24	Ethylene-responsive transcription factor
Solyc03g116100.3	1.63	1.12	MYB transcription factor 31
Solyc08g082210.3	1.53	1.25	AP2/EREBP transcription factor
Solyc08g066660.1	1.23	1.19	Ethylene-responsive transcription factor TINY

TABLE 3 | Common DEG datasets of tomato treated with *Trichoderma* strains P1 or T22 and grown at 20°C, showing opposite regulation sign.

Gene ID	P1 FC	T22 FC	Description
Oxidativeburst/detoxification			
Solyc08g074683.1	2.87	−1.31	Polyphenol oxidase precursor
Solyc12g006760.1	−1.52	3.92	Glutathione S–transferase zeta 1
Solyc03g080100.3	−1.16	1.17	Heavy metal transport/detoxification superfamily protein
JA pathway			
Solyc04g079730.1	2.72	−1.10	Allene oxide synthase
Solyc12g010030.2	4.17	−1.53	Leucine aminopeptidase
Solyc00g187050.3	3.89	−1.49	Leucine aminopeptidase 2
Solyc07g007250.3	4.62	−1.14	Metallocarboxypeptidase inhibitor
Solyc05g047670.1	2.63	−3.75	Protein Ycf2
ET pathway			
Solyc04g077490.3	1.55	−1.04	AP2–like ethylene–responsive transcription factor
Solyc06g053710.3	1.49	−1.19	Ethylene receptor homolog (ETR4)
SA pathway			
Solyc07g005370.3	−1.58	1.45	Pathogenesis-related (PR)- protein 10
Defense-related pathway			
Solyc02g084850.3	4.43	−2.08	Abscisic acid and environmental stress-inducible protein TAS14
Solyc06g074710.1	4.24	−2.23	Hydroxycinnamoyl-CoAshikimate/quinate hydroxycinnamoyltransferase
Solyc04g049750.3	−1.16	2.56	Pentatricopeptide (PPR) repeat protein
Solyc04g011767.1	−1.14	1.14	Pentatricopeptide repeat-containing protein
Solyc10g085900.2	−1.14	1.1	Tetratricopeptide repeat (TPR)-like superfamily protein
Solyc06g068130.3	1.72	−1.87	Tetratricopeptide repeat (TPR)-like superfamily protein

Interestingly, both P1 and T22 treatments downregulated the genes associated with the cell wall, such as expansin-like protein, wall-associated receptor kinase-like 20, pectinesterase, and xyloglucan endotransglucosylase/hydrolase (**Supplementary Table 3B**).

A remarkable opposing effect on tomato transcriptomic profiles determined by the two different species of *Trichoderma* was evident for 73 genes, which were downregulated in P1 and upregulated in T22 samples (**Figure 9**; **Supplementary Table 3C**), and for 48 genes with opposite signs in the available datasets (**Figure 9**; **Supplementary Table 3D**).

Table 3 showed groups of DEGs in common between P1 and T22 samples, showing opposite signs of the gene regulation in the experimental plants. Notably, JA pathway is fundamentally prompted by P1, while in T22, not a single gene associated with this hormonal pathway was retrieved. Similarly, ethylene signaling is stimulated by P1, while it is repressed by T22; SA pathway showed an opposite trend, as can be inferred by the PR-10 transcription level (**Table 3**).

Tomato genes univocally upregulated or downregulated by P1 or T22 are shown in **Supplementary Table 4**. *T. atroviride* P1 induced specifically 1,190 transcripts. The transcript with the highest fold change encodes for threonine deaminase, a late defense gene involved in the resistance against chewing insects (Kang et al., 2006; Gonzales-Vigil et al., 2011). Other transcripts coding for proteins with similar functions are those related to leucine aminopeptidase 2, arginase 2, and several classes of proteinase inhibitors. Interestingly, the transcript coding for the hypersensitive response assisting protein is strongly upregulated (**Supplementary Table 4A**). Several genes involved in ROS scavenging and detoxification are also modulated in their expression (protein detoxification, laccase, NADH dehydrogenase subunit F, superoxide dismutase 1 and 3, and hydrogen peroxide-induced 1). The alteration of early signals in P1 samples is clearly supported by the pathway analysis carried out by MapMan, which identified 79 gene codings for receptor-like kinases, where most of them are upregulated (**Supplementary Table 5**). In addition, among P1-specific genes are included transcripts coding for several classes of defense-related transcription factors, such as WRKY transcription factor 3, 21, 22, bHLH transcription factor, MYB transcription factor 11, 53, 86, GRAS family transcription factor, zinc finger transcription factor 54, and AP2-like ethylene-responsive transcription factor. Genes univocally downregulated by P1 included S-adenosyl-L-methionine-dependent methyltransferases, S-adenosyl-L-methionine, salicylic acid carboxyl methyltransferase 1, pathogenesis-related protein 1, chitinase, β-glucosidase, defensin, subtilisin, thaumatin, and osmotin (**Supplementary Table 4B**).

Genes specifically modulated by T22 include 781, and 401 upregulated genes and 380 downregulated genes (**Supplementary Tables 4C,D**). Functions and ontologies of genes modulated by T22 and P1 are superimposable, despite players are different. T22-specific genes showing high fold-change code for phenylalanine ammonia lyase 4, ethylene-responsive transcription factor, and several heat shock proteins

Class I (**Supplementary Table 4C**). Gene coding for several classes of PR proteins are also induced (chitinase, pathogenesis-related protein 1 and 5), as well as genes involved in SA biosynthesis (phenylalanine ammonia lyase 4 and S-adenosyl-L-methionine-dependent methyltransferases). Ethylene pathway is fundamentally down-represented, since it is observed in the repression of 1-aminocyclopropane-1-carboxylic acid synthase-2, several transcripts coding for ethylene-responsive transcription factor, AP2-like ethylene-responsive transcription factor, and ethylene response factors D2 and 3. A similar trend is observed for the following JA pathway: transcripts coding for lipoxygenase A and D, JA-ZIM domain proteins, and proteinase inhibitors are all downregulated (**Supplementary Table 4D**).

An overview of the impact at the cellular level of the used strains of the two *Trichoderma* species is shown in **Figure 10**. Specific genes of the two interactions are organized in functional categories according to MapMan ontologies (**Figure 10**). *T. atroviride* P1 showed a stronger impact on tomato transcriptome, affecting the expression of a higher number of genes than *T. afroharzianum* T22. Most of the presented functional categories were induced by P1, while repressed by T22, despite the analysis involves specific genes for both interactions (**Figure 10**).

Differential Response of Defense Genes at Different Temperatures During *Trichoderma*-Tomato Interaction

In order to assess the combined effect of the *Trichoderma* strain treatments and the environmental temperatures on the interaction with tomato and the subsequent plant defense response, an expression analysis of a selected group of defense genes was carried out. At 20°C, a greater activation of defense genes was almost exclusively noted in the P1-treated plants (**Table 3**, **Figure 11**). Nine late defense genes were significantly induced in P1-treated plants when compared to the control (Map Kinase 1, germacrene C-synthase, wound-induced proteinase inhibitor I and II, Kunitz type proteinase inhibitor, threonine deaminase, leucine aminopeptidase A, and phenylalanine ammonia lyase). Instead, in the T22 samples only two genes of threonine deaminase and S-adenosyl methionine were moderately induced in comparison to the control (**Figure 11**).

The transcriptional analysis of plants grown at the higher temperature showed an opposite trend for the same gene set, in which the T22 samples were mainly upregulated when compared to the control, even though the regulated genes were slightly different (**Figure 12**); those genes showing transcriptional rates significantly different from the control included Map Kinase 1, Ap2 ethylene responsive factor, hydroperoxide lyase, germacrene C-synthase, subtilisin, and phenylalanine ammonia lyase.

DISCUSSION

The use of microorganisms in agriculture as biocontrol agents represents one of the most promising and widespread tools for the sustainable management of plant diseases (Howell, 2003; Harman et al., 2004; Ab Rahman et al., 2018). Beneficial microbes contribute to the improvement of crop quality and yield, concurring to the establishment of useful plant microbiota, which promotes growth, nutrient availability, and resistance against pathogens (Vinale et al., 2008; Castillo Lopez and Sword, 2015; Poveda et al., 2020; Sinno et al., 2020). Numerous microbial strains are components of biological products promoting plant growth and/or inducing disease resistance, and, along with their secondary metabolites may represent very important bioactive components for the development of effective formulations (Hermosa et al., 2013; Rouphael et al., 2015; Woo and Pepe, 2018). Moreover, another possible effect by beneficial microbes is the induced resistance response in the plant, a priming of plant defenses from their interaction that results in a faster and/or a stronger protective response upon subsequent contact or infection by a pathogen (Conrath, 2011; Hermosa et al., 2012; Conrath et al., 2015). Several strains of fungi belonging to the genus *Trichoderma* are not only effective biocontrol agents of plant pathogens, but also stimulators of this priming effect (Lorito et al., 2010).

Despite the continuous research for new formulations, to find effective combinations of microbial strains with different plant species/cultivars, optimal mixtures and doses of microbes plus bioactive molecules to obtain a broad spectrum of biocontrol results on diverse crops, little attention has been dedicated to the environmental abiotic factors that can influence the interaction with the plant.

Temperature is of pivotal importance in the modulation of biological processes, and its connection to geographic location clearly generates spatial heterogeneity that needs to be taken into account when handling living organisms. This aspect is even more relevant if we consider the severe environmental stress associated with global warming and the extreme conditions to which we are increasingly exposed. Climatic changes strongly influence plant physiology and the agroecosystem; thus crop yield and production have an important impact on the world economy. Empirical data indicate that the productivity of many crops worldwide has already declined markedly, as a result of rising temperatures. In the case of maize, for example, global production has declined by 3.8–12.5% over the past three decades (Lobell et al., 2011; Tigchelaar et al., 2018). Considering that global temperatures are forecasted to increase from 2.6 to 4°C before the end of this century (Change, 2014; Rogelj et al., 2016), this trend will have a remarkable impact on the living organisms and their interactions. The severity of the presented scenario does not include other environmental factors/conditions that are predicted to aggravate the situation, that is, the effect of rapid thermic excursions, precipitation and frost, plus CO_2 release. In this scenario, further studies characterized by an increasing tendency to adopt policy measures (e.g., EU Directive 128/2009) aiming to reduce the use of synthetic pesticides, while promoting integrated pest management (IPM) strategies as a valid alternative, means that plant beneficial microbials are an important tool for a sustainable plant health promotion at a global level. However, their profitable use requires an in-depth understanding of the effects that climatic conditions may have on

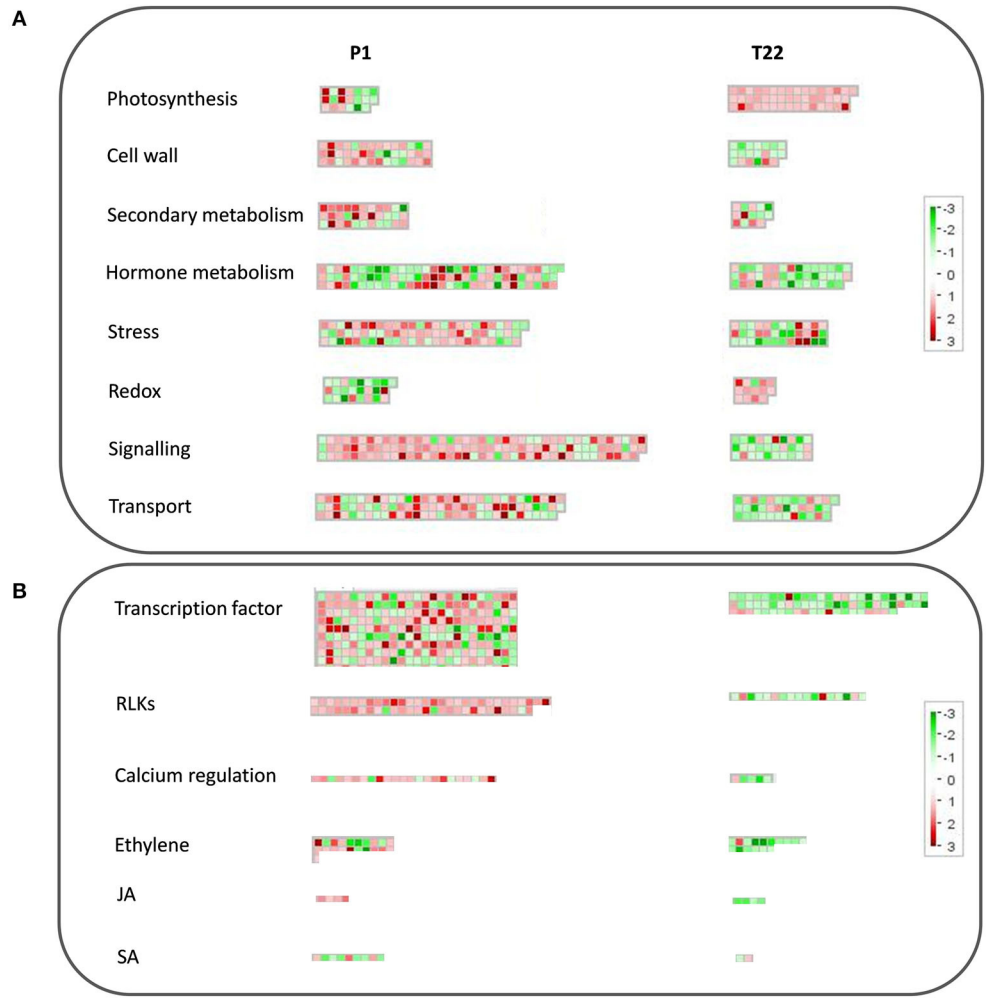

FIGURE 10 | MapMan analysis of tomato transcriptomic profiles imposed by *T. atroviride* P1 or *T. afroharzianum* T22 inocula at 20°C. Organization of tomato by differentially expressed genes (DEGs) in functional categories according to the MapMan ontology across the interaction with spores of P1 or T22. **(A)** Overview of functional categories related to the main cellular processes**; (B)** Overview of the most relevant defense-related regulation processes. Each box represents a transcript; red color indicates upregulation, while green indicates downregulation. Boxes are grouped based on the ontological classification.

their efficiency in controlling pests and disease agents, in order to select the most appropriate strains to a specific environment.

To address this goal, we studied the endogenous defenses of tomato plants as affected by two specific *Trichoderma* species strains. It is expected that *T. atroviride* P1 should have a better performance at lower temperatures, given its original reasons for selection (Tronsmo, 1989) and the profitable use for post-harvest protection in cold storage (Tronsmo, 1991). The beneficial effects of these fungi on tomato plants have been already reported (Harman et al., 2004; Tucci et al., 2011; Nandini et al., 2017; Chen et al., 2019; Coppola et al., 2019a,b), but without special consideration to the effect of temperature, which may have an important impact both on fungal development and on the plant-*Trichoderma* interactions which may be relevant for crop protection.

Different growth was observed for *T. afroharzianum* T22 and *T. atroviride* P1 at 20 and 25°C, both in *in vitro* and *in vivo* test conditions. The lower temperature (20°C) fostered P1 growth and its capacity to colonize tomato plants, whereas T22 germinated and grew more abundantly at the higher temperature (25°C). This is well in line with available experimental evidence supporting that *T. atroviride* P1 is a cold-adapted isolate (Hjeljord et al., 2000), and corroborating the importance of temperature in modulating the fungal development, as already reported for other *T. atroviride* strains (Daryaei, 2014; Daryaei et al., 2016).

The difference in fungal growth observed in the present study, along with data on the induction of insect resistance and the underlying transcriptome reprogramming, well account for the temperature-dependent biocontrol activity observed for the two fungal strains considered. Based on our previous transcriptomic data from experiments with Dwarf San Marzano tomato performed at 20°C and treatments with *T. atroviride* P1 (Coppola et al., 2019a) and *T. afroharzianum* T22 (Coppola et al., 2019b), we were able to choose a representative gene set involved in

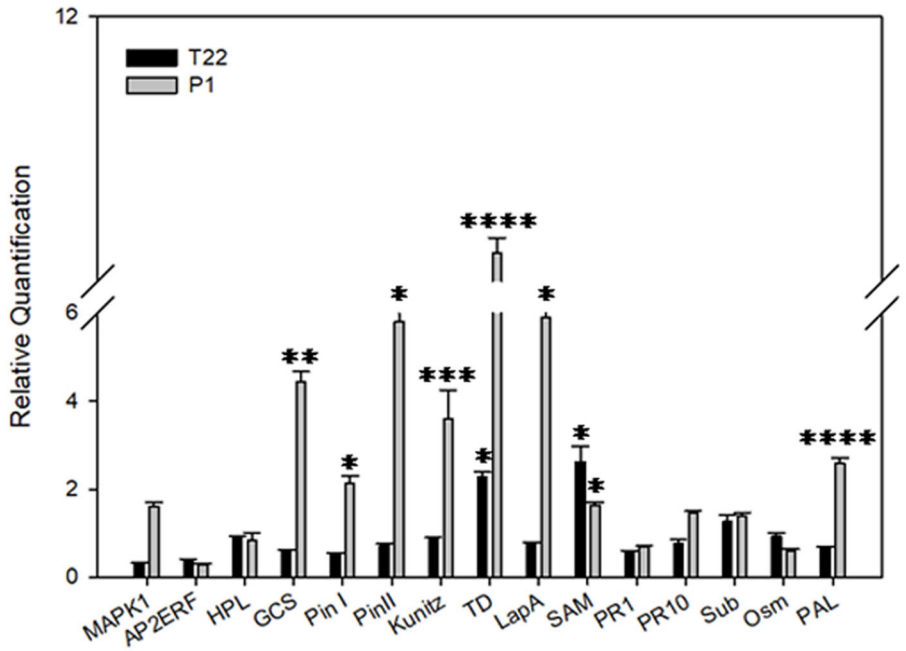

FIGURE 11 | Relative quantification of defense-related genes by real-time PCR (RT-PCR). Relative quantities (RQ) of defense genes in plants treated with *T. afroharzianum* T22 and *T. atroviride* P1 at 20°C. Data are calibrated to the untreated control sample (RQ = 1). Bars represent SE. Asterisks indicate statistically significant differences compared to the control condition (Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$). *MAPK1*, Map Kinase 1; *AP2ERF*, Ap2 Ethylene responsive factor; *HPL*, hydroperoxide lyase; *GCS*, germacrene C-synthase; *Pin I* and *Pin II,* wound-induced proteinase inhibitor I and II; *Kunitz,* Kunitz type proteinase inhibitor; *TD*, Threonine deaminase; *LapA*, leucine aminopeptidase A; *SAM,* S-adenosyl methionine; *PR1-PR10*, pathogenesis-related proteins 1 and 10; *Sub*, Subtilisin; *Osm*, Osmotin; *PAL*, Phenylalanine ammonia lyase.

defense-related pathways and analyze their transcriptional profile by qRT-PCR, by comparing the two plant-fungus associations to determine any temperature-dependent effects.

The present findings were consistent with the expected beneficial effects of *Trichoderma* on the activation of tomato endogenous defense processes: the upregulation of transcripts coding for several families of transcription factors and genes associated with oxidative burst and involved in ethylene signaling are indicative of the instauration of a primed state (Conrath, 2011; Broekgaarden et al., 2015; González-Bosch, 2018). A common gene upregulated in the T22- or P1-treated tomato, encodes for purple acid phosphatases 17 (PAPs17), an enzyme that is involved in the plant regulation of phosphorous uptake but also contributes to other biological functions including peroxidation, ascorbate recycling, mediation of salt tolerance, and regulation of cell wall carbohydrate biosynthesis (Ravichandran et al., 2013). PAPs carry predicted signal peptides and, presumably, are secreted; however, the biological function of these proteins in the extracellular space is unknown (Kaffarnik et al., 2009). The *Arabidopsis* PAP5 is involved in the basal resistance against several plant pathogens (Ravichandran et al., 2013) and its optimal level is crucial for mounting complete basal resistance against pathogens (Ravichandran et al., 2015).

The activation of multiple responses may be derived from the action of several *Trichoderma* elicitors that induce resistance *via* different parallel signaling pathways (Hermosa et al., 2013). The interaction of *Trichoderma* (P1 or T22) with tomato could be responsible for the downregulation of genes related to plant cell wall synthesis: the fungus secretes endoglucanases and other cellulolytic enzymes in order to invade and colonize plant tissues (Klose et al., 2015; Sonoda et al., 2019). Both datasets from tomato treated with either of the *Trichoderma* demonstrated a repression of transcripts encoding for enzymes involved in the process of vegetative cell wall reconstruction that may reflect a strategy shared by these fungi to colonize the plant.

P1-treated plants showed upregulated genes related to JA production, which supports the negative performance on the moth larval growth, development, and adult reproduction. On the contrary, the JA as well as the ethylene pathway was not induced in T22-treated plants, while the SA pathway was induced, as shown by the upregulation of genes involved in both synthesis and signaling of this plant hormone. This was consistent with evidence from bioassays with the aphids, in which their survival and reproduction were significantly impaired when plants interacted with the fungus. Aphid susceptibility to defensive pathways controlled by different plant hormones has been a matter of debate (Avila et al., 2012; Studham and MacIntosh, 2013; Duhlian et al., 2020). Both synergistic and inhibitory aspects of the cross-talk among JA, SA, and ET pathways have been reported (Morkunas and Gabryś, 2011),

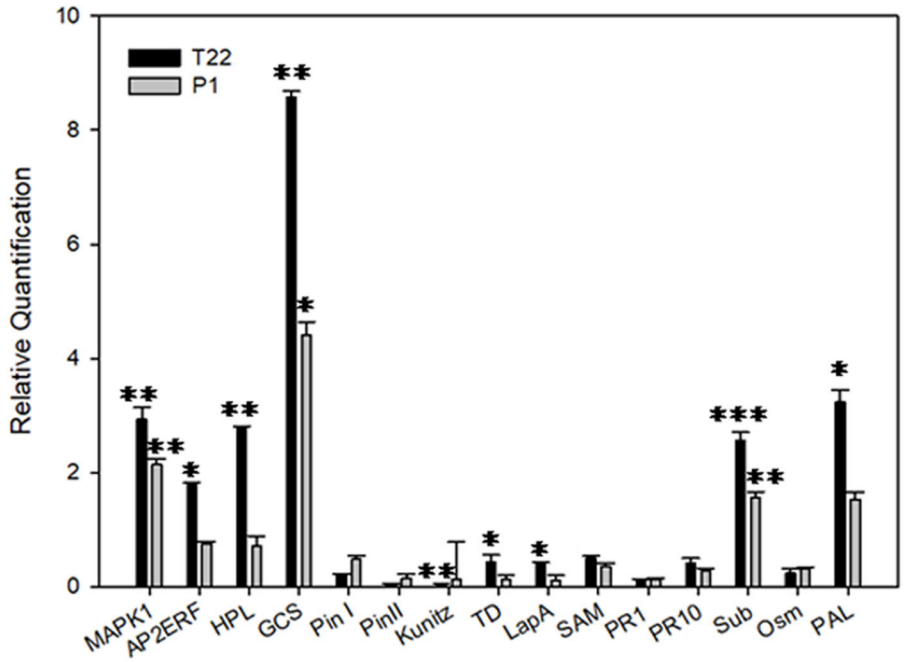

FIGURE 12 | Relative quantification of defense-related genes by real-time PCR (RT-PCR). Relative quantities (RQ) of defense genes in plants treated with *T. afroharzianum* T22 and *T. atroviride* P1 at 25 °C. Data are calibrated to the CTRL sample (RQ = 1). Bars represent SE. Asterisks indicate statistically significant differences compared to the control condition (Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). *MAPK1*, Map Kinase 1; *AP2ERF*, Ap2 ethylene responsive factor; *HPL*, hydroperoxidelyase; *GCS*, germacrene C-synthase; *Pin I* and *Pin II*, wound-induced proteinase inhibitor I and II; *Kunitz*, Kunitz type proteinase inhibitor; *TD*, threonine deaminase; *LapA*, leucine aminopeptidase A; *SAM*, S-adenosyl methionine; *PR1-PR10*, pathogenesis-related proteins 1 and 10; *Sub*, subtilisin; *Osm*, osmotin; *PAL*, phenylalanine ammonia lyase.

a complex scenario in which the role of aphid effectors and suppressors of host-defense responses make the comprehension very difficult (Escudero-Martinez et al., 2020). Thus, the reduction of aphid survival observed in P1-treated plants at the low temperature appears to be associated with the upregulation of a wide group of ethylene-related genes, which is different from that observed in the T22-treated plants. Several studies have examined the role of ethylene in plant–aphid interactions, and aphids have been shown to induce an ethylene burst in several different plant species (Miller et al., 1994; Argandona et al., 2001; Mantelin et al., 2009). Moreover, the role of ET against aphids has been separated by the influence of JA; for instance, Louis et al. (2015) demonstrated a subtle hormonal equilibrium between JA- and ET-enabled maize to respond differentially against chewing and phloem-feeding insects. The maize insect resistance 1 (mir1) gene product, a cysteine (Cys) proteinase that is a maize key defensive protein, is induced by JA-ET and active against chewers. Mir1-mediated resistance to corn leaf aphid (CLA; *Rhopalosiphum maidis*) is independent of JA but regulated by the ET-signaling pathway (Louis et al., 2015). These findings are consistent with the evidence collected on P1 plants ascribable exclusively to ET.

Interestingly, the relative expression of selected plant defense genes clearly mirrors the performance of the insect at different temperatures. In fact, at lower temperatures, P1-tomato plants have a more pronounced negative impact on *Spodoptera* fitness, compared to the T22-tomato plants. These effects are likely induced by the observed higher expression of genes coding for enzymes that interfere with protein digestion/assimilation in insects. Similarly, the negative impact of P1-tomato plants on the longevity of aphids at low temperature can be due to the enhanced transcription rate of *Phenylalanine ammonia lyase* (*PAL*) gene, coding for a key enzyme in the biosynthesis of salicylic acid that is involved in the defense against aphids and pathogens. This conclusion was corroborated by the reverse results observed with the two *Trichoderma* at higher temperature in terms of control efficacy, as supported by a similar gene expression profile, characterized by opposite trends between P1 vs. T22. Indeed, *PAL* is upregulated in T22-tomato plants (in addition to the PR protein, subtilisin), accounting for the higher resistance against aphids, while the lower performance of *S. littoralis* was associated with the disruption of digestion induced by a moderate induction of transcripts coding for several defense molecules controlled by the jasmonate pathway.

Taken into consideration all of our data, the results demonstrated that a better performance of *T. atroviride* P1-tomato interaction occurred at 20°C, potentiating the native defense tools of the cultivar Dwarf San Marzano: active against Lepidopteran and aphids *via* the enhancement of JA-

and/or ET-mediated responses. Conversely, *T. afroharzianum* T22 reached its best performance at 25°C, promoting plant endogenous defenses mainly mediated by SA. The targeted effect of root colonization of *Trichoderma* species could be an interesting consideration in a future investigation.

In conclusion, our results clearly indicate that the outcome of a plant–*Trichoderma* interaction is strongly influenced by temperature; such information is most useful in allowing a rational selection of species/strain, better adapted to different climatic zones and to local environmental conditions, for a more effective and predictable use of these important biocontrol agents.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

Animal subjects involved in the study were insect pests (aphids and caterpillars) that we manage on agricultural crops using biocontrol agents (BCA).

AUTHOR CONTRIBUTIONS

MCD, FP, RR, SLW, and ML contributed to the study design. MC, EC, DM, and ID performed the experiments and analyzed the results. MCD, SLW, and RR supervised the experiments. MC and MCD wrote the first draft of the manuscript. SLW, RR, and FP revised the first draft and wrote new sections. All authors contributed to revise the manuscript, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.678830/full#supplementary-material

REFERENCES

Ab Rahman, S. F. S., Singh, E., Pieterse, C. M., and Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Sci.* 267, 102–111. doi: 10.1016/j.plantsci.2017.11.012

Adnan, M., Islam, W., Shabbir, A., Khan, K. A., Ghramh, H. A., Huang, Z., et al. (2019). Plant defense against fungal pathogens by antagonistic fungi with *Trichoderma* in focus. *Microb. Pathog.* 129, 7–18. doi: 10.1016/j.micpath.2019.01.042

Alfiky, A., and Weisskopf, L. (2021). Deciphering Trichoderma–plant–pathogen interactions for better development of biocontrol applications. *J. Fungi* 7:61. doi: 10.3390/jof7010061

Argandona, V., Chaman, M., Cardemil, L., Munoz, O., Zuniga, G., and Corcuera, L. (2001). Ethylene production and peroxidase activity in aphid-infested barley. *J. Chem. Ecol.* 27, 53–68. doi: 10.1023/A:1005615932694

Avila, C. A., Arévalo-Soliz, L. M., Jia, L., Navarre, D. A., Chen, Z., Howe, G. A., et al. (2012). Loss of function of fatty acid desaturase7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. *Plant Physiol.* 158, 2028–2041. doi: 10.1104/pp.111.191262

Battaglia, D., Bossi, S., Cascone, P., Digilio, M. C., Prieto, J. D., Fanti, P., et al. (2013). Tomato below ground–above ground interactions: *Trichoderma longibrachiatum* affects the performance of *Macrosiphum euphorbiae* and its natural antagonists. *Mol. Plant Microbe Interact.* 26, 1249–1256. doi: 10.1094/MPMI-02-13-0059-R

Bazghaleh, N., Prashar, P., Woo, S., and Vandenberg, A. (2020). Effects of lentil genotype on the colonization of beneficial *Trichoderma* species and biocontrol of *Aphanomyces* root rot. *Microorganisms*. 8:1290. doi: 10.3390/microorganisms8091290

Broekgaarden, C., Caarls, L., Vos, I. A., Pieterse, C. M., and Van Wees, S. C. (2015). Ethylene: traffic controller on hormonal crossroads to defense. *Plant Physiol.* 169, 2371–2379. doi: 10.1104/pp.15.01020

Cai, F., and Druzhinina, I. S. (2021). In honor of John Bissett: authoritative guidelines on molecular identification of *Trichoderma*. *Fungal Divers.* 107, 1–69. doi: 10.1007/s13225-020-00464-4

Castillo Lopez, D., and Sword, G. A. (2015). The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (*Gossypium hirsutum*) and negatively affect survival of the cotton bollworm (*Helicoverpa zea*). *Biol. Control.* 89, 53–60. doi: 10.1016/j.biocontrol.2015.03.010

Change, I.C. (2014). *Synthesis Report.* Pachauri, RK & Meyer LA: Core Writing Team. (IPCC, 2014)

Chen, S.-C., Ren, J.-J., Zhao, H.-J., Wang, X.-L., Wang, T.-H., Jin, S.-D., et al. (2019). *Trichoderma harzianum* improves defense against *Fusarium oxysporum* by regulating ROS and RNS metabolism, redox balance, and energy flow in cucumber roots. *Phytopathology* 109, 972–982. doi: 10.1094/PHYTO-09-18-0342-R

Conrath, U. (2011). Molecular aspects of defense priming. *Trends Plant Sci.* 16, 524–531. doi: 10.1016/j.tplants.2011.06.004

Conrath, U., Beckers, G. J., Langenbach, C. J., and Jaskiewicz, M. R. (2015). Priming for enhanced defense. *Annu. Rev. Phytopathol.* 53, 97–119. doi: 10.1146/annurev-phyto-080614-120132

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Del-Val, E., and Larsen, J. (2018). The root endophytic fungus *Trichoderma atroviride* induces foliar herbivory resistance in maize plants. *Appl. Soil Ecol.* 124, 45–53. doi: 10.1016/j.apsoil.2017.10.004

Coppola, M., Cascone, P., Chiusano, M. L., Colantuono, C., Lorito, M., Pennacchio, F., et al. (2017). *Trichoderma harzianum* enhances tomato indirect defense against aphids. *Insect Sci.* 24, 1025–1033. doi: 10.1111/1744-7917.12475

Coppola, M., Cascone, P., Di Lelio, I., Woo, S. L., Lorito, M., Rao, R., et al. (2019a). *Trichoderma atroviride* P1 colonization of tomato plants enhances both direct and indirect defense barriers against insects. *Front. Physiol.* 10:813. doi: 10.3389/fphys.2019.00813

Coppola, M., Diretto, G., Digilio, M. C., Woo, S. L., Giuliano, G., Molisso, D., et al. (2019b). Transcriptome and metabolome reprogramming in tomato plants by *Trichoderma harzianum* strain T22 primes and enhances defense

responses against aphids. *Front. Physiol.* 10:745. doi: 10.3389/fphys.2019.00745

Corrado, G., Alagna, F., Rocco, M., Renzone, G., Varricchio, P., Coppola, V., et al. (2012). Molecular interactions between the olive and the fruit fly *Bactrocera oleae*. *BMC Plant Biol.* 12:86. doi: 10.1186/1471-2229-12-86

Daryaei, A. (2014). *Conidium* "fitness" in *Trichoderma*. Lincoln University. Ph.D. thesis. Available online at: http://hdl.handle.net/10182/6493

Daryaei, A., Jones, E., Ghazalibiglar, H., Glare, T., and Falloon, R. (2016). Effects of temperature, light and incubation period on production, germination and bioactivity of *Trichoderma atroviride*. *J. Appl. Microbiol.* 120, 999–1009. doi: 10.1111/jam.13076

Di Lelio, I., Varricchio, P., Di Prisco, G., Marinelli, A., Lasco, V., Caccia, S., et al. (2014). Functional analysis of an immune gene of *Spodoptera littoralis* by RNAi. *J. Insect Physiol.* 64, 90–97. doi: 10.1016/j.jinsphys.2014.03.008

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., et al. (2011). *Trichoderma*: the genomics of opportunistic success. *Nat. Rev. Microbiol.* 9, 749–759. doi: 10.1038/nrmicro2637

Duhlian, L., Koramutla, M. K., Subramanian, S., Chamola, R., and Bhattacharya, R. (2020). Comparative transcriptomics revealed differential regulation of defense related genes in *Brassica juncea* leading to successful and unsuccessful infestation by aphid species. *Sci. Rep.* 10, 1–14. doi: 10.1038/s41598-020-66217-0

Escudero-Martinez, C., Rodriguez, P. A., Liu, S., Santos, P. A., Stephens, J., and Bos, J. I. (2020). An aphid effector promotes barley susceptibility through suppression of defense gene expression. *J. Exp. Bot.* 71, 2796–2807. doi: 10.1093/jxb/eraa043

Ferreira, F. V., and Musumeci, M. A. (2021). *Trichoderma* as biological control agent: scope and prospects to improve efficacy. *World J. Microbiol. Biotechnol.* 37:90. doi: 10.1007/s11274-021-03058-7

Ferreira, J. H. S., Matthee, F. N., and Thomas, A. C. (1991). Biological control of *Eutypa lata* on grapevine by an antagonistic strain of *Bacillus subtilis*. *Phytopathology* 81, 283–287. doi: 10.1094/Phyto-81-283

Gonzales-Vigil, E., Bianchetti, C. M., Phillips, G. N., and Howe, G. A. (2011). Adaptive evolution of threonine deaminase in plant defense against insect herbivores. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5897–5902. doi: 10.1073/pnas.1016157108

González-Bosch, C. (2018). Priming plant resistance by activation of redox-sensitive genes. *Free Radic. Biol. Med.* 122, 171–180. doi: 10.1016/j.freeradbiomed.2017.12.028

Harman, G. E. (2000). Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T22. *Plant Dis.* 84, 377–393. doi: 10.1094/PDIS.2000.84.4.377

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). *Trichoderma* species—opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Hermosa, R., Rubio, M. B., Cardoza, R. E., Nicolás, C., Monte, E., and Gutiérrez, S. (2013). The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *Int. Microbiol.* 16, 69–80. doi: 10.2436/20.1501.01.181

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 158), 17–25. doi: 10.1099/mic.0.052274-0

Hjeljord, L., and Tronsmo, A. (1998). "*Trichoderma* and *Gliocladium*. biological control: an overview," in *Trichoderma & Gliocladium: Enzymes, Biological Control and Commercial Applications, Vol. 2*, eds G. E. Harman, C. P. Kubicek (Bristol, PA: Taylor & Francis Inc.), 131–151.

Hjeljord, L. G., Stensvand, A., and Tronsmo, A. (2000). Effect of temperature and nutrient stress on the capacity of commercial *Trichoderma* products to control *Botrytis cinerea* and *Mucor piriformis* in greenhouse strawberries. *Biol. Control.* 19, 149–160. doi: 10.1006/bcon.2000.0859

Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* 87, 4–10. doi: 10.1094/PDIS.2003.87.1.4

Kaffarnik, F.A., Jones, A.M., Rathjen, J.P., and Peck, S.C. (2009). Effector proteins of the bacterial pathogen *Pseudomonas syringae* alter the extracellular proteome of the host plant, *Arabidopsis thaliana*. *Mol. Cell Proteomics.* 8, 145–156. doi: 10.1074/mcp.M800043-MCP200

Kang, J.-H., Wang, L., Giri, A., and Baldwin, I. T. (2006). Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid–isoleucine–mediated defenses against *Manduca sexta*. *Plant Cell.* 18, 3303–3320. doi: 10.1105/tpc.106.041103

Klose, H., Günl, M., Usadel, B., Fischer, R., and Commandeur, U. (2015). Cell wall modification in tobacco by differential targeting of recombinant endoglucanase from *Trichoderma reesei*. *BMC Plant Biol.* 15, 1–11. doi: 10.1186/s12870-015-0443-3

Kredics, L., Antal, Z., Manczinger, L., Szekeres, A., Kevei, F., and Nagy, E. (2003). Influence of environmental parameters on *Trichoderma* strains with biocontrol potential. *Food Technol. Biotechnol.* 41, 37–42.

Lace, B., Genre, A., Woo, S., Faccio, A., Lorito, M., and Bonfante, P. (2015). Gate crashing arbuscular mycorrhizas: *in vivo* imaging shows the extensive colonization of both symbionts by *Trichoderma atroviride*. *Environ Microbiol Rep.* 7, 64–77. doi: 10.1111/1758-2229.12221

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCT method. *Methods*. 25, 402–408. doi: 10.1006/meth.2001.1262

Lobell, D. B., Schlenker, W., and Costa-Roberts, J. (2011). Climate trends and global crop production since 1980. *Science* 333, 616–620. doi: 10.1126/science.1204531

Lorito, M., Woo, S. L., Harman, G. E., and Monte, E. (2010). Translational research on *Trichoderma*: from'omics to the field. *Annu. Rev. Phytopathol.* 48, 395–417. doi: 10.1146/annurev-phyto-073009-114314

Louis, J., Basu, S., Varsani, S., Castano-Duque, L., Jiang, V., Williams, W. P., et al. (2015). Ethylene contributes to maize insect resistance1-mediated maize defense against the phloem sap-sucking corn leaf aphid. *Plant Physiol.* 169, 313–324. doi: 10.1104/pp.15.00958

Mantelin, S., Bhattarai, K. K., and Kaloshian, I. (2009). Ethylene contributes to potato aphid susceptibility in a compatible tomato host. *New Phytol.* 183, 444–456. doi: 10.1111/j.1469-8137.2009.02870.x

Martínez-Medina, A., Fernandez, I., Lok, G. B., Pozo, M. J., Pieterse, C. M., and Van Wees, S. C. (2017). Shifting from priming of salicylic acid-to jasmonic acid-regulated defenses by *Trichoderma* protects tomato against the root knot nematode *Meloidogyne incognita*. *New Phytol.* 213, 1363–1377. doi: 10.1111/nph.14251

Miller, H., Neese, P., Ketring, D., and Dillwith, J. (1994). Involvement of ethylene in aphid infestation of barley. *J. Plant Growth Regul.* 13:167. doi: 10.1007/BF00226033

Morkunas, I., and Gabryś, B. (2011). Phytohormonal signaling in plant responses to aphid feeding. *Acta Physiol. Plant.* 33, 2057–2073. doi: 10.1007/s11738-011-0751-7

Mukherjee, P. K., and Raghu, K. (1997). Effect of temperature on antagonistic and biocontrol potential of shape *Trichoderma* sp. on *Sclerotium rolfsii*. *Mycopathologia* 139, 151–155. doi: 10.1023/A:1006868009184

Muvea, A. M., Meyhöfer, R., Subramanian, S., Poehling, H.-M., Ekesi, S., and Maniania, N. K. (2014). Colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci*. *PLoS ONE.* 9:e108242. doi: 10.1371/journal.pone.0108242

Nandini, B., Hariprasad, P., Shankara, H. N., Prakash, H. S., and Geetha, N. (2017). Total crude protein extract of *Trichoderma spp.* induces systemic resistance in pearl millet against the downy mildew pathogen. *Biotech.* 7:183. doi: 10.1007/s13205-017-0816-4

Oliveros, J. C. (2007). VENNY. An interactive tool for comparing lists with Venn Diagrams. Available online at: http://bioinfogp.cnb.csic.es/tools/venny/index.html

Poveda, J. (2021). *Trichoderma* as biocontrol agent against pests: new uses for a mycoparasite. *Biol. Control.* 159:104634. doi: 10.1016/j.biocontrol.2021.104634

Poveda, J., Abril-Urias, P., and Escobar, C. (2020). Biological control of plant-parasitic nematodes by filamentous fungi inducers of resistance: *Trichoderma*, mycorrhizal and endophytic fungi. *Front. Microb.* 11: 992. doi: 10.3389/fmicb.2020.00992

Ravichandran, S., Stone, S., Benkel, B., Zhang, J., Berrue, F., and Prithiviraj, B. (2015). Optimal level of purple acid phosphatase5 is required for maintaining complete resistance to *Pseudomonas syringae*. *Front. Plant Sci.* 6:568. doi: 10.3389/fpls.2015.00568

Ravichandran, S., Stone, S. L., Benkel, B., and Prithiviraj, B. (2013). Purple Acid Phosphatase5 is required for maintaining basal resistance against *Pseudomonas syringae* in Arabidopsis. *BMC Plant Biol.* 13, 1–12. doi: 10.1186/1471-2229-13-107

Rogelj, J., Den Elzen, M., Höhne, N., Fransen, T., Fekete, H., Winkler, H., et al. (2016). Paris Agreement climate proposals need a boost to keep warming well below 2 C. *Nature* 534, 631–639. doi: 10.1038/nature18307

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Horticultu.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Russo, M. L., Pelizza, S. A., Cabello, M. N., Stenglein, S. A., and Scorsetti, A. C. (2015). Endophytic colonisation of tobacco, corn, wheat and soybeans by the fungal entomopathogen *Beauveria bassiana* (Ascomycota, Hypocreales). *Biocontrol Sci. Technol.* 25, 475–480. doi: 10.1080/09583157.2014.982511

Sinno, M., Ranesi, M., and Gioia, L., d'Errico, G., and Woo, S. L. (2020). Endophytic fungi of tomato and their potential applications for crop improvement. *Agriculture* 10:587. doi: 10.3390/agriculture10120587

Sonoda, M. T., Godoy, A. S., Pellegrini, V. O., Kadowaki, M. A., Nascimento, A. S., and Polikarpov, I. (2019). Structure and dynamics of *Trichoderma harzianum* Cel7B suggest molecular architecture adaptations required for a wide spectrum of activities on plant cell wall polysaccharides. *Biochim. Biophys. Acta Gen. Subj.* 1863, 1015–1026. doi: 10.1016/j.bbagen.2019.03.013

Studham, M. E., and MacIntosh, G. C. (2013). Multiple phytohormone signals control the transcriptional response to soybean aphid infestation in susceptible and resistant soybean plants. *Mol. Plant Microbe Interact.* 26, 116–129. doi: 10.1094/MPMI-05-12-0124-FI

Tigchelaar, M., Battisti, D. S., Naylor, R., and Ray, D. K. (2018). Future warming increases probability of globally synchronized maize production shocks. *Proc. Natl. Acad. Sci. U.S.A.* 115, 6644–6649. doi: 10.1073/pnas.1718031115

Tronsmo, A. (1989). *Trichoderma harzianum* used for biological control of storage rot on carrots. *Norwegian J. Agr. Sci.* 3, 157–161.

Tronsmo, A. (1991). Biological and integrated controls of *Botrytis cinerea* on apple with *Trichoderma harzianum*. *Biol. Control.* 1, 59–62. doi: 10.1016/1049-9644(91)90102-6

Tronsmo, A., and Dennis, C. (1978). Effect of temperature on antagonistic properties of *Trichoderma* species. *Trans. Brit. Mycol. Soc.* 71, 469–474. doi: 10.1016/S0007-1536(78)80075-8

Tucci, M., Ruocco, M., De Masi, L., De Palma, M., and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12, 341–354. doi: 10.1111/j.1364-3703.2010.00674.x

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., and Lorito, M. (2008). *Trichoderma*–plant–pathogen interactions. *Soil Biol. Biochem.* 40, 1–10. doi: 10.1016/j.soilbio.2007.07.002

Walsh, U. F., Morrissey, J. P., and O'Gara, F. (2001). *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr. Opin. Biotechn.* 12, 289–295. doi: 10.1016/S0958-1669(00)00212-3

Woo, S. L., and Pepe, O. (2018). Microbial consortia: promising probiotics as plant biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1801. doi: 10.3389/fpls.2018.01801

Woo, S. L., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., et al. (2014). *Trichoderma*-based Products and their Widespread Use in Agriculture. *Open Mycol. J.* 8, 71–126. doi: 10.2174/1874437001408010071

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