Received: 5 September 2019 | Revised: 26 December 2019 | Accepted: 21 January 2020

DOI: 10.1111/mpp.12918

ORIGINAL ARTICLE

Molecular Plant Pathology WILEY

Analogous wheat root rhizosphere microbial successions in field and greenhouse trials in the presence of biocontrol agents *Paenibacillus peoriae* SP9 and *Streptomyces fulvissimus* FU14

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Funding information
R.A. was supported by an Endeavour Postdoctoral Fellowship. This study was financed by Grains Research and Development Corporation (GRDC) project no. UF00008.

Abstract

Two *Pythium*-infested soils were used to compare the wheat root and rhizosphere soil microbial communities from plants grown in the field or in greenhouse trials and their stability in the presence of biocontrol agents. Bacteria showed the highest diversity at early stages of wheat growth in both field and greenhouse trials, while fungal diversity increased later on, at 12 weeks of the crop cycle. The microbial communities were stable in roots and rhizosphere samples across both soil types used in this study. Such stability was also observed irrespective of the cultivation system (field or greenhouse) or addition of biocontrol coatings to wheat seeds to control *Pythium* disease (in this study soil infected with *Pythium* sp. clade F was tested). In greenhouse plant roots, *Archaeorhizomyces*, *Debaryomyces*, *Delftia*, and unclassified Pseudeurotiaceae were significantly reduced when compared to plant roots obtained from the field trials. Some operational taxonomic units (OTUs) represented genetic determinants clearly transmitted vertically by seed endophytes (specific OTUs were found in plant roots) and the plant microbiota was enriched over time by OTUs from the rhizosphere soil. This study provided key information regarding the microbial communities associated with wheat roots and rhizosphere soils at different stages of plant growth and the role that *Paenibacillus* and *Streptomyces* strains play as biocontrol agents in supporting plant growth in infested soils.

KEYWORDS

biocontrol agent, cereals microbiota, endophyte, field trial, greenhouse, next-generation sequencing, plant microbiota, *Pythium*

1 | INTRODUCTION

Wheat is a major cereal crop worldwide representing the main food source in many regions. Intensive cereal cropping has recently shown slight increases in productivity through the use of innovative strategies, mainly molecular genetics, by selecting and improving varieties and increased nutrient enrichment in soils (Charmet, 2011). However, in many cases productivity has been reduced by the spread of soil diseases, soil degradation, and adverse environmental conditions (Lobell, 2009; Liu *et al.*, 2011; Mavrodi *et al.*, 2014; Barnett *et al.*, 2017). Wheat productivity needs to be rapidly increased in order to cater to the demand of

the burgeoning human populations expected during the coming decades (Fisher and Edmeades, 2010).

Microbial communities associated with various plant habitats collectively constitute the plant microbiome (Turner *et al.*, 2013; Reinhold-Hurek *et al.*, 2015; Schlaeppi and Bulgarelli, 2015; van der Heijden and Hartmann, 2016; Chang *et al.*, 2017). This microbiome can support and aid plant development by providing additional nitrogen and other nutrients, competing with plant pathogens, promoting growth, reducing stress effects, and improving plant resistance through the production of hormones and other chemicals (Mendes *et al.*, 2011; 2013; 2015; Ofek-Lalzar *et al.*, 2014; Massart *et al.*, 2015; Schlaeppi and Bulgarelli, 2015). Today, the importance of the microbiome is no longer questioned but the role of certain microbes and its function in the environment remain to be clarified. Monitoring and manipulating the plant microbiome can interfere with food supply, as well as biodiversity, safety, and ecosystem functionality (Massart *et al.*, 2015; Sessitsch and Mitter, 2015; Chang *et al.*, 2017). It is critical, therefore, to understand both the potential and the risks for human health and nutrition.

Soil represents one of the richest microbial ecosystems and a major source for the diversity and stability of the plant microbiome (Mendes *et al.*, 2015; Panke-Buisse *et al.*, 2015; Chang *et al.*, 2017). The rhizosphere represents a fraction of soil diversity with microorganisms under the influence of the plant. It is currently known that the plant microbiome regulation changes over time from the seed stage to the flowering stage; nevertheless the process is expected to be highly dynamic and not predictable (Turner *et al.*, 2013; van der Heijden and Hartmann, 2016). With regard to wheat, some studies have characterized the rhizosphere and root communities at a single time point. Actinobacteria, Bacteroidetes, and Proteobacteria largely dominate (nearly 90%) wheat root bacterial communities (Liu *et al.*, 2011; Rascovan *et al.*, 2016; Granzow *et al.*, 2017) and the roots of other economically relevant plants such as maize, soybean, or cucumber (Peiffer *et al.*, 2013; Ofek-Lalzar *et al.*, 2014; Donn *et al.*, 2015; Edwards *et al.*, 2015; Rascovan *et al.*, 2016).

The relationship between the endogenous rhizosphere microbiota and the plant host is essential to improve strategies for more productive and less degraded soils. From the perspective of microbiome management, it is important to understand which microbes are sensitive to cropping practices and whether they can be associated with other microbes to impact on plant heath, soil competition, and promote specific networking properties (Hirsch and Mauchline, 2012; Mendes *et al.*, 2013; Massart *et al.*, 2015; Schlaeppi and Bulgarelli, 2015; van der Heijden and Hartmann, 2016). Some bacterial endophytes have been shown to impact plant growth and provide protection to the plant (Araujo *et al.*, 2017; 2019). Although the effects on plants tend to be small initially (Yang *et al.*, 2012; Bokati *et al.*, 2016; Araujo *et al.*, 2017; Wemheuer *et al.*, 2017), these valuable organisms can be used as a basis for developing safer ecological approaches to disease management, particularly against root rots that affect a range of cereals (Orakçı *et al.*, 2010; Araujo *et al.*, 2019). Some endophytes were shown to be able to reduce *Pythium* disease on wheat and other cereals, and their potential as biocontrol inoculants has been well documented (Mavrodi *et al.*, 2014; Araujo *et al.*, 2017). Nevertheless, the results of field trials and greenhouse tests often disagree, which can only be partly explained by climatic conditions (Franco *et al.*, 2007; Cuppels *et al.*, 2013; Araujo *et al.*, 2017; Shi *et al.*, 2018). It is possible that different cultivation systems, such as field versus greenhouse, may affect the soil biodiversity and promote taxa changes, even when the same starting soil is used.

In the present study, we detail the endophytic microbiota dynamics (both bacteria and fungi) in roots and rhizosphere soils of a wheat crop system running simultaneously in field and greenhouse cultivation systems. High-throughput amplicon sequencing is an excellent tool to study and characterize microbial communities when comparing rhizosphere soil and intraplant environments. The objectives of the study were focused on the following points: (a) to identify similarities and differences between the microbiota of wheat plants grown in the field and in greenhouse trials; (b) to monitor the root microbiota in different soil types infested with *Pythium* disease; (c) to confirm the impact of biocontrol agents at early and later stages (up to 12 weeks) of plant growth on the microbial population of seeds, roots, and rhizosphere soils; and (d) to monitor *Paenibacillus* and *Streptomyces* interactions within the microbiota organization of wheat roots and rhizosphere soil samples. A schematic diagram of the experimental design can be seen in Figure 1.

2 | RESULTS

2.1 | Wheat plant growth and core bacterial and fungal microbiota

Wheat plants obtained from biocontrol agent-coated seeds showed earlier formation of wheat heads in greenhouse trials and occasional increase in plant shoot length (more evident in greenhouse *Streptomyces fulvissimus* FU14-treated plants) (Figure 2). Plant parameter results obtained from field and greenhouse trials were only slightly different, with the largest difference observed for the root/upper plant length and root accumulation in the soils; field plant roots/upper plant were shorter (Figure 2) and roots predominantly accumulated near the soil surface (where rain water accumulates in the soil), while greenhouse plant roots/tops were longer and roots accumulated in the bottom of the pots (where the water accumulates after being added manually). No significant differences were found between control and *Paenibacillus peoriae* SP9-treated plants (Figure 2).

A total of 7,469 bacterial and 715 fungal unique operational taxonomic units (OTUs) were found in 254 analysed samples, while the analysis of the amplicon sequence variants (ASVs) revealed 20,061 bacterial and 891 fungal ASVs. As the results regarding taxonomic classification were similar for OTUs and ASVs, the results presented below were produced using OTU information. The OTUs were organized in 588 bacterial taxonomic groups (assigned or unassigned at the genus level) and 257 fungal taxa (assigned or unassigned at the genus level), being the diversity indices calculated for the taxonomic

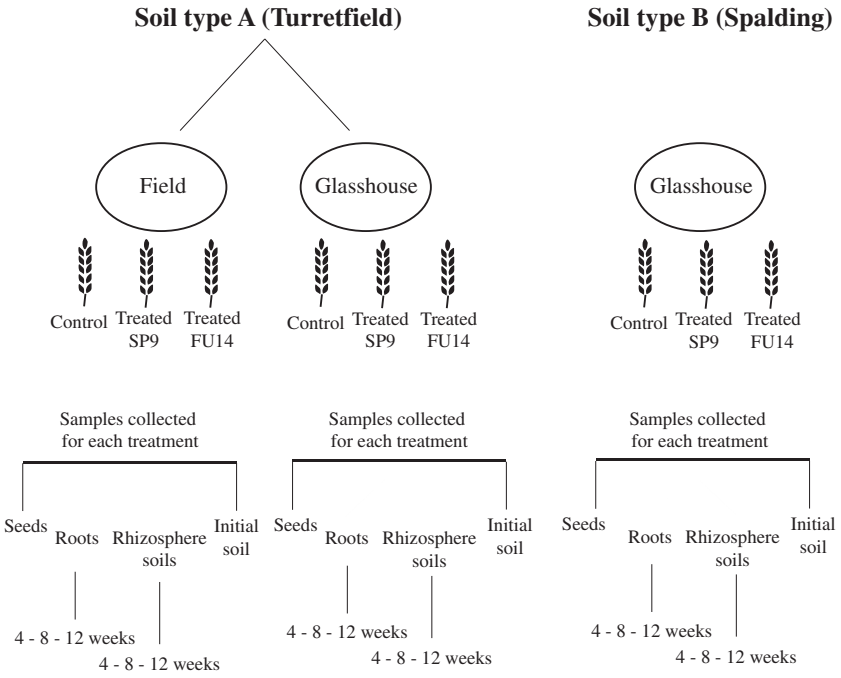

FIGURE 1 Study plan and samples used for microbial molecular analyses

groups classified and unclassified at genus level found in each sample (Supporting Information 1). The rhizosphere soils showed significantly ($p < .05$) more diversity (Shannon index, SI of 5.81) than roots (SI of 4.84) or seeds (SI of 3.79). The most frequent genera found in roots and rhizosphere soils were *Pseudomonas* and *Fusarium/Gibberella* (details for frequent genera in Supporting Information 2). Some other genera were exclusively found in seeds such as *Granulicatella*, in roots such as *Enhydrobacter*, *Kocuria*, and *Propionibacterium*, or in rhizosphere soils such as *Adhaeribacter*, *Kitasatospora*, *Nitrosovibrio*, and *Leohumicola* (more details in Supporting Information 3).

The biodiversity of bacteria and fungi found in control wheat roots and rhizosphere soils was very distinct, with *Pseudomonas* and *Gibberella* dominating in the roots and *Kaistobacter* and *Mortierella* in the rhizosphere soils (biodiversity details in Figure 3 and Supporting Information 4). During the 12-week period, the disease levels in the soils, and consequently on roots, were variable (Figure 2) while the relative abundance of specific bacteria and fungi changed, as showed by the post hoc analyses (Supporting Information 5 and 6). The diversity of bacterial genera increased over the 12-week period (from SI of 5.2 to 5.58; details in Supporting Information 1, 4, and 5), with some bacterial genera dominating the initial stages, for example *Agrobacterium*, *Bacillus*, *Flavobacterium*, *Rhizobium*, and *Rhodoplanes*, whereas other genera increased in the later stages, mainly *Actinoallomurus*, *Aminobacter*, and *Mycobacterium*. Among the fungi, *Alternaria*, *Fusarium/Gibberella*, and *Lewia* were common in 4-week roots, while *Exophiala* was frequent in roots at 12 weeks. Similar results were found using similarity percentages (SIMPER) analysis (Supporting Information 7) reinforcing the above-mentioned bacterial and fungal genera as the most relevant for characterization of wheat root age.

Besides these differences, it was possible to define a core group of bacteria and fungi (classified at the genus level) in the roots at all stages of wheat growth in *Pythium*-infested soils (Figure 3b and Supporting Information 4). The core microbiota found in wheat roots was dominated by the bacterial genera *Arthrobacter*, *Bacillus*, *Bradyrhizobium*, *Flavobacterium*, *Pseudomonas*, *Sphingomonas*, *Sporosarcina*, *Stenotrophomonas*, *Streptomyces*, and *Variovorax*, while the most represented fungi were *Alternaria*, *Aspergillus*, *Candida*, *Cryptococcus*, *Exophiala*, *Fusarium/Gibberella*, *Lewia*, and *Rhodotorula*.

Principal components analyses (PCA) confirmed the relevance of sample type and root age (or crop stage) as the strongest factors separating the samples (Figure 4a,b). Additional tests using analysis of similarities (ANOSIM) and homogeneity of dispersions (PermDISP) showed that the soil type (A or B), the use of biocontrol seed coats, and the culture system (field or greenhouse) did not cause major community shifts either in the rhizosphere soil or the plant roots (Figure 4 and Supporting Information 8). Interestingly, the sterile sand used for the additional control tests of endophytes produced a profile different from the seeds (Figure 4), with *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, and *Stenotrophomonas* found in the sand samples, showing the ability of these bacteria to leave the plant environment and move to the rhizosphere environment.

2.2 | Field versus greenhouse trials

Most of the bacteria and fungi found in the rhizosphere of plants grown in the field versus greenhouse trials, using either soil type A (high texture soil from Turretfield) or soil type B (clay soil from Spalding), were

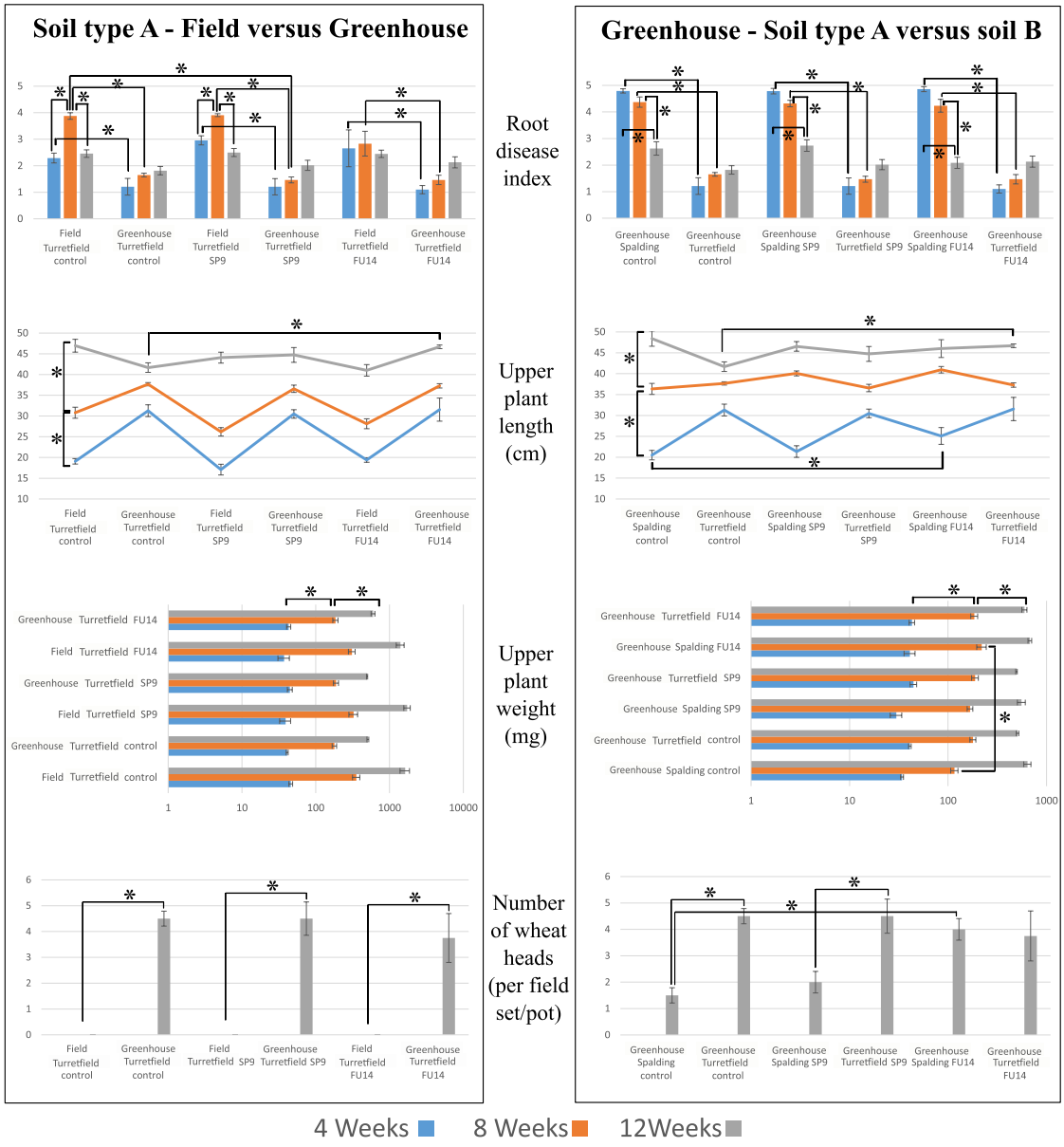

FIGURE 2 Wheat plants obtained after 4, 8, and 12 weeks of crop growth in field and greenhouse trials run at Turretfield (soil type A) and Spalding (soil type B). Plants were grown using control, *Paenibacillus peoriae* SP9, and *Streptomyces fulvissimus* FU14 biocontrol-treated seeds. *$p < .05$

similar (Supporting Information 9 and 10). The bacterial and fungal communities in the rhizosphere soils, as well as the SIMPER profiles, were not distinct when comparing the systems (field versus greenhouse) and soil types (Figure 4c and Supporting Information 11).

Regarding the bacterial and fungal communities within the roots, there were no large differences (Figure 4c), although some occasional differences could still be found in *Janthinobacterium*, *Delftia*, unclassified Pseudeurotiaceae, and a few other taxa using the post hoc comparisons (Figure 5). By detailed comparison of the genera found in the roots of wheat plants obtained from field or greenhouse trials, the taxonomic similarity values (i.e., the same genera were found in both sets of roots) were 74% for bacteria and 53% for fungi when all weeks were considered together. These values were much lower (30% to 66% depending on the week) when each week was considered separately (Supporting Information 12); the differences were mainly observed for genera found in greenhouse plants at 4 weeks that were only seen in the field grown plants at 8 weeks. The taxonomic similarity found in the soil A versus soil B showed values of 58% and 57% for the total bacteria or fungi, respectively, and the values for the independent week were also lower (ranging from 34% to 50%) (Supporting Information 12).

2.3 | Coated wheat seeds and the effect of biocontrol agents

Network analysis was used to clarify the connection among multiple taxonomic groups. The most connected bacteria were

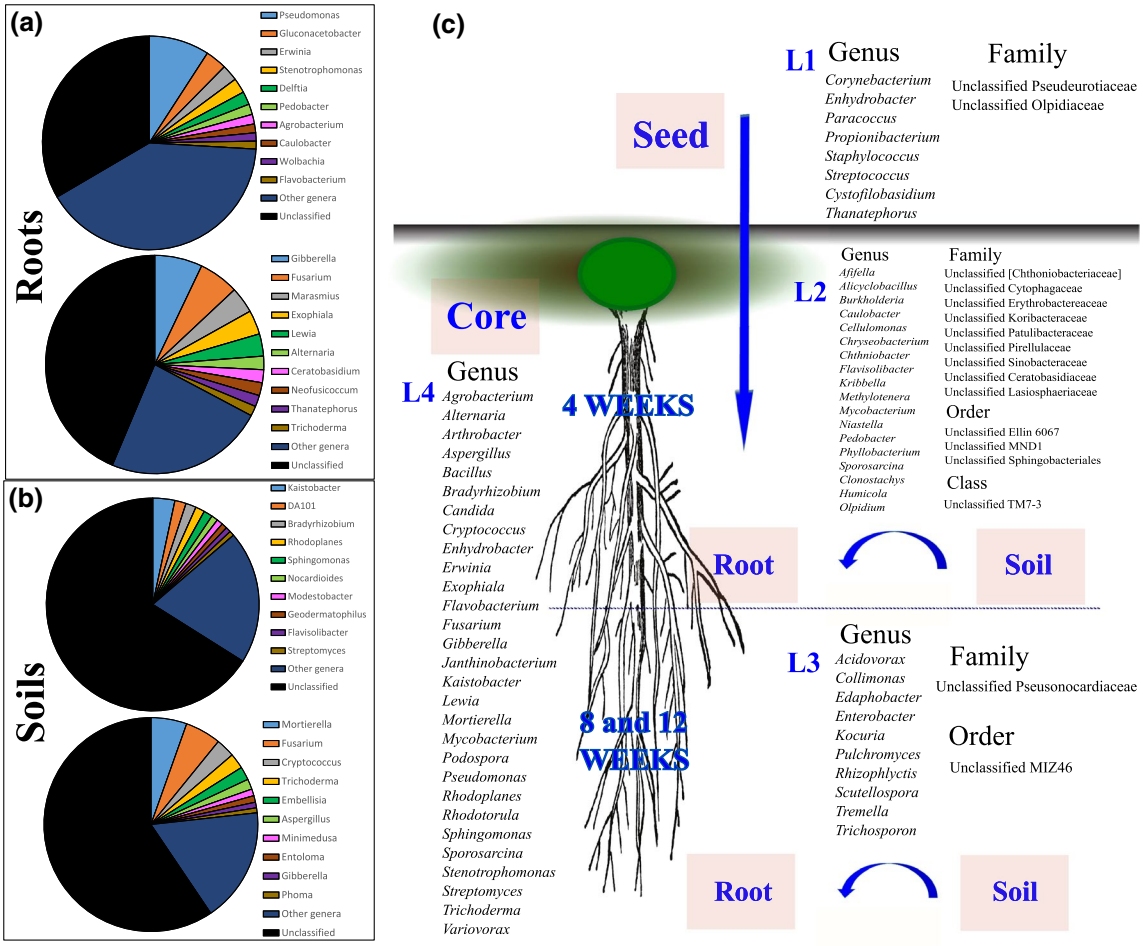

FIGURE 3 Taxonomic groups found in the wheat roots (a) and rhizosphere soils (b) at each stage of the crop. (c) Lists of genera found in: (L1) all seeds (control and coated-seeds) and roots at 4 weeks and not detected in the initial soils and 4-week rhizosphere soils, (L2) all roots and rhizosphere soils at 4 weeks, (L3) all roots and rhizosphere soils at 8 and 12 weeks, and (L4) core genera found in all roots and 4, 8, and 12 weeks. These lists were based on the operational taxonomic units (OTUs) found in each set of samples compared to the other sets

Streptococcus, *Propionibacterium*, *Kocuria*, and *Enhydrobacter*, and the most connected fungi were *Guehomyces*, *Pleiochaeta*, *Dioszegia*, and *Monographella* (Figure 6). *Paenibacillus* and *Streptomyces* were not highly connected in the network, not even when detailed networks were performed for each system, soil type or plant growth in the presence of biocontrol strains (Supporting Information 13 and 14). *Paenibacillus* was connected generally to *Facklamia*, *Yersinia*, and *Psychrobacter*, while *Streptomyces* was connected to *Butyricicoccus*, *Finegoldia*, and KSA1. Curiously, some of the most connected genera in roots, such as *Streptococcus*, *Propionibacterium*, and *Enhydrobacter* could be traced to the seeds, not being detected in the initial soils (Figure 3b).

Control wheat and biocontrol-coated seeds were dominated by the genera *Delftia*, *Erwinia*, *Janthinobacterium*, *Pseudomonas*, *Paenibacillus*, *Staphylococcus*, *Stenotrophomonas*, *Streptomyces*, and *Wolbachia*. Some of these bacteria were then found throughout the sampling times on wheat roots, not being detected in the initial soil or in 4-week-old rhizosphere soils (Figure 3b). The strains added as coats onto seeds dominated the sequences obtained from the DNA extracted using coated seeds (>80% of the recovered sequences were classified as *Paenibacillus* or *Streptomyces*) and the 16S rRNA profile (ASV) was similar among these sequences and the profile of the pure *P. peoriae* SP9 and *S. fulvissimus* FU14 16S rRNA sequence.

Although the effects of the biocontrol strain *S. fulvissimus* FU14 could be observed and measured on wheat plant growth over the weeks, no significant differences were observed on the relative abundance of *Streptomyces* in the roots of FU14-treated plants versus the control roots (Figure 7); similar stability was observed for *Paenibacillus* in the roots obtained from *P. peoriae* SP9-treated plants. Nevertheless, a few other taxa, namely *Pseudomonas* in the plant roots and *Clostridium*, *Diversispora*, and *Steroidobacter* in the rhizosphere soils, showed differences when FU14 was present as biocontrol agent (Figure 7).

The comparison of the ASV profiles was done for *Paenibacillus* and *Streptomyces* and the strain profiles were traced in all the samples (black and green, respectively, for SP9 and FU14 strains in Supporting Information 15) and the profiles compared with the remaining ASVs of *Paenibacillus* and *Streptomyces* obtained from roots and soils throughout the study. In both cases it was possible to monitor the presence of the initial strains in the wheat roots at 4 weeks

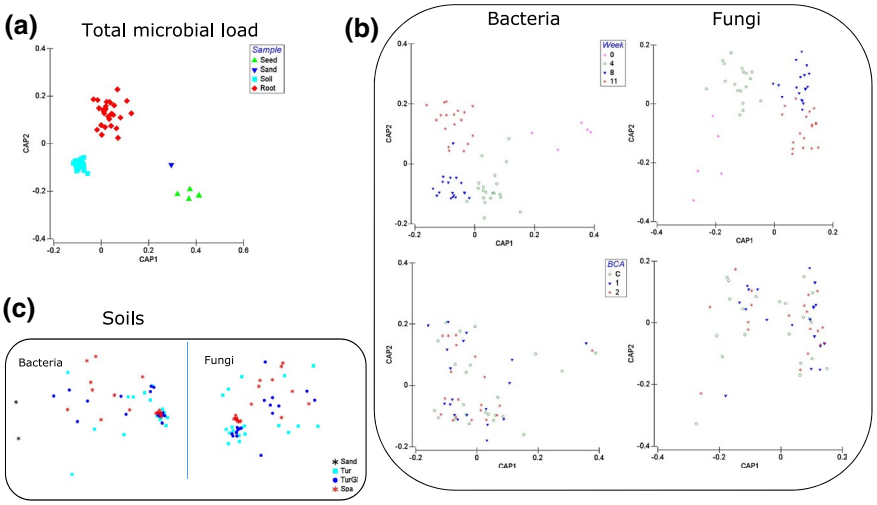

FIGURE 4 Nonmetric multidimensional scaling (NMDS) and canonical analysis of principal components (CAP) of samples: (a) NMDS of bacteria and fungi in all sample types (seed, sand, root, rhizosphere soil), (b) CAP analysis of taxonomic groups found in all root samples, and (c) NMDS analysis of root samples obtained from different systems and soils. Tur, Turretfield field trial; TurGI, Turretfield soil greenhouse trial; Spal, Spalding soil greenhouse trial; sand, in vitro trial using coat sterilized seeds. Analyses done in PRIMER 6 using squared root transformed data, resemblance matrices using Bray–Curtis similarities and dummy variable

(observed in 100% of the samples and replicates), 8 weeks (observed in 45% of the samples), and 12 weeks (only occasionally observed; in less than 10%). In addition, these strains could be observed occasionally (less than 10%) in the rhizosphere soils at 4 weeks but were untraceable in the weeks after.

3 | DISCUSSION

This study reveals for the first time the diversity and dynamics of bacterial and fungal populations found in the roots and rhizosphere soils of the wheat crop grown simultaneously in the field and in greenhouse trials. Two different soil types which were *Pythium*-infested were used and were challenged by adding biocontrol strains as seed coats in order to determine the plant and microbiota responses when exposed to such conditions. The effects observed in the wheat plants regarding root weight, plant length, and increased number of wheat heads in the presence of the FU14 strain were similar to previously published results for other biocontrol strains performed in our and other research laboratories (Cook *et al*., 2002; Franco *et al*., 2007; 2016; Orakçı *et al*., 2010; Yang *et al*., 2012; Cuppels *et al*., 2013; Araujo *et al*., 2017; 2019). In addition, it was possible not only to define a list of OTUs that characterized the core microbiota present in all stages of the crop growth but also a succession of OTUs and taxonomic groups found at particular stages. In fact, the microbial biodiversity in wheat roots shifted mostly in accordance with the crop age: *Fusarium/Gibberella*, *Lewia*, *Alternaria*, *Stenotrophomonas*, and unclassified Dothideomycetes dominated the first weeks, while *Sphingomonas*, unclassified Herpotrichiellaceae, *Exophiala*, and *Pedobacter* were highly abundant in the roots at later stages of wheat crop. Although it had been previously reported that fungal communities could change over time in wheat heads (Hertz *et al*., 2016) and wheat roots (Shi *et al*., 2015), this study shows those changes in microbial communities are not random, following instead a pattern (succession of distinct genera and OTUs). This pattern was independent of the plants being grown in the field or in the greenhouse and also of the soil types employed. The core bacterial and fungal microbiota described in previous reports (Nicolaisen *et al*., 2014; Ofek-Lalzar *et al*., 2014; Donn *et al*., 2015; Bokati *et al*., 2016; Rascovan *et al*., 2016) could partly be seen in the wheat plants but some additional taxonomic groups, especially among fungi, were revealed for wheat root biodiversity. Bacteria were more diverse during the initial stages of the wheat crop cycle (up to the eighth week), while the fungal biodiversity increased in the roots in the subsequent weeks and their role in the plant fitness should be carefully considered. Although such patterns of bacterial versus fungal dominance were suggested before (Coombs and Franco, 2003; Conn and Franco, 2004; Rascovan *et al*., 2016), fungi were believed to gain relevance much later (after the 16th week), being now proposed as an earlier contribution to plant microbiota.

In this study wheat seeds were shown to hold highly diverse native bacterial and fungal communities both internally and externally. Interestingly, some of the microbes described as wheat seed endophytes, namely *Paenibacillus*, *Propionibacterium*, *Pseudomonas*, *Stenotrophomonas*, and *Streptomyces*, match the recently described taxonomic groups in barley seeds (Yang *et al*., 2017). Nevertheless, more than 47% of the bacterial sequences found in seeds were not assigned at family level, leaving a large fraction of unknown bacteria in wheat seeds to be studied. When the external surface of the seeds was sterilized and the endophyte community of seeds monitored by in vitro sand trials, lower biodiversity was observed (over 80% of the taxonomic groups were not detected), affecting mainly the fungal community. *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, and *Stenotrophomonas* were among the mobile bacteria leaving the

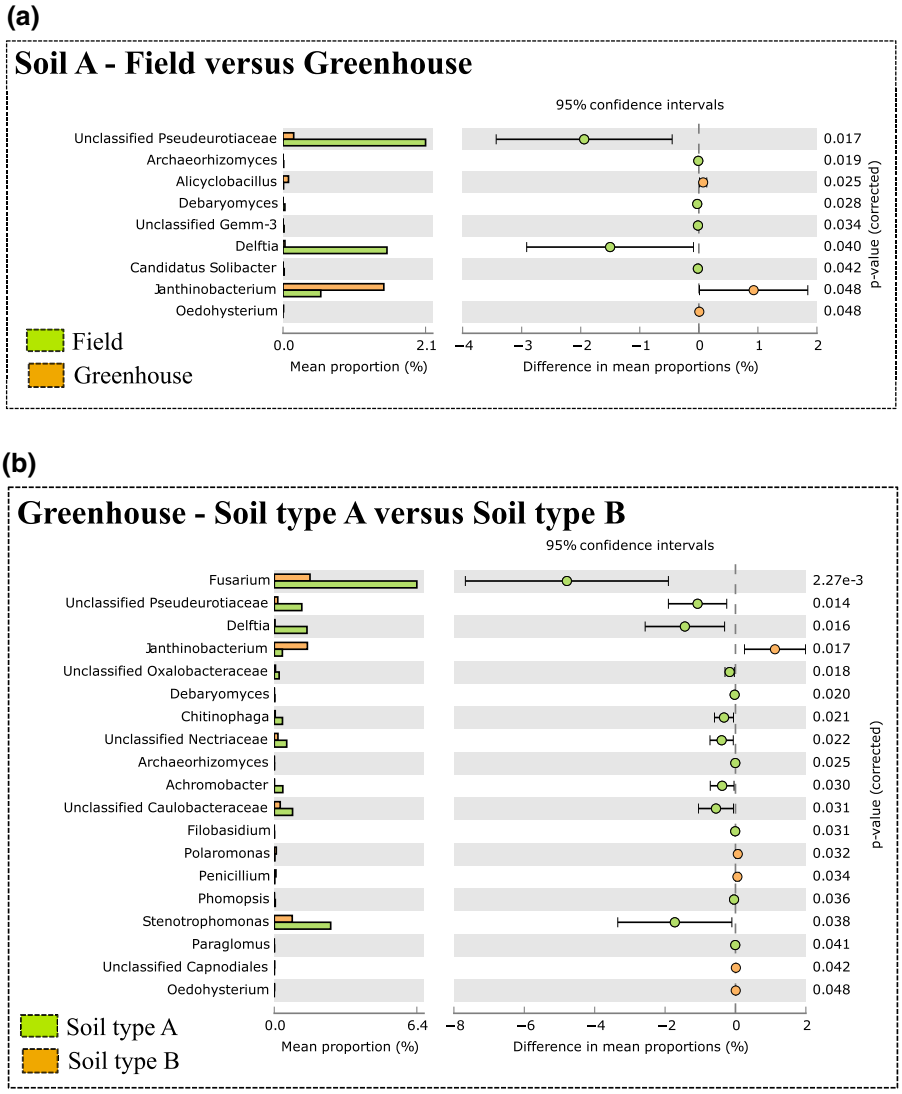

FIGURE 5 Post hoc analyses of (a) root samples obtained from field and greenhouse trials conducted in soil type A and (b) root samples obtained from greenhouse trials conducted in soil type A versus soil type B. These analyses were done in STAMP 2.1.3 using two groups analysis and Welch's *t* test (two-sided, Welch's inverted for confidence interval method)

seed microbiota to the rhizosphere sand environment. Some bacteria found in the seeds and absent in initial and rhizosphere soils were abundant in roots at 4 weeks, for example *Delftia*, *Streptococcus*, and *Staphylococcus*, or in later stages, for example *Sphingomonas*. Taking into account the network analyses it was noticed that some of the most connected genera in roots were acquired from the initial seeds, reinforcing the influence of some taxa, for example *Streptococcus*, *Propionibacterium*, and *Enhydrobacter*, for the overall plant microbiome. These endophytic genera, frequently described as protective for the plant, are capable of producing multiple enzymes, resist plant pathogens, and are highly adaptable to the unique seed environment (Vorobjeva, 1999; Mitter *et al.*, 2017; Raj *et al.*, 2019; Xie *et al.*, 2019), being inherited in wheat seeds. Hence it is suggested some genetic determinants (OTUs) are transmitted vertically and complemented by OTUs obtained from the rhizosphere soils at multiple stages of plant development. For example, *Burkholderia* and *Caulobacter* OTUs, frequently reported in wheat microbiota (Mendes *et al.*, 2013; Turner *et al.*, 2013; van der Heijden and Hartmann, 2016; Sánchez-Cañizares *et al.*, 2017), are acquired from the rhizosphere soil at early stages of wheat growth, as these bacteria are not present in the wheat seed microbiota.

Occasional differences in plant performance can be found when comparing plants grown in field and greenhouse trials (Cuppels *et al.*, 2013; Shi *et al.*, 2018). In this study such differences were observed mostly in roots/tops length and root density in the soil. The microbial communities of roots and rhizosphere soils were highly similar in both cultivation systems. The exception was found in particular taxonomic groups and OTUs, especially among *Archaeorhizomyces*, *Alicyclobacillus*, *Debaryomyces*, *Delftia*, *Janthinobacterium*, and unclassified Pseudeurotiaceae, but the influence of these particular organisms on wheat plants is still largely unknown. At the OTU level, the differences were much larger and it is possible that some OTUs play an important role in plant fitness (Mendes *et al.*, 2013; Lakshmanan *et al.*, 2014), but such a comparison is still hard to

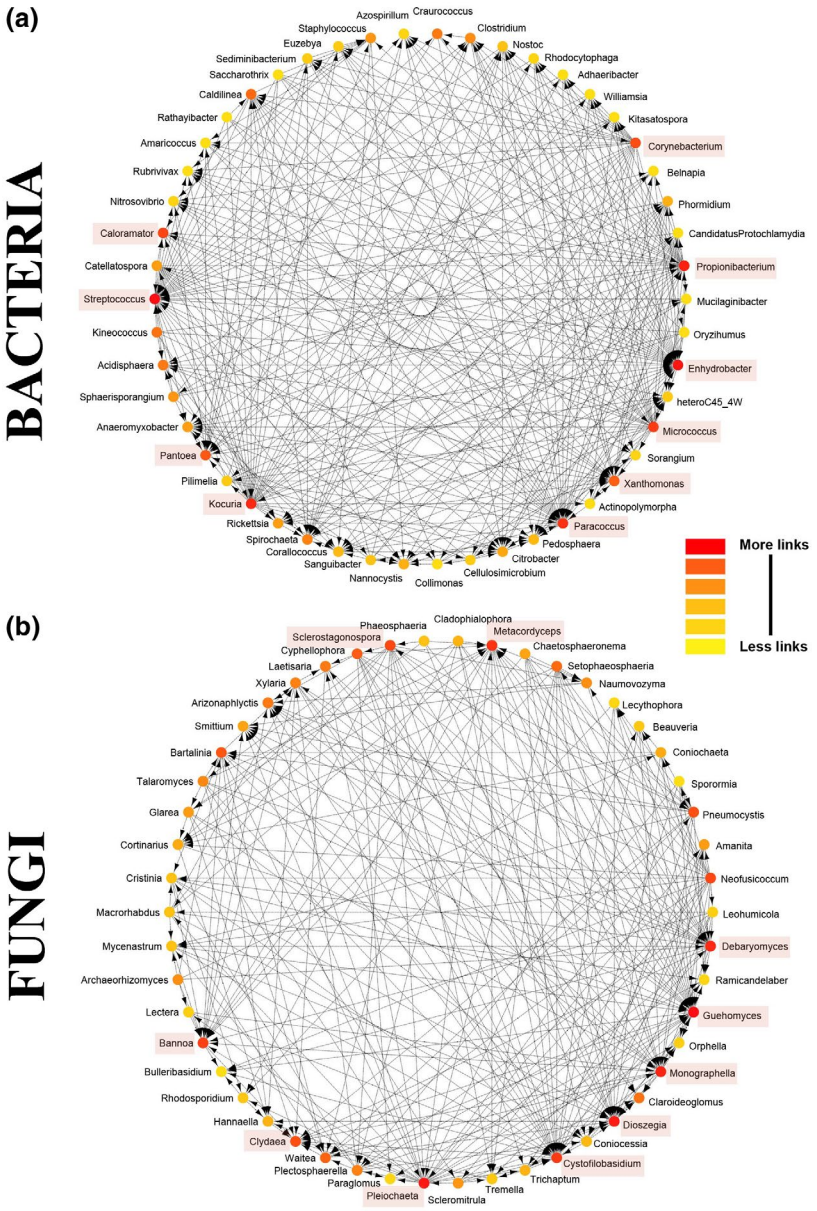

FIGURE 6 Top 50 central genera of bacteria (a) and fungi (b) obtained by network analyses of the community structure (from yellow to red the number of connections is increased and the genera were found in the centre of the complete network shown in Supporting Information 13). Network analysis was conducted using molecular ecological network analysis pipeline (MENA; http://ieg4.rccc.ou.edu/MENA/) to generate the networks with a cut-off of 0.84, Cytoscape environment to visualize networks, and cytoHubba application with maximal clique centrality (MCC) scores to select the top 50 genera for relevance in roots and rhizosphere soil samples. Bacteria and fungi were analysed in separate files

achieve as the rare OTUs (abundance lower than 0.5%) still change drastically among systems, soil types, and even among replicates. The comparison of two soil types showed some taxonomic similarities in the wheat roots and rhizosphere soils after 3 months but also differences, especially associated with rare OTUs. Environmental conditions and rare OTUs may explain the differences found in field versus greenhouse trials.

Biocontrol agent-coated seeds may promote the growth of wheat plants in normal or *Pythium*-infested soils, as shown by some studies in the past (Franco *et al.*, 2007; 2016; El-Tarabily *et al.*, 2009; 2010; Mavrodi *et al.*, 2014) and confirmed by this study. Two biocontrol strains were selected based on their effect on wheat plants tested in planta in previous studies (Barnett *et al.*, 2017). *Pythium* is responsible for root rot disease in wheat and other crops, resulting in the constant presence of spores at different phases even when a break crop is used, such as a cereal–legume–canola crop rotation (Pankhurst *et al.*, 1995). *Pythium* grows fast, continuously reinfecting the growing roots, weakening the plant, and enabling infection by other pathogens, such as *Rhizoctonia* and *Gauemannomyces* (take all). Fungicides, such as metalaxyl, have been used as a treatment; however, they only provide partial control (Cook *et al.*, 2002). Biological control employing certain strains, such as the strains *P. peoriae* SP9 and *S. fulvissimus* FU14 used in this study, can be advantageous against these diseases (Alabouvette *et al.*, 2006; Barnett *et al.*, 2006; Conn *et al.*, 2008; El-Tarabily *et al.*, 2010; Mavrodi *et al.*, 2014; Araujo *et al.*, 2017; 2019; Schlatter *et al.*, 2017) with generally low impact on microbial communities of the roots and rhizosphere soils during the 3-month period. *Streptomyces* was one of the most abundant and genetically diverse taxa in this study, and it is frequently described as an excellent biocontrol agent for multiple plants such as wheat, barley, cucumber, and potato (Prévost *et al.*, 2006; El-Tarabily *et al.*, 2010; Araujo *et al.*, 2017). Biocontrol strains added to the seeds were consistently isolated from the roots up to the eighth week, earlier than previously suggested (Conn and Franco, 2004), and rarely seen in rhizosphere soils. The high

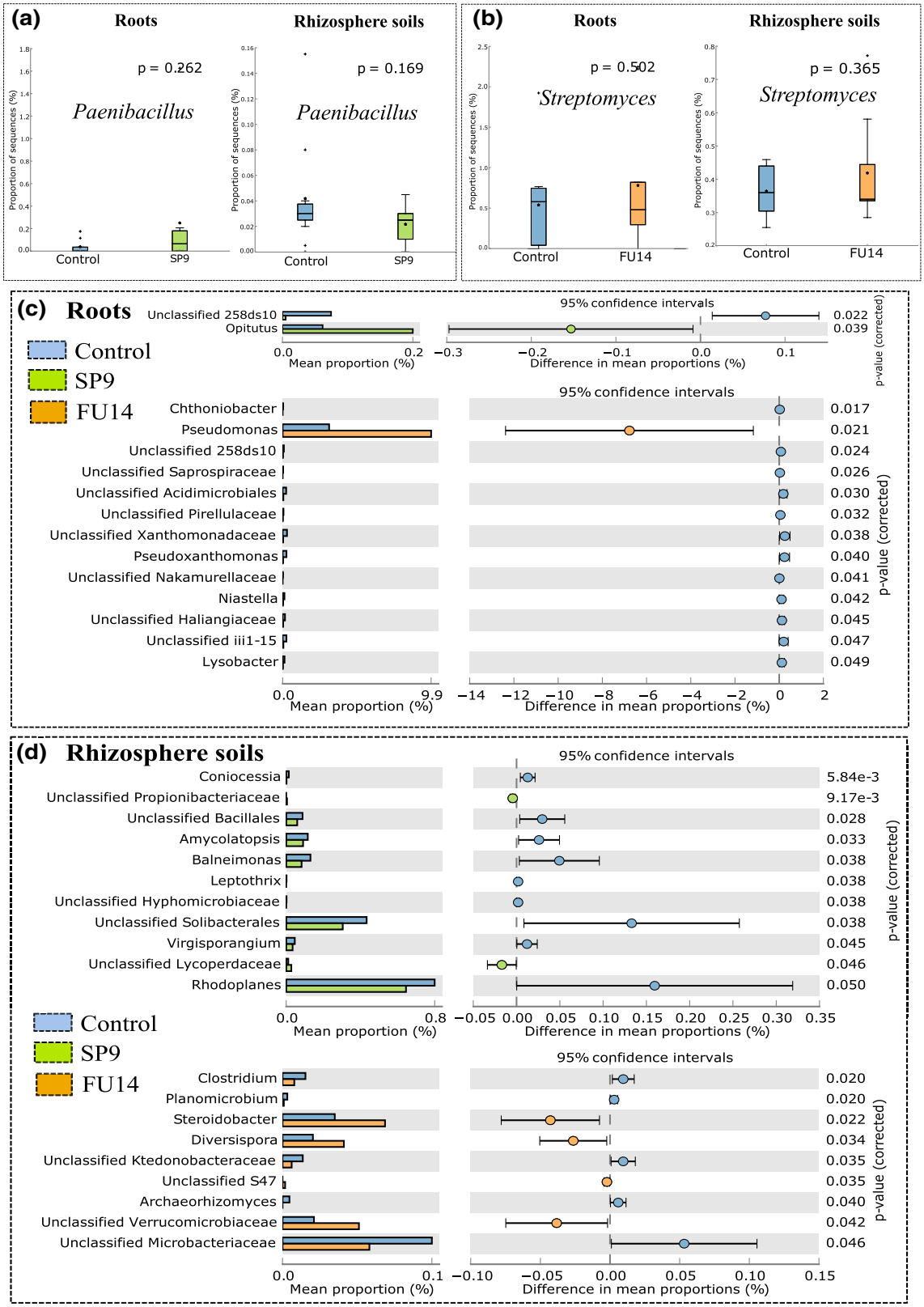

FIGURE 7 Relative abundance of (a) *Paenibacillus* and (b) *Streptomyces* in root and rhizosphere soils collected from control, *Paenibacillus peoriae* SP9-, and *Streptomyces fulvissimus* FU14-treated plants. Post hoc analyses of (c) root samples obtained from control, SP9-, and FU14-treated plants, and (d) rhizosphere soil samples obtained from control, SP9-, and FU14-treated plants. These analyses were done in STAMP 2.1.3 using two groups analysis and Welch's *t* test (two-sided, Welch's inverted for confidence interval method)

genetic diversity of the microbes in the soil certainly contributes to the dilution of the biocontrol agents initially added.

In the present study the physiological benefits of the biocontrol seed coating can be seen in terms of disease control, plant length, and early formation of wheat heads, as previously shown (Mavrodi *et al.*, 2014; Araujo *et al.*, 2017; 2019). Seed-coated inoculants are present in abundance in wheat plants for the first 8 weeks and promote plant health in soils with early disease pressure and by priming the plant defence response (Conn and Franco, 2004) to prolong the effects of biocontrol strains. However, additional strategies may be used to prolong their presence in planta, such as extra inoculations by injection in the soil or by applying sprays (Obradovic *et al.*, 2004; Prévost *et al.*, 2006; El-Tarabily *et al.*, 2010; Yang *et al.*, 2012; Cuppels *et al.*, 2013; Araujo *et al.*, 2017); these strategies were not tested in this work. Soils with high disease pressure may limit the development of wheat plants but these effects may be attenuated by high microbial diversity that adds sustainability and more predictability to the crop system. It needs to be proven if such alternatives can also be used to mitigate other soil problems, for example chemical contamination, besides their application to disease-infested soils.

This study showed a recognizable succession of bacteria and fungi within the wheat root environment starting with seed microorganisms, enriched by the rhizosphere communities over time, and turning roots into a vibrant and diverse microbial environment. The consistency of microbial communities found in roots and rhizosphere soils, irrespective of the cultivation system (field or greenhouse), soil type or addition of biocontrol coating on seeds, were major findings in this study. Neither the biocontrol organisms nor the pathogenic agents had major effects on the root and rhizosphere microbiota, even though they had measurable effects on the physiology of the plant. Against our initial question, *Paenibacillus* and *Streptomyces* were not central genera in the microbial community structure, playing a limited and lateral role in the plant and soil ecosystems. However, it is important to mention that these genera are well-known metabolite and antibiotic producers (Davide Spadaro, 2005; Babalola *et al.*, 2009; Orakçı *et al.*, 2010; Suprapta, 2012; Franco *et al.*, 2016; 2017; Araujo *et al.*, 2017): the release of these compounds to the root and rhizosphere environments may affect the growth of many bacteria and fungi, causing indirect fluctuations of such populations. This study provided key information on the microbes (bacteria and fungi) that modulate wheat root diversity at different stages of growth, both in the field and in the greenhouse, and under disease stress. As more information is offered by extensive analyses of microbial communities, it will become possible to clarify the role of each specific organism, and even of particular OTUs, present in the plant roots.

4 | EXPERIMENTAL PROCEDURES

4.1 | Biocontrol cultures and seed coating

The biocontrol strains *P. peoriae* SP9 and *S. fulvissimus* FU14 were used in this study; the identification of the isolates was done based on the sequence of the complete 16S rRNA gene. These strains showed protective effects in planta against *Pythium* sp.; the stability and survival curves of these strains were reported previously, showing a large survival rate in wheat seed coating procedures after 4 weeks (Barnett *et al.*, 2017). The strains were identified by their 16S rRNA gene sequences amplified using the universal primers 27F and 1492R (Miller *et al.*, 2013), against the NCBI database, and stored in culture collections at Flinders University and the South Australian Research and Development Institute (SARDI). A suspension of each strain (approx. 10^5 cfu/seed) was prepared in sterile 0.3% (wt/vol) xanthan gum sticker solution and applied to 20 g of wheat seeds (Barnett *et al.*, 2006; 2017). Seeds were allowed to dry at room temperature after coating, the coated seeds were kept stored at room temperature for more than 1 week before being used in greenhouse and field trials. The cfu per seed was assessed immediately after and at 1, 2, and 7 days after application for confirmation of bacterial viability and concentration per seed (c.10^5 cfu/seed).

4.2 | Field trials and pot bioassays in greenhouse

Field trials were carried out on commercial cereal paddocks at Turretfield, South Australia (soil A) with high levels of indigenous *Pythium* sp. inoculum (194 pg *Pythium* sp. clade F DNA/g soil, based on quantitative PCR tests done at SARDI). Field experiments were set up in a split-plot randomized complete block design, with an untreated row or plot next to every biocontrol strain (SP9 and FU14) treated row or plot with four replicates. Field trials consisted of 20 m × 6 row plots, with three rows planted with microbe-treated seeds and three rows with untreated seeds. Plots were machine planted. Prior to planting, a machine seeder was used to cultivate, form rows, and add fertilizer, except no seed was planted. At 4, 8, and 12 weeks, 60 plants were dug up using a shovel, keeping the soil surrounding the roots, and each replicate was stored in an individual sterile plastic bag to be taken to the laboratory. The plants were processed in the laboratory on the same day. Each wheat plant was separated carefully from the soil, the rhizosphere (soil adherent to the roots) collected into a sterile paper bag, and the roots carefully washed in running distilled water. The nodal and seminal roots of wheat plants were cut using sterilized scissors and washed at each time point to remove all the soil and organic matter. The surface of the roots was then sterilized with sodium hypochlorite 2% (for 3 min) and ethanol 70% (for 3 min) and washed three times with sterile water (Barnett *et al.*, 2017); a similar sterilization protocol was used for a set of control seeds. Rhizosphere soils were collected by recovering the small layer of soil on the surface of the roots. Roots were initially collected, gently shaken to discard loosely adhering soil, and the adjacent rhizosphere soil in the root surface collected by shaking the roots vigorously into a sterilized envelope (a sterile spatula was occasionally used in this procedure without damaging the roots; 5–30 g of rhizosphere soil was collected per independent pot/replicate). The plants were assessed for *Pythium* sp. disease on seminal and nodal roots (0–5 disease scale, 0 = healthy roots,

5 = highly diseased roots) (Barnett *et al.*, 2017), tiller number, and shoot length and dry weight. Shoot lengths were measured and then the shoots were dried for 4 days at 60 °C and weighed. The same number of plants per plot was assessed at each time point.

Greenhouse pot trials were prepared in Bedford Park, South Australia using field soil collected at Turretfield, South Australia (soil A, from the same plot where the field trial was conducted, with 194 pg *Pythium* sp. clade F DNA/g soil) and Spalding, South Australia (soil B with 112 pg *Pythium* sp. clade F DNA/g soil). The bulk soil (150 kg) was collected from the top 10 cm of a 100 m^2 section of the field, avoiding the collection of plant material larger than 2 mm. Soil A was mainly characterized by high texture, while soil B showed high levels of clay. The soils were collected from the field, discarding the top 10 cm layer and any plant and other organic materials. The pot experiments were prepared with different amounts of soil: 0.6 kg for 4 weeks, 1 kg for 8 weeks, and 1.125 kg for 12 weeks) and five wheat seedlings were plotted per pot for 4, 8, and 12 weeks; four replicates were run simultaneously for each treatment and control. Wheat seeds from cultivar Yitpi (susceptible to *Pythium* disease) were used covered with 50 g soil and 50 g sand to reduce evaporation. Plants were grown in a greenhouse and the pots watered twice a week to their original starting weight. Replicates were arranged in a randomized complete block design. Plants were collected and treated as described above for field trials and assessed for growth and root disease levels.

Sterilized sand trials were run simultaneously. Control and coat-sterilized seeds (no biocontrol added) were grown for 4 weeks in a sterilized container with sand, water, and essential nutrients. This trial was conducted to monitor the presence of endophytes in the coat-sterilized seeds and to determine which microbes could move to the sand and be recovered after 4 weeks.

Pot bioassays were run simultaneously with the field and sand trials from June to September 2016. Background levels of *Pythium* in the soil were analysed by PreDictaB (SARDI, Urrbrae, SA, Australia; http://www.pir.sa.gov.au/research/services/molecular_diagnostics/predicta_b).

4.3 | DNA extraction, sequencing, and data analysis

Sterilized roots and seeds, rhizosphere soils, and initial seeds were kept at −80 °C until use, randomized, and processed for DNA extraction. A fixed amount of five seeds, 1 g of root or 2 g of rhizosphere soil per test, were subjected to DNA extraction using a cetyltrimethylammonium bromide (CTAB) DNA extraction strategy (Zhang *et al.*, 2010). The final DNA was suspended in Tris-EDTA (TE) buffer. PCR was performed using Kapa HiFi PCR mastermix (Kapa Biosystems) using the following parameters: 95 °C, 10 min, 35 cycles of 95 °C, 30 s; 58 °C, 30 s; 72 °C, 60 s. The bacterial community was targeted by amplification of the V3–V4 region of the 16S rRNA genes using the primers 341f and 806r (Muyzer *et al.*, 1993; Caporaso *et al.*, 2011), while the ITS1 region with ITS1F and ITS2 primers (Gardes and Bruns, 1993) was amplified for the fungal community. The loci-specific primers were incorporated into fusion primers for Illumina dual indexing and incorporation of Illumina adapters (Caporaso *et al.*, 2012). PCR products were cleaned and normalized using a SequalPrep normalization plate (Thermofisher Inc.). The samples were equimolecular pooled and the library quantified with a Kapa library quantification kit (Kapa Biosystems). The DNA pool was sequenced using an Illumina MiSeq system with a MiSeq v. 3 2 × 300 bp sequencing kit. QIIME 1.9 (Caporaso *et al.*, 2010) workflow was used for read merging, de novo OTU picking, and taxonomic assignment (RDP v. 11.4 was used for bacteria and UNITE v. 7.2 for fungi) (Bengtsson-Palme *et al.*, 2013; Cole *et al.*, 2014); the algorithms UCLUST for 16S rRNA and BLAST for ITS were used. Usearch (Edgar and Flyvbjerg, 2015) was used for chimera removal (uchime2) using the ChimeraSlayer database (Haas *et al.*, 2011) for 16S rRNA (file name gold.fa) and UNITE/INSDC representative/reference sequences v. 7.2 (UNITE Community, 2017) for ITS sequences. Sequences with ≥97% identity defined the OTUs following sequence alignment in accordance with the model organism priors *Escherichia coli*; the clustering was produced in two passes of the swarm algorithm v. 2.1.6 (the first pass with an aggregation distance equal to 1 and the second pass with an aggregation distance equal to 3). In parallel, ASVs were identified to improve the resolution up to one nucleotide among the sequences (Callahan *et al.*, 2016), using a previously suggested R pipeline and DADA2 method and RDP (rdp_train_set_16), Greengenes (gg_13_8_train_set_97), and UNITE (UNITE_public_28.06.2017) databases for classification. The minimum abundance of 10 sequences was considered for the OTUs and ASVs included in this study.

4.4 | Statistical and data analysis

Plant and disease data from pot bioassays were analysed as a three-way factorial (5 sampling times × 2 disease levels × 4 seed treatments) randomized complete block design with time fitted as a whole plot using GenStat v. 14 (VSN International Ltd.). Fisher's least significant difference (LSD) was used to compare treatment means as the data were near normally distributed with homogeneity of variance between factors. *Pythium*-disease severity was analysed by Kendall's coefficient of concordance (a nonparametric method). Data and statistical analyses were performed using Excel 2013 (Microsoft Corporation), GenStat v. 14, PRIMER-6 (PRIMER-e), and R (R Core Team, 2017). Bacterial reads classified as "Chlorophyta" at taxonomic order level and "Mitochondria" at taxonomic family level were removed from the final data analysis. The core taxonomical groups were defined as the groups found in all weeks (4, 8, and 12) and in both field and greenhouse trials. Community diversity and distribution analyses were conducted by running ANOSIM one-way analysis (calculating the resemblance and using similarity data type), nonmetric multidimensional scaling (NMDS), cluster analysis, CAP, PermDISP (calculating the resemblance, similarity data type, using root-transformed data, Bray–Curtis similarities, and 999 permutations), and SIMPER analysis (using Bray–Curtis similarities and 90% cut-off for low

contributions). Network analysis was conducted using the molecular ecological network analysis pipeline (MENA) (Deng *et al.*, 2012) to generate the networks with a cut-off of 0.84. Cytoscape (Shannon *et al.*, 2003) was used to visualize networks and cytoHubba (Chin *et al.*, 2014) used to select the top 50 genera with more links in roots and rhizosphere soil samples using maximal clique centrality (MCC) scores. The reads in each sample were converted into percentage values according to the total number of sequences in the sample to eliminate the effect of the final number of reads (Araujo *et al.*, 2019). These values were then transformed using the Hellinger approach—square-root of percentage (Legendre and Gallagher, 2001)—to reduce the effects of overestimation among the most common taxa and the values compared on dissimilarity matrices that could be used for multiple population analyses. Post hoc analyses were done in STAMP v. 2.1.3 (Parks *et al.*, 2014) for multiple groups using one-way ANOVA, Tukey–Kramer (0.95), and eta-squared for effect size, while two groups analysis used Welch's *t* test (two-sided, Welch's inverted for confidence interval method). The Geneious platform (Biomatters Ltd) was used for comparison and alignment of *Paenibacillus* and *Streptomyces* sequences and organization of diversity and phylogenetic trees (Geneious Tree Builder option was chosen using the Tamura–Nei genetic distance model and the neighbour-joining method, and no outgroup was considered).

ACKNOWLEDGEMENTS

R.A. was supported by an Endeavour Postdoctoral Fellowship. This study was financed by Grains Research and Development Corporation (GRDC) project no. UF00008. The biocontrol agents included in this study are protected under the Australian Provisional Application no. 2017901523, filed 27 April 2017, and U.S. Provisional Application no. 62/568,763, filed 5 October 2017. The strains are stored in Flinders University and the South Australian Research and Development Institute (SARDI, Adelaide, South Australia, Australia) freely available for research studies. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture; USDA is an equal opportunity provider and employer.

DATA AVAILABILITY STATEMENT

The dataset supporting the conclusions of this article is available in the NCBI BioProject repository at https://www.ncbi.nlm.nih.gov/bioproject/ under accession PRJNA471385 (SRA study SRP149964).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Araujo R, Dunlap C, Franco CMM. Analogous wheat root rhizosphere microbial successions in field and greenhouse trials in the presence of biocontrol agents *Paenibacillus peoriae* SP9 and *Streptomyces fulvissimus* FU14. *Molecular Plant Pathology*. 2020;21:622–635. https://doi.org/10.1111/mpp.12918