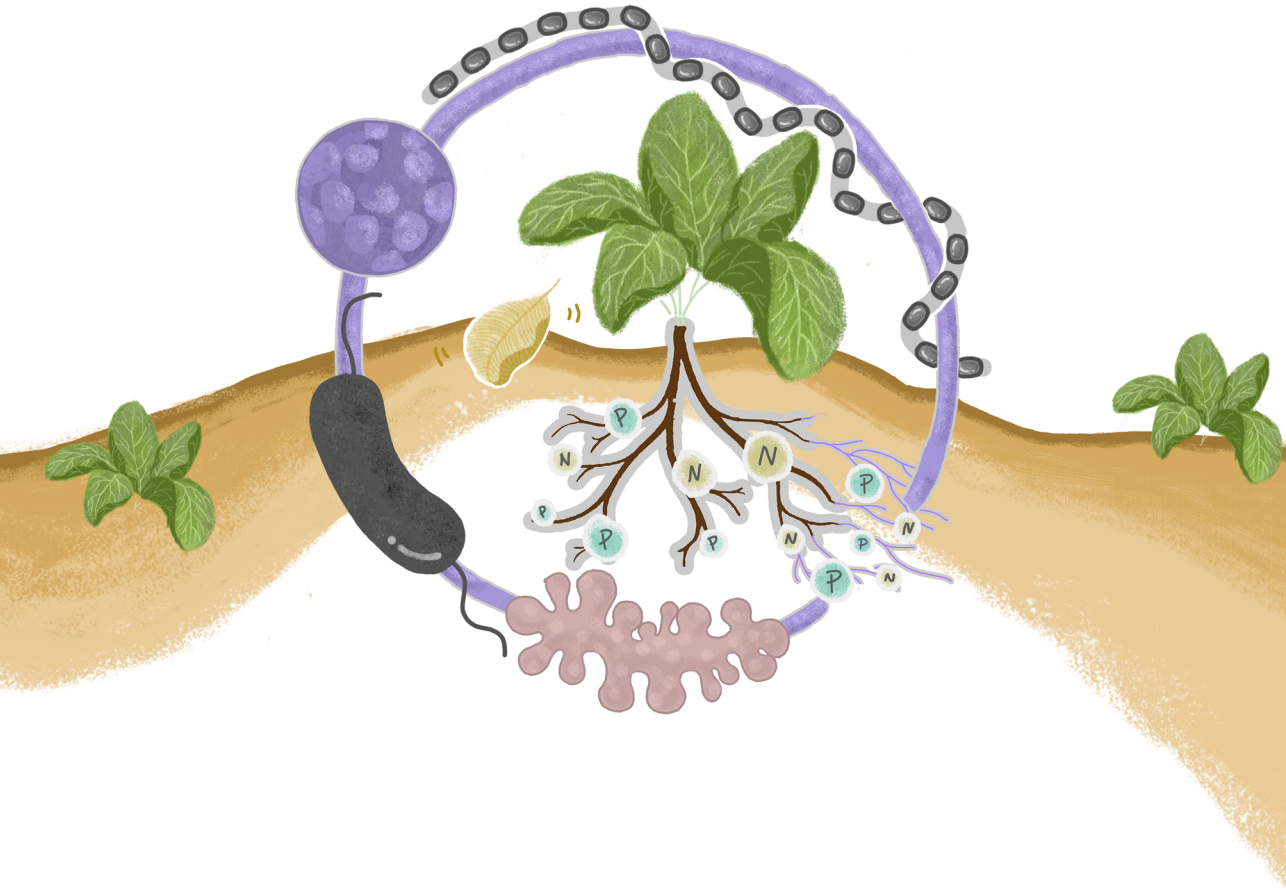
Exploring the role of microbial interactions in soil and rhizosphere and their effects on litter decomposition, mycorrhizal associations, and plant growth



Changfeng Zhang

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De rol van microbiële interacties in bodem en rhizosfeer en hun effecten op afbraak van strooisel, mycorrhizale associaties en plantengroei
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

The soil microbiome

Highly diverse communities of microorganisms including bacteria, archaea, fungi, oomycetes, and protists inhabit the soil. These microbial communities carry out fundamental ecological functions including nutrient cycling (Finzi *et al.*, 2015), sustaining plant biodiversity (van der Heijden *et al*., 1998), influencing plant growth (Lugtenberg & Kamilova, 2009), and enhancing plant resistance to biotic and abiotic stress (Dimkpa *et al.*, 2009; Berendsen *et al.*, 2012). The microorganisms that colonize a specific soil habitat are, together with their genomes, defined as soil microbiomes (Marchesi & Ravel, 2015). Soil microbiomes can be divided into different habitats based on the specific location and substrate that it colonizes (Fig. 1). The soil in the proximity of plant roots is called the rhizosphere and consequently the microorganisms in the rhizosphere comprise the **rhizosphere microbiome** (Berendsen *et al*., 2012). Moreover, soils are colonized by a dense network of fungal hyphae. Similarly, fungal hyphae are also colonized by microbes, and the microbial communities colonizing the surface of hyphae can be called the **hyphosphere microbiome** (Fig. 1). In addition, microbial communities colonize other substrates such as plant litter, which we define here as **the litter microbiome.** Finally, a range of microbes inhabit the soil and are beyond the influence of plants and symbionts. This microbiome is considered a background or indigenous microbiome to compare with the other microbiomes and is defined here as **the bulk soil microbiome**.

The rhizosphere microbiome

The rhizosphere is the narrow zone of a few millimeters of soil that surrounds plant roots (Philippot *et al.*, 2013). In the rhizosphere, microorganisms may receive up to 20% (Bago *et al.*, 2000) of the plant's photosynthetically derived carbon including low-molecular-mass compounds (sugars, organic acids, and amino acids) and polymerized sugar (mucilage). These carbon compounds can shift the plant microbiome by promoting or declining specific microbial taxa. For instance, soils amended with sugars (glucose, sucrose, fructose) that were extracted from the *Pinus radiata* rhizosphere increased 2.5-fold bacterial taxa than control soil where no sugar was added (Shi *et al.*, 2011). In the same study, soil amended with organic acid extracted from the plant rhizosphere caused a 10-22-fold stimulation of bacterial taxa compared to controls without. Metabolite profiling of rhizosphere microbes demonstrated that rhizosphere bacteria have a higher number of organic acid and amino acid transporters in their genomes compared to microbes from control soils (Zhalnina *et al.*, 2018). Polysaccharides that are linked to maize root mucilage, stimulated microbial growth and changed the bacterial community structure (Benizri *et al.*, 2007).

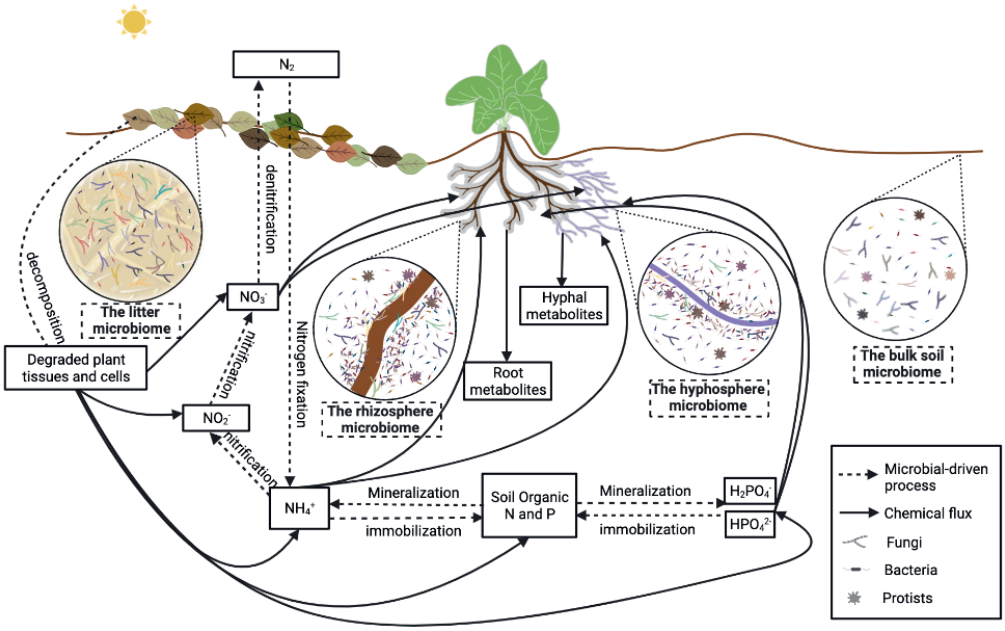

Fig. 1. A diversity of microbes colonizes soil, plant roots, mycorrhizal hyphae, and litter of decaying plants. Microbes drive nitrogen (N) and phosphorus (P) cycles in soil, and help plants take up N and P through root- or mycorrhiza- pathways. Dashed arrows indicate microbial-driven processes in the soil. Line arrows indicate chemical fluxes in the soil. Fungi, bacteria, and protists are represented by the shapes shown in the legend frame. This figure was created with BioRender.com.

Besides primary metabolites (e.g., low-molecular-mass compounds and polymerized sugar), recent studies indicate that secondary metabolites play pivotal roles in the rhizosphere microbiome formation (Jacoby *et al.*, 2020; Hong *et al.*, 2022). For example, plant immune signals and salicylic acid, sculpt the root microbiome likely by differentially gating bacterial taxa colonization (Lebeis *et al.*, 2015). Similarly, triterpenes that have important functions in plant defense also shaped an *Arabidopsis*-specific microbial community (Huang *et al.*, 2019). Benzoxazinoids, the indole-derived metabolites coffering resistance against insect pathogens, also selectively impact on rhizosphere microbiome (Hu *et al.*, 2018; Cadot *et al.*, 2021). Under iron-limiting conditions, *Arabidopsis* increased the production of coumarins, especially scopoletin, and changed the root microbiome, partly due to the antimicrobial effects of the scopoletin on specific plant fungal pathogens (Stringlis *et al.*, 2018). A synthetic community approach manifests that coumarin inhibited *Pseudomonas* colonization on plants by redox-mediated toxicity (Voges *et al.*, 2019). Flavonoids initiate rhizobia nodulation in legumes, and they increase the arbuscular mycorrhiza colonization rate on Chinese tallow trees (Tian *et al.*, 2021). Moreover, flavonoids also preferentially attract *Aeromonadaceae* to enhance plant arabidopsis dehydration resistance (He *et al.*, 2022). Flavones, as one of the important subgroups of flavonoids, enriched *Oxalobacteraceae* promoted plant growth under nitrogen deprivation soils (Yu *et al.*, 2021).

A combination of primary metabolites and secondary metabolites secreted by plant roots are allocated to the rhizosphere and impact the rhizosphere microbiome. These effects result in higher bacterial and fungal numbers in the rhizosphere but lower bacterial/fungal diversity (Foster *et al.*, 1983; Marcial Gomes *et al.*, 2003; Zhang *et al.*, 2017; Bakker *et al.*, 2020). Recently, this "rhizosphere effect" has been shown to also impact protistan groups resulting in a lower protist diversity and a differentially structured protistan microbiome of the rhizosphere compared to bulk soil (Ceja-Navarro *et al.*, 2021; Fiore-Donno *et al.*, 2022). The multiple microbial communities mutualistically or commensurately colonizing the rhizosphere sustain the plant's fitness and growth (Banerjee & van der Heijden, 2023; Hassani *et al*., 2018).

The rhizosphere, the region of soil surrounding a plant's roots, is influenced not only by the plant itself but also by cropping practices and their impact on the rhizosphere microbiome. Understanding these factors is crucial for understanding the dynamics of the rhizosphere. For example, research has shown that pesticides can reduce the abundance of arbuscular mycorrhiza fungi in soil, leading to reduced phosphorus uptake by plants (Edlinger *et al*., 2022). Organic farming practices, such as crop rotation, companion planting, and the use of organic fertilizers, aim to promote sustainability in crop production (Hole *et al*., 2005). Studies have found that soils managed using organic methods tend to have higher microbial diversity compared to conventionally managed soils (Wittwer *et al*., 2021). This greater diversity may provide a larger pool of beneficial microbes that plants can recruit. In fact, research has demonstrated that management practices can significantly affect the bacterial communities in wheat roots, with clear differences observed between microbial communities in organic and conventionally managed fields (Hartman *et al*., 2018). These findings highlight the influence of cropping practices on the composition of the rhizosphere community.

Arbuscular mycorrhizal fungi and the hyphosphere microbiome

The soil is home to a wide range of microbial groups, including bacteria, fungi, protists, and viruses. Among these, the arbuscular mycorrhizal fungi (AMF) are a particularly interesting group of soil fungi. AMF form symbiosis with 80% of terrestrial plants (Smith & Read, 2010). AMF colonize plant roots, but also form extensive networks of extraradical hyphae that forage in the soil and beyond the rhizosphere. Isotopic studies show that plants support arbuscular mycorrhizal fungi (AMF) with 5-20% of their net carbon (Jakobsen & Rosendahl, 1990; Pearson & Jakobsenf, 1993). In return AMF provide their host plants with a range of nutrients in particular phosphorus (P) and micro-elements (van der Heijden *et al*., 2015). In addition, AMF can provide resistance to biotic and abiotic stresses (Jacott *et al*., 2017).

AM fungal extraradical mycelial also exudates considerable amounts of carbon hydrates to the surrounding soil (Wang *et al*., 2022) and by doing that influence the hyphal microbiome. AMF excrete a range of primary metabolites, including formic acid, acetic acid, glucose, and starch-like compounds (Toljander *et al.*, 2007). These mycorrhizal hyphal exudates can increase bacterial growth and vitality, and they promoted the relative abundance of *Gammaproteobacteria* in the bacterial community. Interestingly, fructose, a primary

metabolite from *Rhizophagus irregularis*, can not only be a carbon source consumed by bacteria but also can stimulate the expression of phosphatase of a phosphate-solubilizing bacterium (Zhang *et al*., 2018a). Since mycorrhizal hyphae are extremely thin and fragile, it is difficult to acquire mycorrhizal exudates *in situ.* The abovementioned exudates were collected *in vitro*.

Using hyphal compartments with soil only accessible to fungal hyphae (but not to plant roots), a range of recent studies investigated the hyphosphere microbiome of AMF. For instance, Zhang *et al*. (2018b) demonstrated that AM hyphal surface was significantly different from the bulk soil. In a similar experimental system, where specific AMF species were inoculated, across AM species and soils, consistent bacterial members including *Betaproteobacteriales*, *Myxococcales*, *Fibrobacterales*, *Cytophagales*, *Chloroflexales,* and *Cellvibrionales* were enriched on the extracted AMF hyphae (Emmett *et al.*, 2021).

Some bacteria that are associated with AMF may directly improve the performance of mycorrhiza. For instance, a range of bacteria including *Pseudomonas, Bacillus, Paenibacillus, Rhodococcus,* and *Streptomyces* have been shown to enhance AMF colonization percentage or help AMF to acquire nutrients. These hyphae-associated bacteria are also termed mycorrhiza helper bacteria (MHB; Frey-Klett *et al*., 2007). The positive effects of MHB can be achieved via the production of growth factors, the detoxification of certain substances, or the inhibition of competitors and antagonists (Frey-Klett *et al.*, 2007). MHB may also stimulate the germination of AM fungal spores by degrading chitin and chitosan of fungal spores (Turrini *et al.*, 2018). MHB can colonize the hyphal surface, but also two groups of intracellular endobacteria have been discovered in AM fungal hosts and both groups are obligate endosymbionts. *Burkholderia*-related endobacteria have been found only in members of the family *Gigasporaceae* (Bonfante *et al.*, 1994; Mondo *et al.*, 2012). *Mycoplasma*-related endobacteria on the other hand are more widely distributed among AM fungal families (Macdonald *et al*., 1982). The presence of *Burkholderia*-related endobacteria enhances host fungal hyphae growth and the absence of *Mycoplasma*-related endobacteria improved fungal hyphae growth suggesting the divergent relationship of the AMF and their endobacteria (Lumini *et al.*, 2007; Torres-Cortés *et al.*, 2015; Desirò *et al.*, 2018).

Bacteria not only promote mycorrhiza colonization but have also been found to play functional roles in plant-mycorrhizal symbiosis in two ways. Firstly, phosphate solubilizing bacteria (PSB) can mobilize nutrients from soil minerals. For instance, co-inoculation of PSBs (an *Enterobacter* sp. or a *Bacillus subtilis*) with AMF increased the phosphorus uptake from rock phosphate to onion (Toro *et al.*, 1997). Secondly, diazotrophs fix atmospheric nitrogen and additively promote plant nutrient uptake with AMF. For instance, a sorghum endobacterium (*Azospirillum brasilense*) co-inoculated with AMF promoted plant dry weight, shoot-to-root ratios, and the nitrogen (N) content of plants (Pacovsky *et al.*, 1985). The co-inoculation of the nitrogen-fixing rhizobia and AMF in a model grassland community resulted in increased plant diversity and improved nutrient uptake (van der Heijden *et al.*, 2016). Except for the bacterial communities which actively interact with AMF, soil protists can also interact with AMF. The

addition of the soil protist *Polysphondylium pallidum* combined with *Paenibacillus* sp. strain to the AMF hyphae significantly increased the organic nitrogen utilization of the mycorrhiza (Rozmoš *et al.*, 2021). However, the ecological roles of the hyphosphere-associated bacteria and protists are still insufficiently explored and so far, no studies assessed which protists colonize AMF hyphae.

The litter microbiome

Dead plant material, originating from leaves and roots, is composed of organic material and acts as a food source for a range of organisms, including bacteria and fungi that can decompose litter. The plant litter can be further decomposed and converted into the soil through a humification and mineralization process that is driven by the microorganisms (Berg & McClaugherty, 2008). Unlike the rhizosphere and hyphosphere forming a relatively stable microbiome, the litter microbiome undergoes a dramatic succession with the litter decomposition process. For instance, in the leaf litter, the predominant *Ascomycota* at the early stage of the decomposition will gradually be substituted by *Basidiomycota* or *Ciliophora* dependent on the litter quality (Purahong *et al.*, 2016; Zhan *et al.*, 2021). In the same study, a dramatic succession in the bacterial community was also seen with *Proteobacteria* dominating throughout the litter decomposition processes.

Evidence has revealed that one of the most important factors that determine litter microbiome succession is the quality of litter (Cleveland *et al.*, 2014). For example, fungi can also decompose recalcitrant organic compounds (van der Wal *et al.*, 2013) while bacteria tend to thrive on labile organic matter (Fierer *et al.*, 2012). Fungi are able to secrete specific enzymes to degrade cellulose, hemicellulose, and lignin and subsequently fungi can decompose recalcitrant organic compounds (Aneja *et al.*, 2006; Kuramae *et al.*, 2013; Purahong *et al.*, 2014). A range of fungal strains including *Fusarium* sp., *Aspergillus* sp., and *Penicillium* sp. were identified as keystone taxa in the litter microbiome in a co-occurrence network (Zheng *et al.*, 2021). The individual fungal strains were subsequently inoculated in a straw medium revealing their strong litter decomposition ability and enzyme actives. Even though bacteria and fungi always co-occur in the litter microbiome and both kingdoms respond to the litter component change during decomposition. Bacteria likely play subsidiary roles in litter decomposition as fungi have the majority of enzymes that can break down the recalcitrant component on plant cell walls (Abdel-Hamid *et al.*, 2013). Subsequently, fungi probably stratify the decomposition process from this litter-soil interface (cell wall). This destruction releases nutrients for microorganisms and plants that affect C storage in the soil (Chapin *et al.*, 2011). Bacteria harbor relatively few enzymes that can degrade organic material. For instance, only minor bacterial hydrolases were detected using a proteomics-based analysis of litter decomposition (Romaní *et al.*, 2006; Schneider *et al.*, 2012). Some researchers postulate that bacteria colonize litter and acquire readily accessible substances degraded by fungi (de Boer *et al*., 2005; Romaní *et al*., 2006). Strains of *Streptomyces*, *Nocardia*, *Rhodococcus,* and *Sphingomonas* have been reported to break down lignin individually (Bugg *et al.*, 2011). The contribution of these bacterial strains in a community context to decomposing natural

component litter is still unclear and very few studies experimentally manipulated the abundance of bacteria and fungi as functional microbial groups. To exclusively investigate the contribution of the bacterial or fungal community to litter decomposition is crucial for better understanding the roles of each microbial kingdom in litter decomposition.

Beneficial effects of microbes on plant nutrient uptake and plant growth

The roles of fungi in promoting plant nutrient uptake and plant growth

Plant associate with a wide range of microbes and microbes play a key role in driving plant nutrient acquisition. Members of *Glomeromycota* forming arbuscular mycorrhizae with terrestrial plants help plants' nutrient uptake. Up to 90% of plant P can be acquired by AMF. Some studies also indicate that AMF can acquire N and results are variable (George *et al.*, 1995). Using labeled isotope Govindarajulu *et al.* (2005) demonstrated that N can be taken up by AMF and transported to its host plant. In monoxenic root organ systems, arbuscular mycorrhiza fungi (AMF) can transfer up to 20-50% of the total root nitrogen from the hyphal compartment to the root when inorganic nitrogen is present (Hodge & Storer, 2015). The N uptake through AMF appeared to be linked with the root development of the host. Generally, the N uptake was increased when root growth was limited to a specific area and AMF could explore a further soil volume inaccessible to plant roots (Hodge & Fitter, 2010). However, the AMF-mediated N uptake diminished when the growth area of roots is not restricted. In some studies, neutral or negative effects were found on plant total N with the presence of AMF (Cui & Caldwell, 1996; Tanaka & Yano, 2005). The positive effects of AMF on plant total N were often found under water-limited conditions, probably due to the fact that N molecules become immobile under these conditions, thus limiting the roots' access to N in the dry substrate (Tobar *et al.*, 1994; Azcón *et al.*, 2008).

Phosphorus (P) exists in the soil in a form of phosphate anions which interact with soil cations and are absorbed by clay minerals (Hinsinger, 2001). The amount of P in the soil that is immediately available to plants is often low (Bieleski, 1973) and P availability can limit plant growth. A wide range of studies have shown that AMF enhance plant P uptake and P-uptake has been seen as the main mechanisms by which AMF support plant growth (van der Heijden *et al.*, 2015). In greenhouse experiments, AMF have been reported to increase plant P uptake of onion, maize, chickpea, and Medicago plants (Hattingh *et al*., 1973; Miransari *et al*., 2009; Hoeksema *et al*., 2010; Farzaneh *et al*., 2011; Püschel *et al*., 2021). AMF specific phosphate transporters – GvPT and GiPT have been identified (Harrison & Buuren, 1995; Maldonado-Mendoza *et al.*, 2001). The gene of these transporters is predominantly expressed in the extraradical fungal mycelium suggesting the AMF participation of phosphate uptake at the fungal-soil interface (Karandashov & Bucher, 2005).

The roles of bacteria in promoting plant nutrients uptake and plant growth

The bacteria that are recruited by plants can benefit the host by providing it with nutrients and promoting plant growth by secreting specific metabolites (Jacoby *et al*., 2020). Possibly the most well-known mechanism by which bacteria facilitate nutrient acquisition in plants is via nitrogen fixation. Here, rhizobacteria associated with plant root nodules of leguminous plants fix atmospheric nitrogen (N_2) into nitrate (NO_3^-) via the action of a nitrogenase enzyme (Peix *et al.*, 2015). The host plant may now absorb the nitrate, and in return, the plant supplies the rhizobacteria with reduced carbon in the form of carbohydrates (Schwember *et al.*, 2019). Bacteria can also promote phosphorous uptake. For example, phosphorous is usually present as inorganic phosphate in the soil, bound to minerals as a metal complex (Varga *et al.*, 2020). Phosphate solubilizing bacteria (PSB) can solubilize this inorganic phosphate complex into soluble ionic phosphate (Pi, HPO_4^{2-}, $H_2PO_4^-$), which can be easily taken up by the plant (Alori *et al.*, 2017). Phosphate can also exist as organic phosphorous, which must also first be mineralized by bacteria before uptake into the plant (Alori *et al.*, 2017). These processes may either occur in the root itself, by bacteria colonizing the root (Varga *et al*., 2020), or in the soil. Bacteria also promote plant growth by secreting secondary metabolites including phytohormones, siderophores, etc. The phytohormone of indole-3-acetic acid (IAA) is widespread among bacteria that inhibit the rhizosphere (Patten & Glick, 1996). The IAA promotes root length and area surface, increasing the nutrient uptake capacity of the roots (Ali *et al.*, 2010). The other bacterial phytohormone – gibberellin promotes plant stem elongation and seed germination (Cassán *et al.*, 2009).

Microbial interactions benefit plant growth

Interaction between different microbes can vary widely and include positive, negative, and neutral interactions (Hassani *et al.*, 2018; Thoms *et al.*, 2021). For example, co-inoculation of bacterial, fungal, and oomycetes communities showed the highest Arabidopsis biomass, higher than the individual microbial community members (Durán *et al.*, 2018). In the same experiment, bacterial co-inoculation with fungi rescued the plants' survival compared to plants only inoculated with fungi. Interestingly, negative correlations between bacteria and fungi were dominant in microbial co-occurrence networks suggesting that the competitive interactions are critical for keeping plant-microbial homeostasis and such interactions can promote healthy plant growth. Cooperative interactions were also recognized to benefit plant growth. By manipulating microbial richness in soil, researchers found that higher richness and multi-kingdom microbial communities present more diverse functions to support plant growth than lower microbial richness or mono-kingdom microbial communities (Wagg *et al.*, 2014, 2019).

Thesis outline

Throughout this thesis, I used *Prunella vulgaris* (Prunella) as the experimental model plant. Prunella has been regularly used as a model plant in ecological and evolutionary research (Winn, 1988; Miller *et al*., 1994; Streitwolf-Engel *et al.*, 2001; Qu & Widrlechner, 2011). This plant is small in size and easy to manipulate in a small microcosm. (Chapter 2). Moreover,

Prunella is highly responsive to AMF and as such also suitable model plant for studying plant-mycorrhiza-microbe interactions (Chapter 3 & 4).

Diverse microbial communities colonize soil and plant roots, and the first pieces of evidence suggest that these microbes, as has been shown for fungal hyphae may also themselves be colonized by other microbes (Artursson *et al*., 2006). Despite this, the ecological roles of most microbial communities and individual microbes are not well understood, with most research focusing on bacteria. This thesis focuses on bacteria-fungi interactions and their role in influencing plant growth, litter decomposition, and mycorrhiza functioning. It presents new insights into the role of bacteria and fungi in plant growth and litter decomposition, including the discovery that the selected fungi are the main drivers of litter decomposition and that there are synergistic effects of bacteria and fungi in stimulating plant growth under nutrient poor conditions.

In **Chapter 2**, we isolated bacteria and fungi from plant roots that were grown in agricultural field soil from the Farming Systems and Tillage Experiment (FAST; Wittwer *et al*., 2021). Subsequently, the ecological function of bacteria and fungi was investigated, and it was tested whether bacteria and fungi have synergistic effects on plant growth and litter decomposition. Although many researchers provide evidence for complementarity among bacteria and fungi, experimental evidence supporting this claim is weak and few studies jointly manipulated bacteria and fungi to assess their effects upon plant growth and specific ecosystem functions (e.g., here litter decomposition). By inoculating 41 bacteria and 35 fungi into microcosms, I examined whether bacteria and fungi provide different services to litter decomposition and plant growth (Chapter 2; Fig. 2a). Subsequently, I characterized the root and litter microbiome by amplicon sequencing. By doing this, I aimed to understand whether there are differences between the reconstructed microbial communities on root and litter. And my goal is to find the specific microbes that are abundant in these habitats that may imply important roles of these abundant microbes in the ecological functions.

In the next set of experiments that together comprise **Chapter 3** (Fig. 2b), fungal-bacterial interactions are explored on AMF hyphae extending from Prunella roots. I specifically examined the microbial communities associated with fungal hyphae with special attention to bacteria and protists. I found that specific bacterial groups were consistently enriched on fungal hyphae implying the functional importance of these bacteria to plant-mycorrhiza symbionts. I also examined the effect of different agricultural soil management practices (e.g., AMF occurring in organically versus conventionally managed soils) on AMF and their associated microbiomes. To do this, I compared microbial communities associated with hyphae to those associated with plant roots and bulk soil and examined how management practices influenced the divergence of hyphal, root, and soil microbiomes.

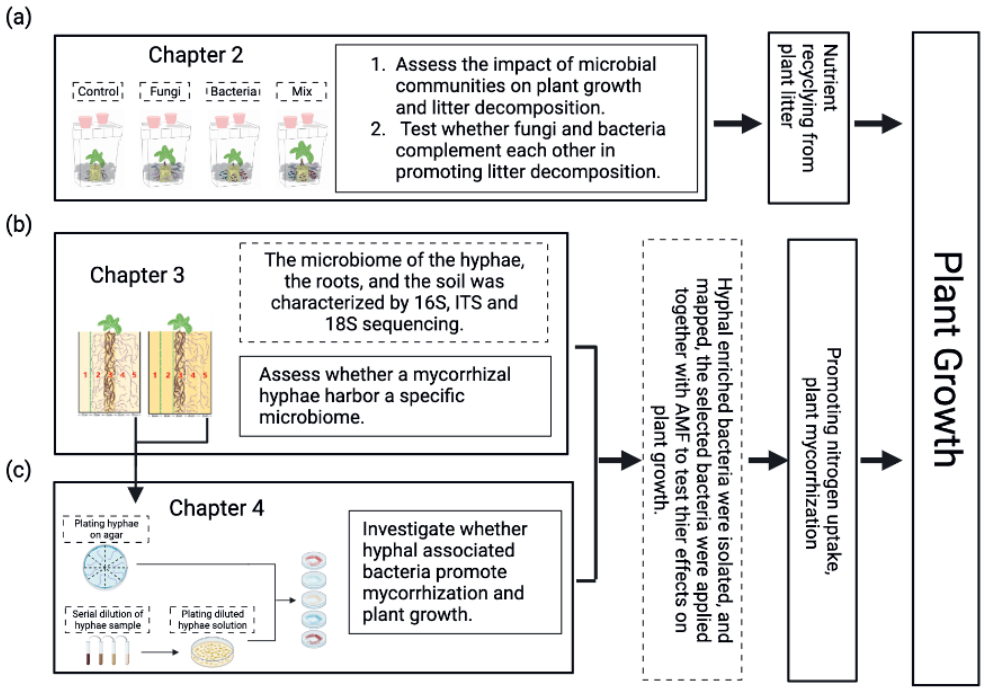

Fig. 2. Schematic overview of the dissertation structure. The approach and main research question are summarized for (a) chapter 2, (b) chapter 3, and (c) chapter 4. The approach is shown in the dash line frames, whereas the research questions are described in the solid line frames.

In **Chapter 4** (Fig. 2c), I isolated and built a collection of bacteria that were adhering to extraradical hyphae. We characterized this collection and were able to identify 5 bacteria that matched with the sequencing data of chapter 3 that were significantly enriched in hyphal samples. I then examined how these mycorrhizal hyphae-associated bacteria impacted plant growth, mycorrhization, and nutrient acquisition. In this way, we identified one bacterium that benefits plant-mycorrhiza growth by mediating plant N uptake. We also sequenced the genome of this hyphae-associated bacteria and searched for specific functional genes (e.g., nitrogen-fixing genes) to identify potential mechanisms for promoting symbiosis growth.

Finally, in **Chapter 5** we discussed the results of this thesis in light of the composition of microbial communities of litter and mycorrhizal microbiome, and the functional roles of these microbiomes in litter decomposition, mycorrhization, and plant growth. In addition, further research directions are proposed, and remaining research questions are discussed.

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Chapter 2

The microbial contribution to litter decomposition and plant growth

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Abstract

Soil and plant roots are colonized by highly complex and diverse communities of microbes. In contrast to knowledge on the composition of microbial communities colonizing a wide range of habitats, the ecological function of many microbes is still poorly understood. It is also unclear to what extent the microbial kingdoms that are most abundantly represented on earth – Bacteria and Fungi – interact and influence important ecosystem functions such as litter decomposition or plant growth. It has been proposed that bacteria and fungi have synergistic effects on litter decomposition, but experimental evidence supporting this claim is weak. In this study, we manipulated the composition of two microbial kingdoms (Bacteria and Fungi) in experimental microcosms. The microcosms were inoculated with synthetic communities of either bacteria (41 strains), fungi (35 strains), or a mixture of bacteria and fungi together, and the effects of these treatments on plant growth and litter decomposition were subsequently compared to control microcosms that were not inoculated. In microcosms that were inoculated with fungi, litter loss was 47% higher than in microcosms that were not inoculated or only inoculated with bacteria. In comparison, inoculation with the bacterial only treatment slightly (9.5%) enhanced decomposition compared to the control treatment. Combined inoculation with both bacteria and fungi did not significantly enhance decomposition and, as such, we found no evidence for complementary effects. Inoculation with fungi also had a positive impact on plant growth after 4 and 8 weeks (480% and 710% growth stimulation respectively), while inoculation with the isolated bacteria did not influence plant growth. After 16 weeks, plant biomass was highest in microcosms where both bacteria and fungi were present. Overall, this study suggests that fungi are the main decomposers of plant litter and that the inoculated fungi contribute to plant growth when microbial abundance is low. Further experimental studies with a wider range of microbes from different microbial kingdoms are necessary to better understand how microbial communities interact and influence plant growth, litter decomposition and ecosystem functioning.

Keywords, litter decomposition, bacteria, fungi, synthetic communities

Introduction

Soil microbes are highly abundant and represent the ‘unseen majority’ on earth, providing one of the largest pools of genetic diversity (Bardgett & van der Putten, 2014; Roesch *et al.*, 2007; Whitman *et al.*, 1998). Moreover, soil communities are fundamental for maintaining important ecosystem processes (Banerjee & van der Heijden, 2022; Wagg *et al*., 2014; Wagg *et al.*, 2019). Bacteria and fungi are dominant members of soil microbial communities, interacting not only with one another, but also with plant roots, as they share the same habitats. These multi-kingdom interactions vary, and synergistic effects on plant growth and health have been repeatedly observed (Etesami *et al.*, 2021; van der Heijden *et al.*, 2015).

A wide range of studies have analyzed the composition and diversity of microbes colonizing the soil and inhabiting plant roots (Lundberg *et al.* 2012; Gaiero *et al.* 2013; Lareen *et al*., 2016;

Fierer 2017; Fitzpatrick *et al.* 2018). While much progress has been made to catalogue such microbial communities, much less is known about the actual functions of individual microbes and microbial communities. Some groups of microbes have been widely investigated (e.g., nitrogen fixing rhizobia bacteria, plant immunity triggering bacteria, a wide range of mycorrhizal fungi, and microbial pathogens) (Garrido-Oter *et al.*, 2018; Pieterse *et al.*, 2021; van Der Heijden *et al.*, 2015; Xin & He, 2013), but the function of the majority of microbes, including a wide range of rhizosphere-inhabiting microbes is still poorly understood. Here, we focus on microbes isolated from *Trifolium* roots (excluding well-known nitrogen fixing bacteria and mycorrhizal fungi), and we test the impact of these bacteria and fungi on litter decomposition and plant growth.

Although a range of studies linked the decomposition of plant litter to the bacterial and fungal communities that colonize litter (Purahong *et al.* 2016; Zheng *et al.* 2021; Mei *et al.* 2022) the relative contribution of bacteria, fungi and their interactions to litter composition are poorly understood. Only very few studies have experimentally manipulated the presence and abundance of bacteria and fungi to assess their roles in litter decomposition. Fungi exude a range of extracellular enzymes (Romaní *et al.*, 2006; Schneider *et al.*, 2010, 2012), and, based on metaproteomics, it was proposed that fungi contribute much more to decomposition and C loss than bacteria (Chen *et al.* 2021; Pascoal & Cássio 2004). This implies that fungi are the main drivers of litter decomposition. However, bacteria do appear to influence the litter decomposition process. Some studies suggest that bacteria complement fungi when decomposing litter (Güsewell & Gessner 2009; Zhao *et al*., 2021), and that certain bacteria contribute to the production of extracellular degrading enzymes in the later stages of decomposition (Kirby 2005). For instance, *Betaproteobacteria* and *Dothideomycetes* showed higher litter degradation capability in larch litter (Sauvadet *et al*., 2019). Moreover, based on network analyses, the bacteria from the genus *Chryseobacterium* have been identified as one of the keystone taxa in litter decomposition processes (Zheng *et al.* 2021). In contrast, other studies found much lower litter degradation activities in bacterial communities (Pascoal & Cássio, 2004; Schneider *et al.*, 2010, 2012).

In order to further investigate the relative contributions of fungi and bacteria to the litter decomposition processes, experimental microcosms filled with sterilized soil, plant litter (leaves from the grass species *Lolium multiflorum*) and planted with the herb *Prunella vulgaris* were inoculated with either a synthetic community of 1) bacteria (41 strains), 2) fungi (35 strains), 3) bacteria and fungi together, or 4) a negative control that did not receive an inoculum. These bacteria and fungi were isolated from the roots of *Trifolium pratense* collected from the long-term Farming Systems and Tillage (FAST) experiment (Wittwer *et al*., 2017; Wittwer *et al.* 2021). An earlier study demonstrated that many of these microbes are abundant in the arable and grassland soil and actively colonize plant roots at this location (Hartman *et al.*, 2017, 2018). The effects of these treatments on plant growth and litter decomposition were assessed every 4 weeks for 16 weeks, and we subsequently used amplicon sequencing to verify which bacterial and fungal taxa established and colonized the plant litter and plant roots.

Materials and methods

Microcosm construction and preparation

Magenta GA-7 boxes were used as experimental microcosms and modified after Hartman *et al.* (2017). The lids of the boxes contained 2 holes (⌀ 1.5 cm) and were sealed with gas-permeable foil for air exchange. The boxes were filled with 90 g calcined clay, marketed as Oil-Dri (Damolin GmbH, Oberhausen, Germany). Two litterbags, each measuring 3 x 4 cm and made of mesh with a 30 µm pore size that can prevent plant roots from accessing the litter, were buried in the substrate in a back-to-back position within the magenta boxes. Each litterbag contained 0.3 g of dried *L. multiflorium* litter. During autoclaving and for short term storage, the magenta boxes (covered with aluminum foil) and lids were placed inside two autoclavable bags, thus providing a double layer of protection and preventing accidental contamination in case one bag was later damaged during the experimental setup. The microcosms were filled with substrate and litterbags, and together with the lids were autoclaved twice for 99 min at 121°C. We plated autoclaved soil substrate onto agar plates and confirmed that the autoclaving protocol successfully deactivated all microbes.

Seed germination for planting

P. vulgaris has been widely used as a model plant in ecological and evolutionary research (Winn 1988; Winn & Gross 1993; Miller & Alice 1994; Streitwolf-Engel *et al*., 2001; Qu & Widrlechner 2011), and its small size fits well for gnotobiotic system construction and manipulation in small microcosms. *P. vulgaris* seeds were surface sterilized for 5 minutes in 70% EtOH, followed by 5 minutes in 5% NaClO, and rinsed 3 times with sterile distilled water. The seeds were sown on 1/2 Murashige and Skoog basal medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 1% sucrose. A maximum of 10 seeds were sown on one plate to prevent cross contamination. After 2 days of stratification at 4°C, the plates were transferred to a climate chamber (Sanyo MLR-352H; Panasonic, Osaka, Japan) under controlled conditions (25°C 16 h, 16°C 8 h). Seedlings with roots of approximately ~0.5 cm in length that were free of visible contamination, but potentially containing endophytes, were selected for planting in the microcosms.

Microbial community creation

We made use of a previously published bacteria collection isolated from naturally collected and climate chamber cultivated roots of *T. pratense* (Hartman *et al.*, 2017). Fungal isolates were isolated from *T. pratense* root fragments describing in the supplementary method. The isolates were sequenced using the primer pair 27F (Lane 1991) and 1401R (Nübel *et al.* 1996) for bacteria and the primer pair ITS5 and ITS4 (White, Bruns, Lee & Taylor 1990) for fungi. The microbial isolates were clustered into operational taxonomic units (OTU) at > 97% sequence similarity and a total of 41 bacterial and 35 fungal OTUs were detected (e.g., for most OTUs several strains were detected). For each OTU, we randomly selected one bacterial or one fungal strain for inoculation of the microcosms (Table S1).

Preparation of the microbial treatment inocula

Four microbial community treatments (*Control*, *Bacteria*, *Fungi*, and *Mix*) were used in our study. We inoculated pure cultures of 41 bacterial strains in the Bacteria (only) treatment. The Fungi (only) treatment received pure cultures of 35 fungal strains. The Mix treatment was inoculated with 41 bacteria and 35 fungi. The Control treatment was not inoculated with any microbes and received sterilized agar plugs to standardize all treatments and to ensure that the addition of microbial inocula did not influence soil nutrient availability.

The selected bacteria and fungi were revived from glycerol stocks stored at -80ºC by plating on Flour Medium agar (FMA; Coombs & Franco, 2003) and Mathur's Medium agar (MMA; Freeman & Katan, 1997), respectively. The bacteria plates were cultured at 28℃ for one to two weeks. The fast growers were stored in 4℃ and subsequently, the fast and slow growers were subcultured at the same period. We scraped off the bacteria colonies of each strain and mixed with 100 µl sterile distilled water. Subsequently, 100 µl of each bacteria suspension was pipetted onto an FMA plate, and the mixture was spread around the plate with a flamed glass spreader. The plates were incubated at 28℃ for up to 2 weeks, or until bacteria colonies had covered the entire plate.

The fungi plates were cultured at 26℃ for one to two weeks to ensure enough growing time for slower growing fungi. Fungi were sub-cultured by taking agar plugs (ø 5 mm) from each strain and transferring it to a new MMA plate. The sub-cultured plates were incubated at 26℃ for up to two weeks, or until fungi hyphae covered the entire plate. Faster growing isolates were stored at 4℃ until use. Three replicate plates per bacterial and fungal isolate were plated to ensure enough biomass for inoculum creation.

The microbial inoculum for each microcosm was created independently. One agar plug (5 mm ø) of each strain was added into a sterile 50 ml Falcon tube for each microcosm. Therefore, 41 bacteria plugs were added in per tube in the bacteria treatment. In addition, the bacteria treatment received 35 sterile MMA plugs to ensure equal nutrient additions across all treatments. Similarly, the inoculum for the fungi treatment included 35 fungi plugs and 41 sterile FMA plugs for the nutrient adjustment. The mix treatment contained 35 bacteria plugs and 41 fungi plugs, and each control treatment microcosm was inoculated with 35 sterile FMA plugs and 41 sterile MMA plugs.

Subsequently, 20 ml of sterile 15% Hoagland solution (Table S2) was added to the tube and the contents were blended with a sterile laboratory blender (Polytron, Kinematica, Lucerne, Switzerland; setting 3 for 30 seconds). The head of the blender was surface sterilized by submersing in 70% ethanol for 10 min, then in 5% sodium hypochlorite for 20 minutes. The head of the blender was then rinsed 3 times with sterile distilled water. The blender was surface sterilized between inoculum preparation of the different treatments to prevent cross contamination. The efficiency of the surface sterilization procedure was verified by plating 100 µl of the water used for rinsing the blender on FMA and MMA and checking for microbial

growth. After blending the plugs and Hoagland solution mixture into a slurry, the slurry volume in each tube was adjusted to 45 ml with 15% Hoagland solution to create the inoculum for each microcosm.

Microcosm assembly

The inoculation of the microcosms was performed in a sterile laminar flow cabinet. 45 ml of inoculum was poured evenly over the surface of the substrate in the microcosm, followed by another 45 ml of sterile 15% Hoagland solution to ensure enough water and nutrients for plant growth. Two pre-germinated seedlings were sown in the substrate with a sterile spatula. The microcosms were closed with the lids and then sealed with parafilm. The microcosms were randomly distributed across the shelves of the climate chamber (25℃, 16 h light, 16℃, 8 h dark; 70% relative humidity). Every week, the microcosms were randomly reallocated to new positions in the climate chamber to minimize any effects of environmental variability.

Harvest

A total of 96 microcosms were set up, and microcosms were harvested after 4, 8, 12 and 16 weeks (4 treatments * 4 timepoints * 6 replicates = 96 microcosms). Harvesting was performed in a sterile laminar flow cabinet. Above-ground plant biomass was cut using a sterile scalpel, dried in paper bags for 48 h at 60℃, and weighed. The plant roots that loosely attached to Oil-Dri were shaken gently and collected using sterile tweezers, placed into 50 ml tubes, and immediately frozen at -20℃. The litterbags were removed by sterile tweezers. One litterbag from each microcosm was rinsed with distilled water to remove substrate particles, dried in paper envelopes at 60ºC for 48 hours and weighed. The other litterbag from each microcosm was placed in a sterile 50 ml tube and stored at -20℃. The remaining growth substrate was collected in a 50 ml tube and stored at -20℃.

Quantification of active microorganisms in microcosms by serial dilution

At the 8^{th} week and 16^{th} week harvests, 1 g of substrate was sampled from each microcosm and serially diluted on FMA and MMA plates to quantify the active bacteria and fungi, respectively. The substrate was mixed with sterile 0.9% saline water, vortexed for 1 min, and serially diluted to 10^{-6}. For each microcosm, 50 µl of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was spread on FMA and MMA plates separately. The colony forming units (CFU) were calculated after 3 days until 7 days.

Litter and root microbiome profiling

Because our root sampling method did not discriminate between the rhizoplane (root surface) or the endosphere (root interior) compartments, we refer generally to the sampled unit as “root microbiome”. After 8 weeks, the litter and root microbiomes were characterized by conducting 16S rRNA gene and ITS amplicon sequencing. Litter and root samples were lyophilized for 48 h. DNA was extracted from litter and root samples using the NucleoSpin Soil DNA extraction kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the

manufacturer's instructions. Extracted DNA was quantified using Qubit® (1.0) Fluorometer and the Tapestation (Agilent Technologies, Santa Clara, CA USA).

16S and ITS PCR and library preparation

We amplified the V3 and V4 region of the 16S rRNA gene using PCR primers 341F and 806R (Takahashi *et al*., 2014), targeting a single amplicon of approximately ~460 bp. The concentration of DNA samples was diluted to 5 ng/µl and used in a two-step PCR amplification protocol. The first PCR reaction was processed on a thermocycler (Hybaid, Ashford, UK) using the KAPA HiFi HotStart ReadyMix (F. Hoffmann-La Roche AG, Basel, Switzerland) PCR system with the cycling conditions in Table S3. Each sample was amplified in a 15 µl reaction volume containing 1.5 µl DNA template, 7.5 µl KAPA, 1.5 µl of 2 µM concentrated forward and reverse primers, 1.5 µl 2.5 µM pPNA primer and 1.5 µl 2.5 µM mPNA primer (Lundberg *et al.*, 2013). The primers were adapted with a 0-7 base heterogeneity spacer to enhance sequence diversity (Wu *et al.* 2015). The resulting PCR products were purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK) according to the manufacturer's instructions. The purified PCR products were then used as template DNA in the second PCR (Table S3). Each sample was amplified in a 25 µl reaction volume containing 2.5 µl DNA template, 12.5 µl KAPA, 2.5 µl 2 µM forward and reverse primers, and 5 µl MilliQ-purified water. The primers were adapted with an error-tolerant 6-mer barcode to allow pooling of the multiplexed PCR products. The resulting PCR products were then cleaned up using AMPure XP beads. Afterwards, we loaded 5 µl of each sample on an agarose gel to check for correct amplicon size and used a Qubit® (1.0) Fluorometer to quantify the DNA concentration in each sample. Each library of 5 µl 4 nM DNA was pooled together.

For ITS amplicon library preparation, we targeted the ITS1 region yielding a ~300 bp amplicon using primers ITS1F (Gardes & Bruns 1993) and ITS2 (Op De Beeck *et al.* 2014). We prepared the ITS library following the same protocol as for the 16S rRNA gene amplification. In short, the diluted 5 ng/µl DNA was first amplified in a 15 µl reaction volume containing 2.5 µl 1 µM forward and reverse primer, 7.5 µl KAPA, and 10 ng DNA template. The PCR products were purified using AMPure XP beads and the resulting DNA was used as template in the second PCR using the same conditions for the 16S mentioned above. Both PCR cycling conditions are shown in Table S3. The PCR products were cleaned up with AMPure XP beads and DNA concentration was quantified by Qubit® (1.0) Fluorometer. Equal PCR product amounts (5 µl 4 nM) were pooled together. The 16S library and ITS library were mixed together and sequenced on Illumina MiSeq Sequencer (Illumina, San Diego, USA) using a paired-end 300 bp V3 kit at Utrecht Sequencing Facility (www.useq.nl).

Sequence data processing

We employed the Qiime2 environment (version 2019.07, https://qiime2.org/) for sequence processing. The quality of the paired-end sequences was assessed using the *demux* plugin. Primers of imported sequences were removed via Cutadapt (Martin 2011). The paired-end sequences were merged using vsearch join-pairs script, allowing the joining of staggered read

pairs to retain as many sequences as possible (Rognes *et al*., 2016). Deblur (Amir *et al.* 2017) was used to filter, denoise sequences, trim sequences to a common length (16S: 269 bp, ITS: 200 bp), and remove chimeras. Sequences were then classified into actual sequence variants (ASVs). 16S and ITS ASVs were taxonomically annotated using a pre-trained naive Bayes classifier (Werner *et al.* 2012) against the Greengenes reference database (release 13_5, 99% OTUs) (McDonald *et al.* 2012) and the UNITE (v8, 04.02.2020, 99% OTUs) (Kõljalg *et al.* 2013) databases, respectively. From this taxonomic assignment, 16S ASVs annotated as mitochondria and chloroplast were removed. The filtered sequences were subsequently clustered to OTUs at 97% sequence similarity. The denoised sequences of the bacterial community were then rarefied to 1000 sequences per sample (Figure S2). To preserve the low sequence depth of fungal community in control and bacteria treatments, we did not rarefy the fungal sequence. The raw sequencing data were deposited at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) by the study accession PRJEB54741.

Rediscovery of inoculated strains in the microcosms

To identify which of the of the inoculated bacterial and fungal strains established in the microcosms, we mapped the sequences of the inoculated microbes to corresponding OTU sequences of the community profiling. The 41 bacterial sequences and 35 fungal sequences were aligned and trimmed based on their 16S rRNA v3-v4 region and ITS1 region using ClustalW (Thompson, Gibson & Higgins 2003) in MEGA X (Sudhir *et al*., 2018), respectively. The trimmed sequences were imported to Qiime2 and used as query sequences to map with clustered OTUs using the 'quality-control exclude-seqs' script (Camacho *et al.* 2009) at > 97% sequence similarity (Table S4, S5).

Statistical analyses

All statistical analyses were conducted in R version 4.0.2 (R Foundation for Statistical Computing & Austria. 2020). Differences in community composition between the bacterial and fungal communities in the different microbial treatment and sample types were tested by pairwise permutational analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities using the *adonis* function in the *vegan* package (Jari *et al.* 2019) with 999 permutations. Plant productivity was assessed for variation among treatments by ANOVA and followed by a Tukey HSD test. Two-way repeated measures ANOVA were used to test the effect of microbial treatment on litter decomposition over time. All bioinformatic files generated by qiime2 were imported to R by qiime2R package (Jordan 2018). The bacterial and fungal OTUs were rarefied from 0 to 20,000 (bacterial) and from 0 to 60,000 (fungal) sequences per sample and the observed OTUs were plotted at each rarefaction level using phyloseq package (McMurdie & Holmes 2013) and ggplot2 (Wickham 2016). The OTUs that positively associated to one or a combination of microbial treatments were determined by a correlation-based indicator species analysis with the R package *indicspecies* (De Cáceres & Legendre 2009). The observed OTUs were calculated in qiime2 by the diversity core-metrics-phylogenetic script, and the differences across microbial treatments and sample types were determined by Two-way ANOVA in R.

The observed OTUs' variation within treatments and sample type were determined by ANOVA and t-test respectively.

Results

In this study, we assessed the effects of the microbial treatments on plant growth and litter decomposition every 4 weeks for 16 weeks and subsequently, we used amplicon sequencing to characterize the plant litter and root microbiome profiling.

Fungi as main decomposers

To assess the relative contribution of bacteria and fungi to litter decomposition, we investigated litter loss in litter-filled mesocosm inoculated with bacteria (treatment "Bacteria"), fungi (treatment "Fungi"), a mix of bacteria and fungi (treatment "Mix") and a control treatment without the addition of bacteria and fungi (treatment "Control"). At each time point, litter loss in the two treatments with fungi (Fungi and Mix) was significantly higher than treatments without inoculated fungi (Figure 1). Litter loss in the Bacteria treatment did not differ from the Control, and litter loss in the Fungi treatment did also not differ from the Mix. This result indicates that fungi were the main decomposers in the microcosms. A two-way repeated measures ANOVA test (Table S6) showed that litter loss increased with time in the Bacteria and Mix compared to the Control treatment, indicating that bacteria do have some influence on decomposition over time. Litter loss in the Bacteria and Mix treatments increased from week 8 to week 12 (Table S6), perhaps suggesting that bacteria may contribute to litter decomposition at a specific stage. The Mix treatment showed a significantly stronger litter loss rate compared to other treatments (Figure. S1), suggesting that bacteria and fungi interactions have dynamic effects on litter decomposition.

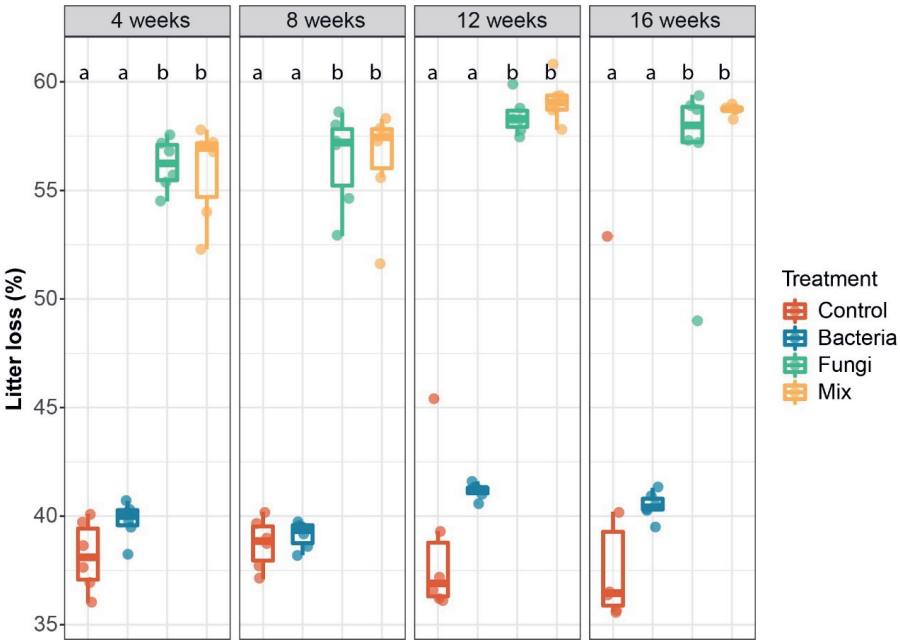

Figure 1. Percentage litter loss across microbial treatments with time. Significance differences are represented by letters ($p < 0.05$, one-way ANOVA and Tukey HSD test).

A mixture of bacterial and fungal communities enhances plant growth

The effects of inoculation on plant biomass varied with time (Figure 2, Table S6). At the 4- and 8-week harvest, inoculation of fungi had significantly enhanced plant biomass compared to the Control or the treatment where only bacteria were inoculated. However, the positive effect of fungal inoculation on plant biomass was no longer observed after 12 and 16 weeks. Interestingly, co-inoculation of bacteria and fungi resulted in the highest biomass at the final harvest. Additionally, differences in plant biomass between microbial treatments diminished over time. At 16 weeks, the Bacteria and Fungi treatments had a similar plant biomass, while biomass in the Mix treatment was significantly higher than the Control treatment.

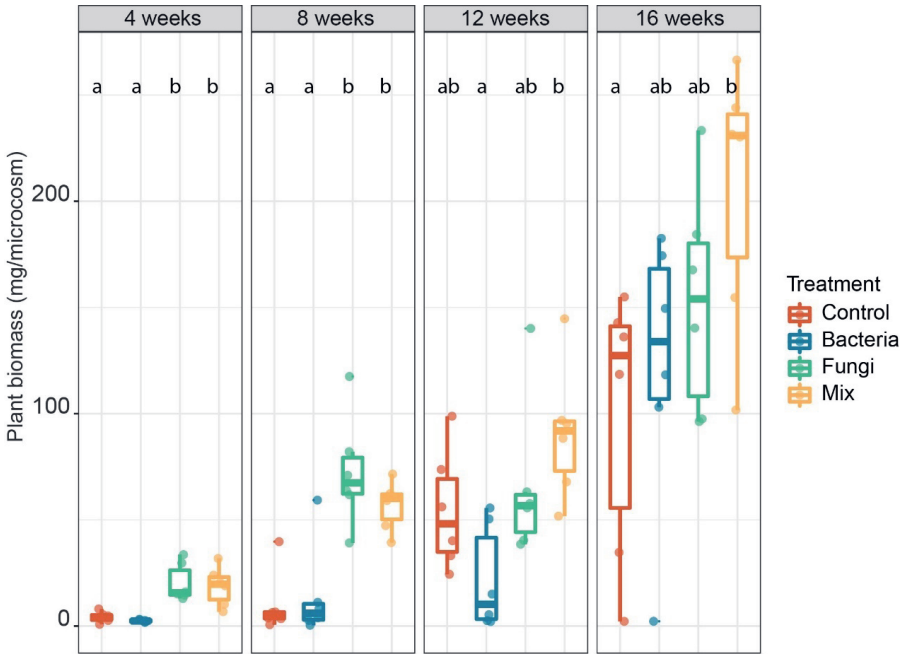

Figure 2. Plant biomass response to microbial treatments. Significance differences are represented by letters ($p < 0.05$, one-way ANOVA and Tukey HSD test).

More active bacteria and fungi detected in microbe inoculated treatments

Autoclaved soil was plated on agar plates after one week to confirm that autoclaving had successfully sterilized the microcosm system. We did not detect the growth of microbes on these agar plates. The results suggested that the autoclaved substrate was completely free of microbes. To determine whether active microbes survived in the microcosms after 8 and 16 weeks of plant growth, we plated serial dilutions of subsamples of the substrate on agar-solidified medium and counted the colony forming units (CFUs; Figure 3). The abundance of bacterial CFUs was significantly higher (on average 5.1 ~ 5.7 times) in treatments inoculated with bacteria (Bacteria and Mix) compared to treatments not inoculated with bacteria (Control and Fungi). However, we also noted bacterial CFUs in the Control and Fungi treatments, indicating some bacterial contamination (e.g., from plant endophytes or introduced during the experiment) had occurred during the experiment. Fungal CFU counts were significantly higher in fungi inoculated treatments (Fungi and Mix) than in the non-fungi inoculated treatments (Control and Bacteria). Overall, fungal CFU counts in Control and Bacteria were below detection limits, except for two replicates in the Control (Figure 3b).

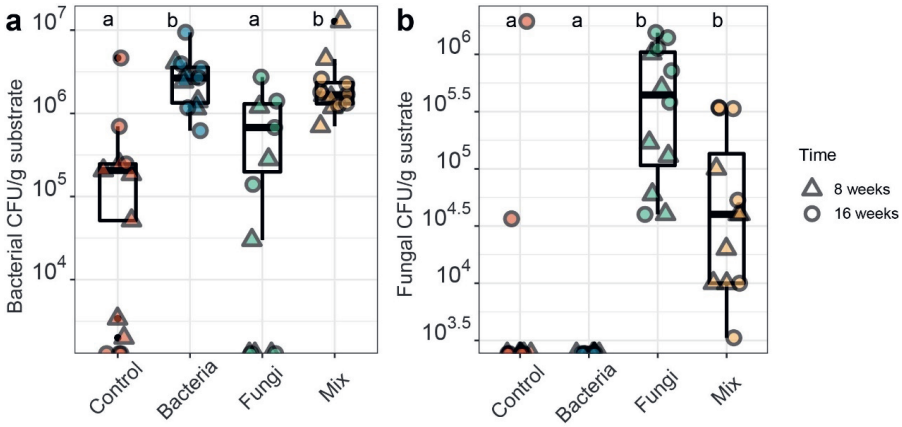

Figure. 3. Higher CFU numbers present in bacteria and fungi inoculated treatments. (a) The bacterial CFUs compared among microbial treatments. **(b)** The fungal CFUs compared among microbial treatments. Harvest time points are indicated by different symbols. The treatments are indicated by colors. The Kruskal Wallis test and the Dunn's post-hoc test ($p < 0.05$, Table S7) were performed to determine the significant differences between microbial treatments that is indicated by different letters in the boxplots.

Bacteria and fungi inoculated treatments forming specific rhizosphere and litter consortium

We employed 16S rRNA gene and ITS amplicon sequencing to profile the diversity and community composition of bacterial and fungal communities colonizing litter and root samples and to verify which inoculated bacteria and fungi established in the microcosms. After quality filtering of raw sequences reads, we obtained 280,925 bacterial and 889,376 fungal sequences.

Bacterial inoculation significantly increased bacterial OTU (bOTU) richness, which was on average 2.3 ~ 2.6 times higher in the Bacteria and Mix treatments compared to the Control or the Fungi (Figure 4; Table S8). In the Bacteria treatments, bacterial richness of the root samples was significantly higher (11.6%) than in the litter samples (Figure S3). Similarly, OTU richness in fungal inoculated treatments was higher compared to the Control or Bacteria. We detected very few fungal sequences in non-fungal (Control and Bacteria) microcosms (Figure S2b), corroborating the serial-dilution results in which fungal CFUs were lower than the detection limits in the large majority of microcosms where no fungi were inoculated (Figure 1b). Fungal OTU (fOTU) richness did not differ between the Fungi and Mix (Figure 4b) and was generally higher in root samples than in the litter samples (Figure S3, Table S9).

For a more in-depth analysis of differences in the structure of the bacterial and fungal communities in the different treatments, principal coordinate analysis (PCoA) and pairwise PERMANOVA on Bray-Curtis dissimilarities were performed. We noted clear separation between microbe-inoculated treatments and non-microbe-inoculated treatments in the ordination space (Figure 4c & d) and pairwise PERMANOVA testing confirmed the significant differences between microbial treatments (Table S10 & S11). Within microbe-inoculated

treatments, sample type appeared to drive differences in community composition, as we noted root and litter samples separated from each other on axis 1 (Fig. S4 & S5).

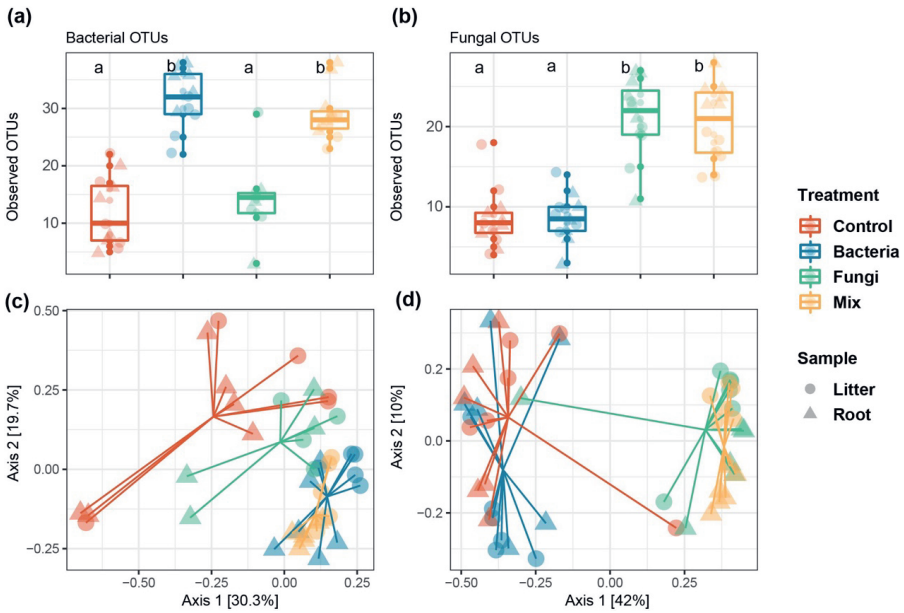

Figure. 4 Microbial community diversity across treatments. (a) Bacterial OTU richness across all treatments. Significance differences between treatments are indicated with letters ($p < 0.05$, ANOVA and Tukey's Honest HSD test). **(b)** Fungal OTU richness across all treatments ($p < 0.05$, ANOVA and Tukey's Honest HSD). **(c)** The bacterial community PCoA based on Bray-Curtis distances. **(d)** The fungal community (PCoA) based on Bray-Curtis distances. Samples are color coded by treatments. The sample types are indicated by different symbols.

Rediscovering fungal and bacterial inoculates in the microbial communities

In a next step, we determined which of the inoculated fungal and bacterial taxa established and could be detected in the microcosms. For this, the sequences of the 35 inoculated fungi were mapped to the representative OTU sequences of the fungal community profiles at 97% sequence similarity. Similarly, the 41 sequences of the inoculated bacteria were mapped to the bOTU sequences (Figure 5). In all root and litter samples, we detected 51 bOTUs and 32 fOTUs (Table S4, S5). The 35 inoculated fungal sequences matched to 26 fOTUs in the community profile. Thus, 74% of the inoculated fungi established and could be rediscovered. The most abundant fOTUs belonged to the phylum *Ascomycota* (24 fOTUs). One fOTU belonged to the Basidiomycota, and one fOTU belonged to *Mucoromycota*. These taxa were nearly exclusively present in the Fungi inoculated treatments (Fungi and Mix) (Figure 5). In litter samples, 10 fOTUs were abundant in both fungi inoculated treatments (Figure 5a). In the root samples, more fungal taxa were detected, with 15 fOTUs enriched in fungi inoculated treatments (Figure 5a).

In the bacterial community, 28 out of the 41 inoculated bacteria were rediscovered in the community profiles. The 28 bacterial sequences matched to 27 bOTUs. 18 bOTUs were significantly more relatively abundant in the inoculated treatments compared to the control treatment, and this was especially clear for the root microbiome (bOTUs in red dashed frame in Fig. 5b). In the litter samples, we noted that one inoculated *Bradyrhizobiaceae* (bOTU 17) was abundant in both bacteria inoculated treatments. However, we also observed four inoculated bOTUs (bOTU 38, 33, 28, 44) that were present in low abundance in all bacterial inoculated treatments. 14 bOTUs (bOTUs in blue dashed frame in Fig. 5b) were generally found in all treatments. These bOTUs belonged to *Proteobacteria* (11 bOTUs), *Bacteroidetes* (2 bOTUs) and *Actinobacteria* (1 bOTU). Four bOTUs belonging to the *Proteobacteria* (bOTU15, bOTU11, bOTU10, bOTU2, Figure 5b) were also abundant in the control treatment, suggesting these bOTUs were contaminants of the microcosms.

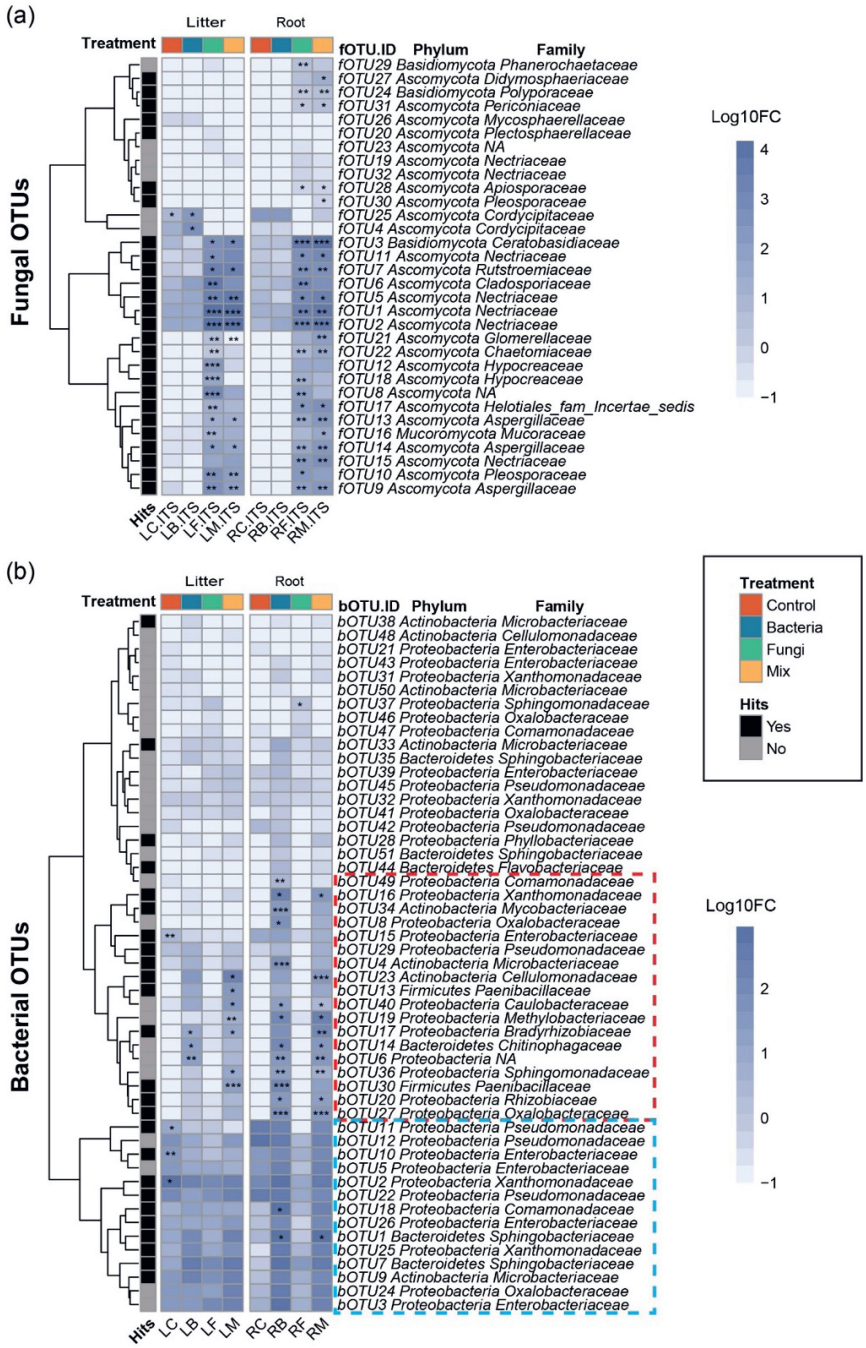

Figure 5. Relative abundance of bacterial and fungal OTUs in litter (L) and root (R) samples inoculated with fungi (F), bacteria (B), fungi and bacteria (M) or non-inoculated controls (C). Black colored boxes (Hits) refer to OTU sequences that are similar to inoculated bacterial or fungal while grey colored boxes (Non-hits) are OTUs that are not similar to the inoculated bacteria and fungi. The asterisk in the heatmap cells indicate OTUs that are significantly and positively correlated to one

or more treatments. **(a)** Litter and root fungal OTUs. **(b)** Litter and rhizosphere bacterial OTUs. Only OTUs presents in at least 3 samples are shown here. Red dashed frame indicates the abundant bOTUs of bacterial inoculated treatments of root microbiome. Blue dashed frame indicates bOTUs abundant in all treatments of litter microbiome. The dendrogram is based on hierarchical clustering. Litter, rhizosphere and microbial treatments are represented by different colors.

Discussion

Fungi as main drivers of litter decomposition

This study demonstrates that fungi are the main decomposers of plant litter in our experimental system. Litter decomposition in treatments inoculated with fungi was 47% higher compared to the control treatment or the treatment where only bacteria were inoculated. A number of studies also suggest that bacteria contribute to litter decomposition and produce extracellular enzymes that can degrade lignocellulose, a main component of plant litter (Adhi *et al*., 1989; Lin *et al*., 2012). However, we did not observe strong evidence for this. Our study is in line with other studies, which identify fungi as main drivers of litter decomposition due to their ability to produce a range of extracellular enzymes (Bugg *et al.*, 2011; Schneider *et al.*, 2012). However, so far, very few studies have obtained direct experimental evidence for the role of fungi and directly manipulated microbial communities (e.g., bacterial and fungal communities) to investigate the main drivers and identify complementarity. Further studies should also include protists or soil invertebrates to better understand the decomposition process.

Of the 35 inoculated fungi, sequences of 10 fungal OTUs (fOTU1, fOTU2, fOTU3, fOTU5, fOTU7, fOTU9, fOTU10, fOTU13, fOTU14, fOTU21) from 7 genera were found enriched in litter samples and we hypothesize that these fungi likely grew on the litter and contributed most to its decomposition. *Fusarium* is the most abundant genus in litter samples including fOTU1 (*Fusarium solani*), fOTU2 (*Fusarium oxysporum*) and fOTU5 (*Fusarium proliferatum*). Although these and other *Fusarium* spp. are mostly studied for their plant pathogenic lifestyle (Dugan, Hellier & Lupien 2003; Ohara *et al*., 2003; Ma *et al.* 2013), the plants that received fungal inoculation that included these fOTUs did not exhibit any disease symptoms and plant growth was promoted in the beginning of our experiment. *F. solani, F. oxysporum and F. proliferatum* have been shown to possess moderate lignin-degrading capacities (Perestelo, Carnicero, Regalado & Rodri 1997; Lozovaya *et al.* 2006; Kgd, Jm, Ct & Jr 2015) and we thus hypothesize that they also promoted litter decomposition in our study. Fungal OTU9 in our study was annotated as *Aspergillus fumigatus,* a fungal species which has been reported to decompose lignocellulose biomass (Song & Fan, 2010;Jiang *et al.*, 2014). Moreover, also *Penicillium* and *Alternaria* spp. have been demonstrated to decompose lignin and cellulose (Song & Fan 2010). This suggests that *Penicillium* fOTU14 and *Alternaria* fOTU10, which were both enriched in our litter samples, share a similar function as those strains. In a recent study, strains from the abovementioned genera (*Fusarium, Aspergillus, Penicillium)* were identified as keystone taxa in litter decomposition process of three different land use types (Zheng *et al.* 2021b). These keystone strains enhanced microbial complexity and showed high enzyme activities of litter decomposition. We found *Rhizoctonia* fOTU3 enriched in the litter,

in contrast to (Ivarson 1974) who reported that *Rhizoctonia* sp. had low survival ability on litter during a 45-month period. Additionally, there is no previous evidence of a role of the genera *Zalerion* or *Colletotrichum* in litter decomposition, and it is therefore difficult to deduce the contributions of the enriched fOTU7 (*Zalerion* sp.) and fOTU21 (*Colletotrichum* sp.) to the results we observed in our experiment. To further investigate how these enriched fungal OTUs decompose plant litter, metatranscriptomic sequencing could be performed to determine the activity of functional genes involved in decomposition.

Microbial effects on plant growth

Litter decomposition is an important process for nutrient cycling in natural ecosystems (Hättenschwiler *et al*., 2005; Krishna & Mohan 2017; Floudas *et al.* 2020). During decomposition, the C: N ratio decreases, and inorganic nutrients are released into the surrounding environment (Crowther *et al*., 2012). In our experiment, we saw the strongest litter mass loss during the first 4 weeks in the two treatments inoculated with fungi. The increase of plant growth in microcosm with fungi after 4 and 8 weeks may therefore be related to increased nutrient release from the decomposing material. Moreover, previous studies also suggest that microbes (e.g., fungi) can promote plant growth by exuding plant-growth-promoting compounds or liberate (micro) nutrients (Hayat, Ali & Amara 2010).

Interestingly, after 16 weeks, we found that shoot biomass was highest in the Mix treatment pointing to synergistic effects of bacterial and fungal communities. Several studies indicate that bacteria and fungi can complement each other and provide different limiting nutrients to plants resulting in enhanced plant biomass (e.g., van der Heijden *et al.*, 2015). We also observed the highest microbial richness in the Mix treatment, and this may have contributed to increased plant biomass as observed in earlier works. The investigation of the interkingdom microbial interactions suggests that the bacteria are essential for plant survival and protection against root-derived filamentous eukaryotes (Wagg *et al.* 2014, 2019; Durán *et al.* 2018). In our case, the fungi probably released more plant available nutrients to the surrounding soil, while the bacteria may have benefited plant growth in other ways, e.g., by secreting plant growth hormones (Bartoli *et al.* 2022; Poonam Pandurang 2021).

One of the inoculated bacteria, bOTU17, which was enriched in litter of both the Bacteria and Mix treatment, belongs to family *Bradyrhizobiaceae*, a genus known to be involved in N fixation and in N cycling (Meng *et al.*, 2018). It has also been shown that members of *Bradyrhizoium* are capable of the degradation of recalcitrant compounds like lignocellulose and lignin and can contribute to litter decomposition (Gołębiewski *et al.* 2019).

Establishment and rediscovery of the inoculated microbes in the synthetic microbial communities

In both bacterial and fungal communities, we found that over 60% of the inoculated isolates were rediscovered. The 16S rRNA gene sequences of these isolates matched with OTU read sequences detected through microbiome profiling of litter and root samples. Some of the

inoculated isolates were not detected, likely because these microbes were unable to grow or survive in the microcosm. The microcosm was designed to create a gnotobiotic system, providing an environment for studying plant-microbe interactions. We use calcined clay as a substrate for plant growth in the microcosm. The physicochemical differences between the calcined clay in the microcosms and natural soil likely exerted a selective pressure on the inoculated microbes and probably favored those that could quickly adapt to the new growth conditions.

In this experiment, we observed that a higher number of bacteria and fungi colonized plant roots compared to litter. This is likely due to the fact that plants exude carbon-rich nutrients, which create favorable conditions for microbial growth in the root microbiome. Previous studies have shown that the microbial populations in the rhizosphere are denser on plant roots than in the surrounding soil (Bakker *et al.*, 2020). Additionally, the microbes used in this study were initially isolated from plant roots, which may have made them more adapted to survive in the rhizosphere than on litter. However, we also detected many of the inoculated taxa in soil samples (Hartman *et al.* 2018), indicating that further research is needed to fully understand the role of litter in microbial decomposition. Future studies should specifically include microbes isolated from litter to better understand their contribution to the decomposition process.

We detected various bacteria in the Control treatment, which should have been free of microbes. Four *Proteobacteria* OTUs were significantly abundant in the Control, suggesting that these OTUs are major contaminants in our system. The autoclaved substrate was checked before it was added to the microcosm, confirming that our sterilization protocol was successful. The plant seedlings were sterilized and pre-germinated on agar plates. These sterile seedlings with no surrounding microbes were transplanted into the microcosm and the Control treatments were prepared first to prevent cross contamination. Thus, the contaminating microbes were most likely introduced after the preparation and assemblage of the microcosms. It is possible that, condensation, which formed on gas exchange film at the top of the microcosms, could act as passage for airborne bacteria to access the microcosms.

In conclusion, this study provides experimental evidence that fungi are the main decomposers of plant litter. This paves the way for a deeper understanding of fungi and bacteria interactions and community succession during litter decomposition, which could eventually be used to develop microbial solutions to enhance litter decomposition and nutrient cycling in agroecosystems.

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Authors' contributions

C.Z., C.E.S., and M.G.A.v.d.H. conceived and designed the experiments. C.Z. and S.D.P. performed the experiments. K.H. isolated the bacteria and fungi used in the manuscript. C.Z. analyzed the data. C.Z., R.L.B., and M.G.A.v.d.H. wrote the manuscript.

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Supplementary method

Fungal isolation and identification

Individual plants of *Trifolium pratense* were collected from the Farming Systems and Tillage (FAST) experiment (Wittwer *et al.* 2021) and five separate growth chamber experiments (Hartman 2018). Upon harvest, the plants were removed from their pots and the roots were shaken to remove bulk soil. In addition, naturally collected individuals were excavated from the field with a hand shovel, shaken to remove bulk soil, and placed in a plastic bag. In the lab, all root samples were rinsed with distilled H_2O to remove the loosely adhering soil particles, and 3-5 mm root fragments were cut from the lateral roots into a dish of sterile distilled H_2O with a pair of flamed scissors. In a sterile laminar flow cabinet, the root fragments were surface sterilized by agitating in 95% EtOH for 15s, 30% H_2O_2 for 15s, and finally two separate rinses in sterile distilled H_2O.

Three sterilized *Trifolium* root fragments per plate were placed on modified MMA or Malt Extract agar (MEA) (Sigma Aldrich, St.Louis, MO USA) plates amended with 15 µg/mL oxytetracycline (Sigma Aldrich, St. Louis, MO USA) to inhibit bacteria growth. All plates were incubated at 25 ºC until single hyphae were visible on the plate surface. Small fragments of individual hyphae were cut from the plates with a sterilized scalpel and sub-cultured at least three times on MMA or MEA plates. The isolates were subsequently re-plated for PCR-based taxonomy identification (see below) or preserved to create the fungi reference stock. For this, re-plated isolates were allowed to grow until fungal biomass covered the plate. In a sterile laminar flow cabinet, ten plugs of each isolate were punched out from the plate with a flamed cork borer (ø 2.5 mm). Five plugs were placed in a 2 mL cryogenic tube (Thermo Scientific, Waltham, MA, USA) containing 50% glycerol (v/v final) and stored long-term at -80 °C. The other five plugs were placed in a 2 mL cryogenic tube (Thermo Scientific, Waltham, MA, USA) containing sterile distilled H_2O and stored at room temperature in the dark.

A small amount of fungal biomass from each isolate was scraped from the surface of the agar plate and placed in a sterile 1.5 mL tube. Fungal DNA was extracted with the REDExtract-N-Amp Plant PCR Kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. The extracted DNA was used as a template in PCR reactions. Each 20 µL PCR reaction per isolate contained 10 µL REDExtract-N-Amp PCR Ready Mix (Sigma Aldrich, St. Louis, MO, USA), 400 nM of each primer ITS5 and ITS4 (White, Bruns, Lee & Taylor 1990) 4 µL of template DNA, and the remaining volume sterile distilled H_2O. All reactions were performed in an iCycler instrument (BioRad, Hercules, CA, USA) with the cycling conditions of 2 min at 95 ℃, followed by 35 cycles of 1 min at 94 ℃, 1 min at 54 ℃ and 1 min at 72 ℃ and a final extension of 10 min at 72 ℃. PCR amplicons were verified on a 1% agarose gel. The reactions were purified and sequenced using the Sanger method with ITS5 as the sequencing primer by Microsynth AG (Balgach, Switzerland).

The resulting AB1 sequencing files were converted into FASTQ file format using EMBOSS v6.6.0(Rice, Longden & Bleasby 2000). Sequences were quality filtered by trimming 50 bp

from the 5' and 3' ends and then progressively trimming nucleotides from both ends at a mean Phred score <25 (window size 5, step size 2). Finally, sequences <400 bp or with a mean Phred score <30 were discarded. Quality filtering was performed using PRINSEQ v0.20.4 (Schmieder & Edwards 2011). Quality sequences were used for taxonomy assignment using the RDP classifier against the UNITE database v7 (Abarenkov *et al.* 2010) as implemented in QIIME v1.8 (Caporaso *et al.* 2010).

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Supplementary figures

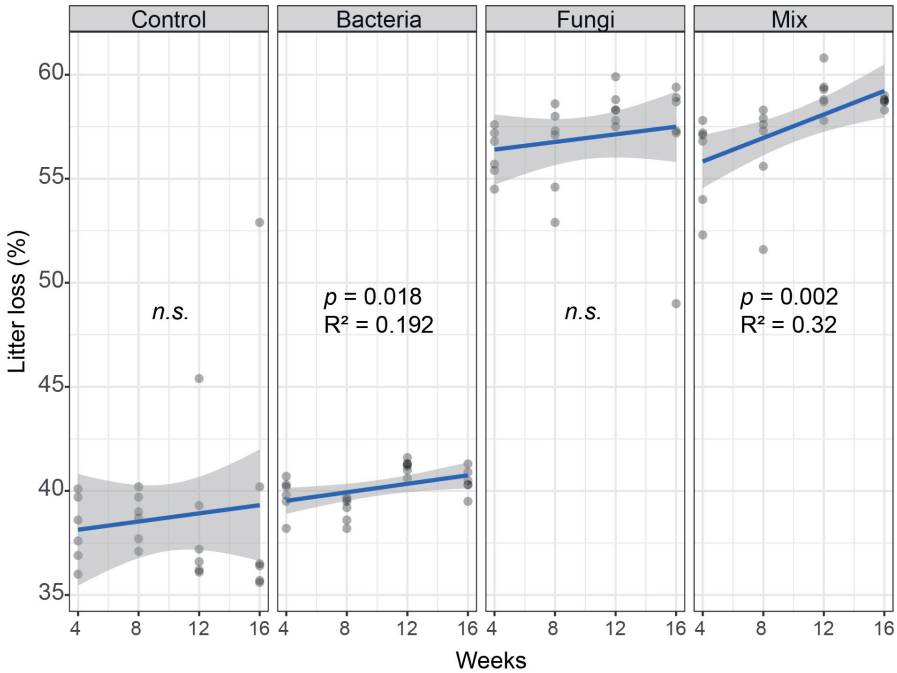

Figure S1. Litter loss increased with time in Bacteria and Mix treatment.

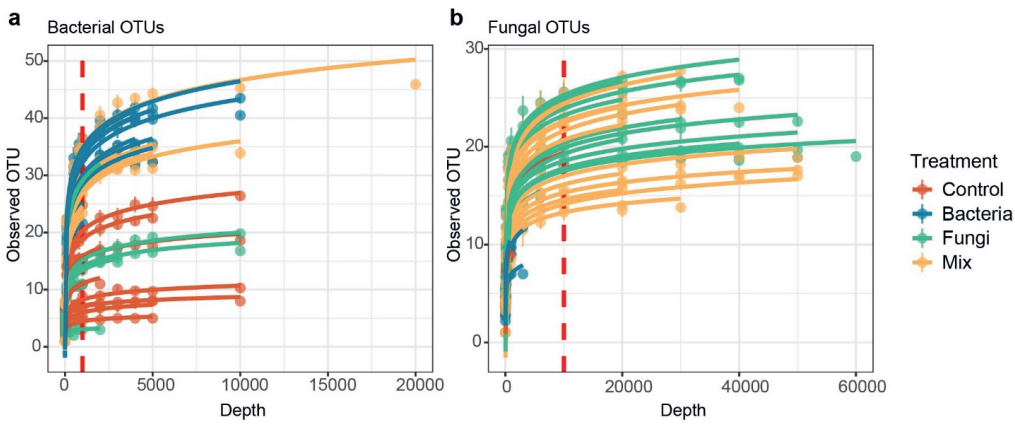

Figure. S2. Rarefaction cure of bacterial and fungal communities. The microbial treatments are depicted by four colors. The red dash lines indicate the selected rarefaction depth.

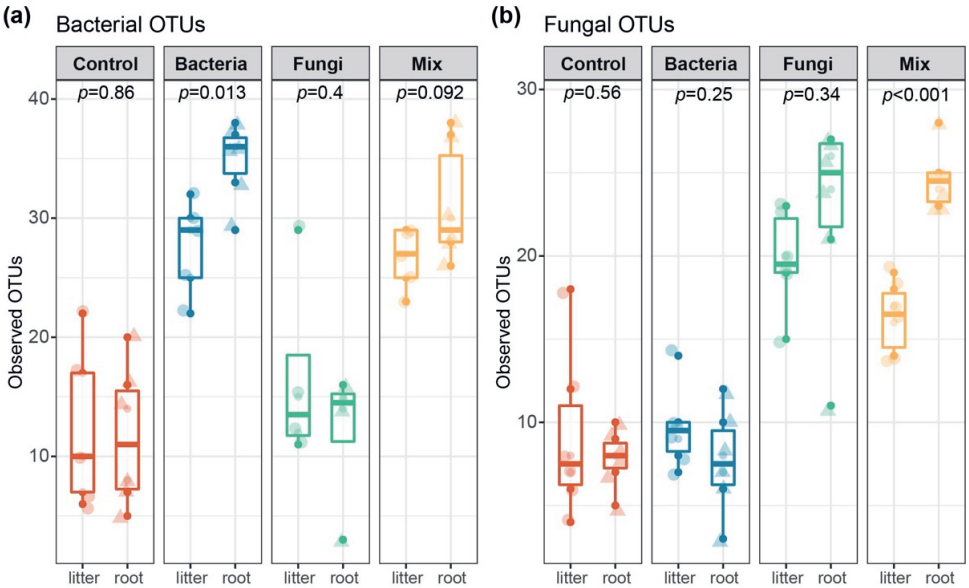

Figure S3. Bacterial and fungal OTUs comparison within microbial treatments between sample types. The significance levels were determined by t-test. The results of two-way ANOVA of the effects of the treatments and sample types are shown in Table S8.

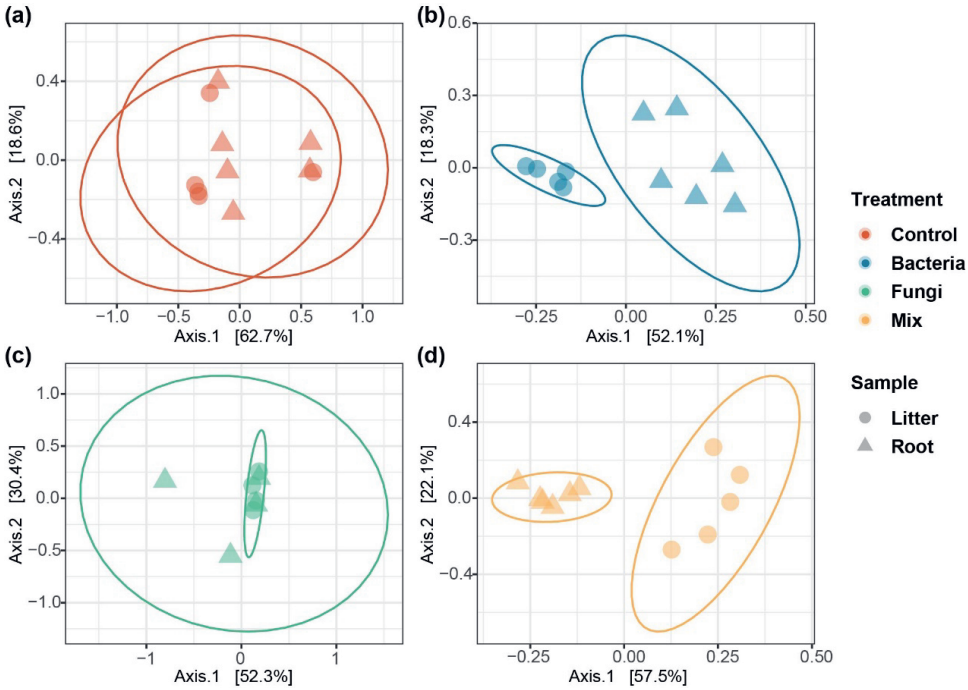

Figure S4. Sample type effects on bacteria inoculated treatments. (a) The bacterial communities of Control treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 1.268, R^2 = 0.123, *p*-value = 0.234). **(b)** The bacterial communities of Bacteria treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 7.980, R^2 = 0.469, *p*-value = 0.003). **(c)** The bacterial communities of Fungi treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 0.974, R^2 = 0.139, *p*-value = 0.463). **(d)** The bacterial communities of Mix treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 10.558, R^2 = 0.539, *p*-value = 0.005).

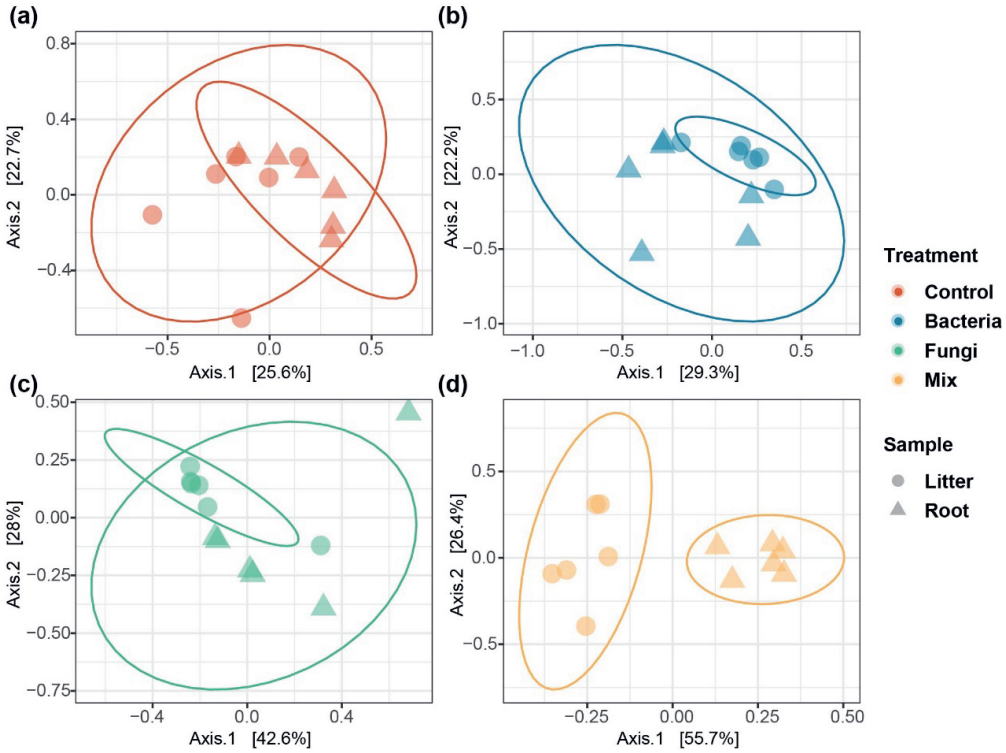

Figure S5. Sample type effects on fungi inoculated treatments. (a) The fungal community of Control treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 2.091, R^2 = 0.173, *p*-value = 0.014). **(b)** The fungal community of Bacteria treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 2.529, R^2 = 0.202, *p*-value = 0.009). **(c)** The fungal community of Fungi treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 2.563, R^2 = 0.204, *p*-value = 0.017). **(d)** The fungi community of Mix treatment PCoA based on Bray-Curtis distances (PERMANOVA by sample type, pseudo-F = 10.953, R^2 = 0.523, *p*-value = 0.004).

Supplementary tables

Table S1 Taxonomy of selected bacteria and fungi for creating synthetic communities.

(A) Selected bacteria isolates for creating bacterial inoculum.

Isolate	Phylum	Class	Order	Family	Genus	Species
KHB083	Actinobacteria	Actinobacteria	Micrococcales	Cellulomonadaceae	Oerskovia	Oerskovia turbata
KHB067	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens
KHB064	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Herbiconiux	Herbiconiux moechotypicola
KHB058	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Microbacterium	Microbacterium gilvum
KHB020	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Microbacterium	Microbacterium terregens
KHB070	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	Micromonospora	Micromonospora matsumotoense
KHB036	Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	Mycobacterium	Mycobacterium tusciae
KHB098	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium aquidurense
KHB034	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium hydatis
KHB111	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter boryungensis
KHB103	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter trunci
KHB102	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter rubeus
KHB200	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus subtilis
KHB121	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus simplex
KHB012	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium
KHB119	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	Paenibacillus sp. HA34
KHB088	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	Paenibacillus alginolyticus
KHB090	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus argenteus
KHB115	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	JVGV_s
KHB112	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	Bradyrhizobium ganzhouense
KHB085	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	Bradyrhizobium_AUGA_s
KHB114	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Cupriavidus	Cupriavidus basilensis
KHB010	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	Variovorax boronicumulans
KHB060	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	Serratia liquefaciens
KHB052	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter	Enterobacter cloacae
KHB044	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	Pantoea agglomerans
KHB017	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia	Erwinia rhapontici
KHB110	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Collimonas	Collimonas fungivorans
KHB093	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	KB906725_s
KHB030	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium lividum
KHB082	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium	Mesorhizobium shangrilense
KHB188	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas rhodesiae
KHB172	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas canadensis
KHB142	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas mediterranea
KHB107	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas frederiksbergensis
KHB076	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas putida
KHB148	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium zeae
KHB005	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium leguminosarum
KHB113	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella	Dyella japonica
KHB080	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rudaea	Rudaea cellulosilytica
KHB055	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	Stenotrophomonas maltophilia

(B) Selected fungi for creating fungal inoculum.

Isolate	Kingdom	Phylum	Class	Order	Family	Genus	Species
KHF00069	Fungi	Ascomycota	Sordariomycetes	Incertae sedis	Apiosporaceae	Apiospora	Apiospora montagnei
KHF00124	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	Chaetomium erectum
KHF00091	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Metacordyceps	Metacordyceps khaoyaiensis
KHF00086	Fungi	Ascomycota	Sordariomycetes	Incertae sedis	Glomerellaceae	Colletotrichum	Colletotrichum destructivum
KHF00098	Fungi	Ascomycota	Sordariomycetes	Incertae sedis	Glomerellaceae	Colletotrichum	Glomerella lindemuthiana
KHF00052	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	Trichoderma pubescens
KHF00109	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	Trichoderma spirale
KHF00018	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Incertae sedis	Didymella	Didymella exigua
KHF00085	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Incertae sedis	Periconia	Periconia sp 9 MU 2012
KHF00154	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis	Ilyonectria	Ilyonectria mors panacis
KHF00176	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis	Ilyonectria	Ilyonectria macrodidyma
KHF00059	Fungi	Ascomycota	Sordariomycetes	Incertae sedis	Magnaporthaceae	Gaeumannomyces	Gaeumannomyces cylindrosporus
KHF00025	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Gibberella	Gibberella avenacea
KHF00031	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	Fusarium cf solani 9 d DPGS 2011
KHF00032	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	Fusarium oxysporum f sp melonis
KHF00114	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Cylindrocarpon	Cylindrocarpon pauciseptatum
KHF00180	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	Fusarium proliferatum
KHF00199	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Cylindrocarpon	Cylindrocarpon sp D60
KHF00165	Fungi	Ascomycota	Sordariomycetes	Incertae sedis	Plectosphaerellaceae	Verticillium	Verticillium dahliae
KHF00042	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	Alternaria sp 3 MU 2012
KHF00147	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Drechslera	Drechslera sp BAFC 3419
KHF00050	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	Aspergillus fumigatus AF138287
KHF00072	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	Aspergillus sclerotioniger
KHF00146	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	Penicillium crustosum
KHF00057	Fungi	Ascomycota	Unidentified	Unidentified	Unidentified	Unidentified	uncultured Zalerion
KHF00135	Fungi	Ascomycota	Unidentified	Unidentified	Unidentified	Unidentified	uncultured Ascomycota
KHF00167	Fungi	Ascomycota	Dothideomycetes	Pleosporales	unidentified	unidentified	Pleosporales sp 2 MU 2012
KHF00184	Fungi	Ascomycota	unidentified	unidentified	unidentified	unidentified	uncultured Ascomycota
KHF00013	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Rhizoctonia	Rhizoctonia sp AG K
KHF00009	Fungi	Basidiomycota	Agaricomycetes	Polyporales	Polyporaceae	Funalia	Coriolopsis trogii
KHF00020	Fungi	Unidentified	Unidentified	Unidentified	Unidentified	Unidentified	uncultured fungus
KHF00026	Fungi	Unidentified	Unidentified	Unidentified	Unidentified	Unidentified	fungal sp GFI 146
KHF00040	Fungi	Unidentified	Unidentified	Unidentified	Unidentified	Unidentified	fungal sp GZ 2010b
KHF00125	Fungi	Unidentified	Unidentified	Unidentified	Unidentified	Unidentified	uncultured fungus
KHF00149	Fungi	Zygomycota	Incertae sedis	Mucorales	Mucoraceae	Mucor	Mucor moelleri

Table S2 20ml 15% Hoagland solution added to each microcosm

Macronutrients	**Concentration (mM)**
KNO_3	0.9
$(NH_4)H_2PO_4$	0.3
Ca(NO3)2•4H2O	0.6
$MgSO_4•7H_2O$	0.15
Micronutrients	**Concentration (µM)**
KCl	7.5
H_3BO_3	3.75
$MnSO_4•H_2O$	0.3
$ZnSO_4•7H_2O$	0.3
$CuSO_4•5H_2O$	0.075
$(NH_4)_6Mo_7O_{24}•4H_2O$	0.075
$C_{10}H_{12}FeN_2NaO_8$	3

Table S3 Two-step PCR cycling conditions used to amplify the 16S and ITS sequences.

	16S Community Profiling						**ITS Community Profiling**					
	First step PCR			**Second step PCR**			**First step PCR**			**Second step PCR**		
	Temp.	Time	Cycle	Temp.	Time	Cycle	Temp.	Time	Cycle	Temp.	Time	Cycle
1	95℃	3 min	1	95℃	3 min	1	95℃	3 min	1	95℃	3 min	1
2	95℃	30 sec	25 cycles	95℃	30 sec	10 cycles	95℃	30 sec	25 cycles	95℃	30 sec	8 cycles
3	75℃	10 sec		55℃	30 sec		55℃	30 sec		55℃	30 sec	
4	55℃	30 sec		72℃	30 sec		72℃	30 sec		72℃	30 sec	
5	72℃	30 sec		72℃	5 min	1	72℃	5 min	1	72℃	5 min	1
6	72℃	5 min	1	10℃	Hold		10℃	Hold		10℃	Hold	
7	10℃	Hold										

Table S4 Taxonomy table of bOTUs.

Feature.ID	bOTU.ID	Kingdom	Phylum	Class	Order	Family	Genus	Species
dd5554a054f66f8f4a294c534cd1afe7	bOTU1	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	gossypii
2048ac57ace3583c7e252430999c5ae7	bOTU2	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	maltophilia
b52ca05ab199b3b277b4c0cda2bead57	bOTU3	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter	cloacae
23792c8f03d9459b495d0f4c3df84f85	bOTU4	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Curtobacterium	*NA*
c65f1fddebd0cf2b46727a42d0f4ab9e	bOTU5	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Rahnella	aquatilis
8171c0441d0b79540429cac2c31958cf	bOTU6	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	*NA*	*NA*	*NA*
6209c81faf4714251a53f5ae2e7d61ef	bOTU7	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	*NA*
30c4edfa384d53219d5bd48d3c794287	bOTU8	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Collimonas	*NA*
81ef26452f15492af67d7f5c30b954d6	bOTU9	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	*NA*
1b5b3ec8288e79da239b63942d12e52e	bOTU10	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*NA*	*NA*
ab30088bdc4980dbc30b9aeb8cfb53f3	bOTU11	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	veronii
1e02756796bfdfad045a9029f8d228a5	bOTU12	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*NA*
e5fc89c32e09245d60836800b46ab2fa	bOTU13	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	*NA*
4e2e6c963132cb3294a1195c80e06f5d	bOTU14	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Sediminibacterium	*NA*
a4fb4cdf769ed77316f6920aa74f49c2	bOTU15	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Gluconacetobacter	*NA*
b421af9fdfee94bc161340719d5f37b0	bOTU16	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rudaea	cellulosilytica
57f1e5f801006c948837a51395ac4b19	bOTU17	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*NA*	*NA*
100a5319b8c16490ed59161867f81a0a	bOTU18	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	paradoxus
ed34390276bc7b72af32b27e60910587	bOTU19	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	*NA*
ccc04f9b263a3243e79ed63a5144d84d	bOTU20	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	*NA*
166c692a9c84c4cb8e9b3369472c1adb	bOTU21	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Rahnella	aquatilis
e4e3cdaaa5e275454f3b06b76a0ccb7c	bOTU22	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*NA*
ebe2138829c19c433d7d86eb95154089	bOTU23	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*NA*	*NA*
7c68e0db557e8b1f6ddc7bd7d4b62dff	bOTU24	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	lividum
917534d5d11d5422cfffa79fd400080c	bOTU25	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rhodanobacter	*NA*
87911445cd69835ff2d59c77342df7b9	bOTU26	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*NA*	*NA*
8a9df622c4c8573d3f9f807584c84b63	bOTU27	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Cupriavidus	*NA*
04598e9ab30714a064c2d81e3f1fb05b	bOTU28	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*NA*	*NA*
b27b751ffa96218fe641665691afd001	bOTU29	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*NA*
627e66c8628e42897673708ce189dd21	bOTU30	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	chondroitinus
0ab6484995b48f542a73d83ac72b2c0a	bOTU31	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rhodanobacter	*NA*
ad6c481536783548b592aeb548c9a36c	bOTU32	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	*NA*
25d19205bb69b4886425e10846360849	bOTU33	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*NA*	*NA*
65634f9015850be731b83abc837e107e	bOTU34	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium	*NA*
07d78e67a64fc8f88a5be951493f6717	bOTU35	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	*NA*
e9865cbd0d2721093c30ddae25385abf	bOTU36	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	*NA*
5575faab203057c9377c22d3110de843	bOTU37	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	*NA*
4100ce5b659108262d7b148464b20877	bOTU38	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	*NA*
ed6f49b786b289372d460c80bfefe8ad	bOTU39	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter	*NA*
1ca272b41b51149c10a1a9bfc0e9661f	bOTU40	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*NA*	*NA*
ba1fb0ef1c71c36a252fbee949cb4671	bOTU41	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	lividum
3bee251da6623395461d52841f6db049	bOTU42	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*NA*

0b856f0fbca535761b52a5344dad157d	bOTU43	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*NA*	*NA*
f2a9ef6c5ad9f6c7404a5cdd89ecd5a7	bOTU44	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	frigidarium
ad14f69dcbc264d1ce2a9f49a38ca7db	bOTU45	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	veronii
07e1033e789760fa1d0b79aa883fa31f	bOTU46	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia	*NA*
43ed10e4a61683b88aa843c3ecb0cad6	bOTU47	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	*NA*
18444c5c2f38cb91f530cb2b930b2f98	bOTU48	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Oerskovia	*NA*
7ad67061ad745f2fb0c4cd753a0ede2b	bOTU49	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	paradoxus
76b3d062644e3feb4893ea080c54a7c0	bOTU50	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	maritypicum
8902e45c901b3cb6ffb1c14177ee0a0b	bOTU51	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	gossypii

Table S5 Taxonomy table of fOTUs).

Feature.ID	fOTU.ID	Kingdom	Phylum	Class	Order	Family	Genus	Species
9eea5a87908c2a9cf0aa8dbd3b16ad41	fOTU1	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	Fusarium_solani
cb993cc989ac21dc6ad2c44910c1433d	fOTU2	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	Fusarium_solani
03ccc8a6679bb1a24c973895e1cd3e19	fOTU3	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Rhizoctonia	Rhizoctonia_solani
f24da9a67281fd80cba1bfa0938ae2d7	fOTU4	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	Beauveria	Beauveria_pseudobassiana
46cddb42ac2322d13c009f77ba7ecbae	fOTU5	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	Fusarium_solani
3ec34ea7bdbb85ef757b8080a23552ab	fOTU6	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	Cladosporium_tenuissimum
9e54f151a29715552979ef52d771acab	fOTU7	Fungi	Ascomycota	Sordariomycetes	Lulworthiales	Lulworthiaceae	Zalerion	uncultured Zalerion
7a7ac411a1d71146d7c7df81c4aa7528	fOTU8	Fungi	Ascomycota	Dothideomycetes	*NA*	*NA*	*NA*	*NA*
74bda2d131d2c0b1e59b51ed3a1d082a	fOTU9	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Aspergillus_flavus
4a8bd9839d6c4f7d9237bbd3629f4cbf	fOTU10	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	Alternaria_alternata
4be51c4e8c9615eafd443b9d2a9cf001	fOTU11	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Cylindrocarpon	unidentified
804b00d968009ef2b1689db21b637b5e	fOTU12	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	Trichoderma_longisporum
c18bdd266f60102adbc39153cfd0e825	fOTU13	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Aspergillus niger
66af1741c222f1fa00eaa585dec21959	fOTU14	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	Penicillium_commune
ecaa07bc77649faea7a527b569bffeda	fOTU15	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Gibberella	Gibberella_avenacea
bfb578d4a3bc059d65f8e6719bd89936	fOTU16	Fungi	Mucoromycota	Mucoromycetes	Mucorales	Mucoraceae	Mucor	Mucor_moelleri
597e3d6821832678ea441321d78a2e62	fOTU17	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiales_fam_Incertae_sedis	Cadophora	Cadophora_orchidicola
40aa1123a6d3a299f104cb898fd82d1f	fOTU18	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	Trichoderma asperellum
47c1d98328dde0e4f6cb4682bca0bf5a	fOTU19	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	Fusarium_oxysporum
2ed09a4ca8da6e0f6e752b3c54c941e4	fOTU20	Fungi	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	Verticillium	Verticillium_nubilum
236718890f3fd3dc21acd3eb57a0bd6a	fOTU21	Fungi	Ascomycota	Sordariomycetes	Glomerellales	Glomerellaceae	Colletotrichum	Colletotrichum_fusc um
1fbf4987f7edd865b211548d74f6b4a7	fOTU22	Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Dichotomopilus	Dichotomopilus erectus
b7040cdd43c326751cc1e81df4667a91	fOTU23	Fungi	Ascomycota	Sordariomycetes	Hypocreales	*NA*	*NA*	*NA*
f111ad9def322fc7b7429a38cf6aa48a	fOTU24	Fungi	Basidiomycota	Agaricomycetes	Polyporales	Polyporaceae	*NA*	*NA*
9c84275f823c7a7532884b42e7436e8c	fOTU25	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	Cordyceps	Cordyceps_bassiana
e26ac66242935f3c58e3656898e1ddef	fOTU26	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Mycosphaerella	Mycosphaerella_tassiana
b2b71bd36817c5a497235817fac0885f	fOTU27	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Bimuria	Bimuria novae-zelandiae
39b233dadaab0dba1f9f20a15a420a9c	fOTU28	Fungi	Ascomycota	Sordariomycetes	Xylariales	Apiosporaceae	Arthrinium	Arthrinium_malaysianum
2f8d35020161e4586e7091fffd38c661	fOTU29	Fungi	Basidiomycota	Agaricomycetes	Polyporales	Phanerochaetaceae	Bjerkandera	Bjerkandera_adusta
5b365d270ecd2fc3261eedc9fdd2dcce	fOTU30	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Drechslera	unidentified
553455a5d4db66f26f35c5109f661433	fOTU31	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Periconiaceae	Periconia	Periconia_macrospinosa
3d3994d20f1febff945754e859c7dfb9	fOTU32	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*NA*

Table S6 Litter loss change between time points. Litter loss change by time was calculate by Time point 2 (Litter loss mean value) - Time point 1(Litter loss mean value). The *P* value adjusted by FDR method indicate the significance effect in the two-way repeated measures ANOVA of litter loss difference between two time points. Two Control samples were identified contaminated by fungi were deleted from the analysis.

Treatment	Time point1 (weeks)	Time point2 (weeks)	Litter loss change by time	*P*-adjust	Significance codes
Control	4	8	0.583	0.846	ns
Control	8	12	-0.266	0.846	ns
Control	12	16	-1.467	0.846	ns
Bacteria	4	8	-0.697	0.093	ns
Bacteria	**8**	**12**	**2.034**	**< 0.001**	***
Bacteria	12	16	-0.7	0.093	ns
Fungi	4	8	0.217	0.875	ns
Fungi	8	12	2.016	0.46	ns
Fungi	12	16	-1.683	0.46	ns
Mix	4	8	0.516	0.684	ns
Mix	**8**	**12**	**2.75**	**0.026**	*
Mix	12	16	-0.416	0.684	ns

Table S7 Kruskal Wallis and Dunn's post-hoc test determine the CFUs differences across microbial treatments. For bacterial communities, the CFUs were found significant difference among microbial treatments (chi-squared = 23.6, df = 3, p-value < 0.001). For fungal communities, CFUs were found significant difference (chi-squared = 34.8, df = 3, p-value <0.001) among four microbial treatments.

Bacterial CFUs		Fungal CFUs	
Comparison	***p*-value**	**Comparison**	***p*-value**
Bacteria - Control	0.001	Bacteria - Control	0.416
Bacteria - Fungi	0.002	Bacteria - Fungi	< 0.001
Control - Fungi	0.919	Control - Fungi	< 0.001
Bacteria - Mix	1	Bacteria - Mix	0.001
Control - Mix	0.004	Control - Mix	0.017
Fungi - Mix	0.005	Fungi - Mix	0.214

Table S8 Two-way ANOVA determine the observed OTUs differences across microbial treatments and sample types. Shown are the treatment, sample type and their interaction effect on observed OTUs.

	Bacterial OTU number			Fungal OTU number		
Factor	**F**	**Df**	***P*-value**	**F**	**Df**	***P*-value**
Treatment	8.87	3	<0.001	18.25	1	<0.001
Sample type	7.43	1	0.010	21.70	1	<0.001
Treatment * Sample type	3.19	3	0.036	4.29	1	0.052

Table S9 ANOVA and Tukey HSD test determine the OTU richness differences across microbial treatments. The bacterial communities were found significant different among treatments (df = 3, Std. Error = 5.706). The fungal communities were found significant different among treatments (df = 1, Std. Error = 4.074)

	Bacteria				Fungi			
Comparison	**Mean Difference**	**Lower Bound**	**Upper Bound**	***p*-value**	**Mean Difference**	**Lower Bound**	**Upper Bound**	***p*-value**
Bacteria-Control	19.545	13.004	26.086	<0.001	0.167	-4.318	4.652	0.999
Fungi-Control	2.375	-4.753	9.503	0.806	12.750	8.265	17.235	<0.001
Mix-Control	17.091	10.549	23.632	<0.001	12.000	7.515	16.485	<0.001
Fungi-Bacteria	-17.170	-24.298	-10.042	<0.001	12.583	8.098	17.068	<0.001
Mix-Bacteria	-2.455	-8.995	4.087	0.745	11.833	7.348	16.318	<0.001
Mix-Fungi	14.715	7.588	21.844	<0.001	-0.750	-5.235	3.735	0.969

Table S10 Bacterial community pairwise PERMANOVA results on Bray-Curtis dissimilarities testing the microbial treatment effects and sample type effects.

Factors	pseudo-F	R^2	*P*-value
Treatment			
Control - Bacteria	7.697	0.278	0.006
Control - Fungi	2.449	0.126	0.306
Control - Mix	7.577	0.275	0.006
Bacteria - Fungi	3.495	0.171	0.006
Bacteria - Mix	1.976	0.090	0.582
Fungi - Mix	3.809	0.183	0.006
Part			
Litter - Root	4.197	0.097	0.001

Table S11 Fungal community pairwise PERMANOVA results on Bray-Curtis dissimilarities testing the microbial treatment effects and sample type effects.

Factors	pseudo-F	R^2	*P*-value
Treatment			
Control - Bacteria	1.088	0.047	0.350
Control - Fungi	11.305	0.339	0.001
Control - Mix	15.294	0.410	0.001
Bacteria - Fungi	12.150	0.356	0.001
Bacteria - Mix	16.374	0.427	0.001
Fungi - Mix	1.363	0.058	0.190
Part			
Litter - Root	2.717	0.056	0.034

Chapter 3

Arbuscular mycorrhiza fungi harbor a microbiome that is distinct from the surrounding soil

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Abstract

The large majority of land plants live in symbioses with arbuscular mycorrhiza fungi (AMF) which form an essential pathway for the exchange of chemicals between plants and soil. Plant roots harbor microbial communities that are distinct from the bulk soil and that affect the success and functioning of both the plant and its fungal symbiont. It is however unclear to what extent mycorrhizae drive the assembly of these plant-associated microbiomes. Here, we used microcosms filled with either organically or conventionally managed soils taken from a long-term experimental cropping field. We compartmentalized these microcosms to create soil sections with roots and AMF and sections with only AMF. By isolating hyphae from the latter section of the microcosms and by subsequent 16S, ITS, and 18S amplicon sequencing, we were able to characterize the fungal, bacterial, and protist communities associated with these mycorrhizal hyphae. After three months of the symbiosis development, the microbial communities of the soil were still affected by preceding organic or conventional management practices, but the microbial communities of the hyphal and root were not. We identified *Rhizophagus irregularis*, *Septoglomus viscosum* and *Funneliformis mosseae* as the AMF that predominantly colonized our experimental plants. The genera *Halangium, Massillia, Pseudomonas, Devosia, SWB02, Cellvibrio,* possible genus 04*, Noviherbaspirillum, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Sulfurifustis, Ohteakwangia, Pseudoxanthomonas,* and *Pseudoduganella* were found to be consistently enriched on the hyphal samples. The protists group, *Hacrobia,* shows significant higher relative abundance in hyphal samples than in soil samples. The identification of microbes that are consistently present on fungal hyphae may help us identify keystone species that are beneficial to plant-mycorrhiza symbiosis.

Key words: arbuscular mycorrhiza fungi, bacteria, protists, organic farming

Introduction

The zone of soil around the root is known as the rhizosphere and recognized as the site where many of the plant's interactions with microbial communities affect plant growth and survival (Philippot *et al*., 2013). The rhizosphere typically harbors a microbial community that is distinct from the surrounding bulk soil and is usually also characterized by a higher density but lower diversity of microbes (Bakker *et al*., 2020; Foster, 1983; Wang *et al*., 2020).The assemblage of rhizosphere microbial communities is driven by root exudates that include primary metabolites (e.g., sugars, amino acids) as well as secondary metabolites (e.g., benzoxazinoids, coumarins, flavonoid; Hu *et al*., 2018; Stringlis *et al*., 2018; Wolinska *et al*., 2021; Zhu *et al*., 2016). Arbuscular mycorrhiza fungi (AMF) live in symbiosis with 80% of terrestrial plants (Brundrett, 2004) and help plants to access distant water and nutrient sources (Chowdhury *et al*., 2022; Drigo *et al*., 2010; Govindarajulu *et al*., 2005; Nuccio *et al*., 2013; Pfeffer *et al*., 1999; Zhang *et al*., 2016). AMF extraradical hyphae extend from the host plant to forage past the rhizosphere. In this way, AMF enlarge the host plant's area of nutrient uptake and form a 'hyphosphere' that goes beyond the rhizosphere.

Like plants, AMF have been shown to secrete metabolites that can have an effect on the microbes that surround them. For example, carbohydrates, carboxylates, and amino acids are dominant metabolites in the AMF exudate (Toljander *et al*., 2007) and potentially help differentiate the microbial community on AMF hyphae from the bulk soil. Moreover, Filion *et al.* (1999) found that soluble exudates of *Rhizophagus irregularis* can have either antagonistic or stimulatory effects on individual fungal and bacterial isolates. Thus, it is likely that AMF hyphae shape the microbiome that surrounds them through the secretion of exudates.

Toljander *et al*. (2006) discovered that cells of some bacterial species had higher affinity to attach to mycorrhizal hyphae than others. Moreover, the affinity of bacteria to attach to AMF hyphae depended also on the fungal species and the vitality of the hyphae. Such specific interactions between bacteria and AMF likely play a role in the assembly of the hyphosphere microbiome.

In recent years, efforts have been taken towards *in situ* experiments in which AMF hyphae were sampled from the soil. Scheublin *et al*. (2010) found that *Oxalobacteraceae* representing a large proportion of the bacteria colonized on AMF hyphae. Zhang *et al*. (2018) found that the bacterial community on AMF hyphae significantly differed from that of the bulk soil. Moreover, the bacterial community with the presents of AMF shows higher alkaline phosphatase activity than the bacterial community in the AMF-excluded samples suggesting these bacteria play a role in the uptake of phosphorus. Emmett *et al*. (2021) tracked the development of bacterial community composition on AMF hyphae in time. They also found that distinct bacterial communities had established on AMF hyphae within 14 days of hyphae access to soil, and identified six bacterial orders including *Betaproteobacteriales*, *Myxococcales*, *Fibrobacterales*, *Cytophagales*, *Chloroflexales*, and *Cellvibrionales* that were consistently enriched on hyphae. A recent high throughput stable isotope probing research found that the phyla of *Myxococcota*, *Fibrobacterota*, *Verrucomicrobiota* and an archaeon genus of *Nitrososphaera* assimilated the most AMF-derived ^{13}C (Nuccio *et al*., 2022). In addition to bacteria, also protists have been found to interact with AMF. Diverse protistan taxa were found enriched or decreased by the presence of AMF (Bukovská *et al*., 2018; de Gruyter *et al*., 2021). Moreover, the protist *Polysphondylium pallidum* was found to increase the rate of AMF nitrogen uptake when applied together with bacteria (Rozmoš *et al*., 2021).

The excessive use of fertilizer and pesticide in conventional agriculture results in pollution and loss of biodiversity (Geiger *et al*., 2010; Thiele-Bruhn *et al*., 2012). Alternatively, organic farming refrains from the use of inorganic fertilizers, rejects synthetic crop protecting agents and is thus associated with much less deleterious effects on the environment (Hole *et al*., 2005). Studies comparing conventional and organic farming system show that organic farming promotes soil biodiversity (Banerjee *et al*., 2019; Wittwer *et al*. 2021). Especially mycorrhiza species were identified as keystone taxa in these organic farming systems underlining their importance in sustainable farming systems (Banerjee *et al.* 2019). Nonetheless organic farming typically results in lower crop yields than conventional practices with high chemical inputs. A

thorough understanding of the soil microbiome and the functioning of key players like AMF within organic farming systems, can help improve the yields of sustainable agricultural practices and lower the environmental degradation that results from food production.

For this reason, we studied the role of AMF in shaping the soil microbiome in organic and conventional farming systems. Using soil from a long-term field experiment that undergone either conventional or organic farming practices, we created compartmentalized microcosms in the greenhouse on which we grew *Prunella vulgaris* plants. *P. vulgaris* has been widely used as a model plant in ecological and evolutionary research that strongly associates with and responds to AMF symbionts(Qu & Widrlechner, 2011; Streitwolf-Engel *et al*., 2001; Miller & Winn, 1994; van der Heijden *et al*., 1998, 2003; Winn, 1988). We subsequently sampled compartments with roots and AMF, with only AMF and with neither of the symbionts. The root, hyphal, and soil microbial communities were subsequently characterized by ITS, 16S, 18S amplicon sequencing. Our results show that the bacterial, fungal, and protistan communities of the hyphal samples are differentially structured to the root and soil microbial communities and that specific bacterial genera are consistently enriched in hyphal samples.

Methods

Soil collection

The organic soil (OS) and conventional soil (CS) used in this study were derived from the Farming System and Tillage experiment (FAST) site (Wittwer *et al*., 2017; Wittwer *et al*. 2021). The FAST site was established in 2009 near Zürich (latitude 47°26′ N, longitude 8°31′ E) and the plots in this field have since undergone either conventional or organic management. The soil was collected in April 2019 and March 2020 for experiment I and experiment II respectively. The top layer of vegetation (2 cm) was removed and a 30 cm depth of soil was excavated from the field. The soil was passed through a 2 mm sieve and stored at 4 ℃ before use.

Description of microcosms and plant growth conditions

Experiment I

Microcosms were constructed of 20×10×19 cm (L×W×H) that were divided in 5 equal compartments (Fig. 1a). The compartments were separated from each other by 30µm nylon filters that allows hyphae to pass through but not roots. COMP1 and COMP2 were separated by a 1µm filter that also blocked hyphae. The middle compartment (COMP3) was filled with 1200 g of a mixture of 30 % non-autoclaved soil (either OS or CS), 4% autoclaved Oil-Dri (Damolin GmbH, Oberhausen, Germany), and 66% autoclaved sand. This compartment acted as soil inoculum. The outer compartments (COMP1, COMP2, COMP4, and COMP5, respectively) were each filled with 1200 g of sterilized outer substrate (8% autoclaved soil (either OS or CS), 6% autoclaved Oil-Dri and 86% autoclaved sand). All autoclaved substrates used in this study were heated to 121℃ for 45 mins twice. Seven replicate microcosms were set up for OS and CS, respectively.

Prunella vulgaris (henceforth Prunella) seeds were vapor-phase sterilized by exposure to chlorine gas for 4 hrs. To this end, chlorine gas was generated by adding 3.2 ml 37% HCl to 100 ml Bleach (Hijman Schoonmaakartikelen BV, Amsterdam, NL). The seeds were sown on half-strength Murashige and Skoog basal agar-solidified medium (Sigma Aldrich, St. Louis, MO, USA). The plates with seeds were subsequently incubated in a climate chamber (Sanyo MLR-352H; Panasonic, Osaka, Japan) under controlled conditions (light 24℃ 16 h, dark 16℃ 8 h). Seven two-week-old seedlings with roots of approximately ~0.5 cm length that were free of visible contaminations were transplanted to the middle compartment of the microcosms. The plants in the microcosms were allowed to grow in greenhouse (Reckenholze, Agroscope, Zürich, CH) with a 16hr photoperiod at 24℃ alternated with 8 h of darkness at 16℃. Plants were watered with 120 ml H_2O 2-3 times per week.

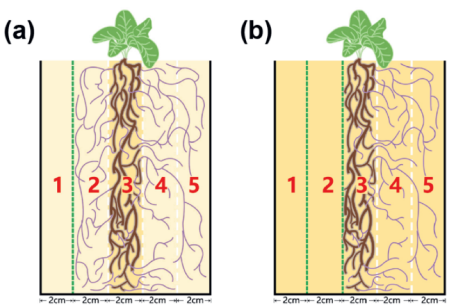

Fig. 1 Schematic representation of 5-compartment microcosm layout in Experiment I and II.

(a) Experiment I. Only COMP3 is filled with 30% of either OS or CS soil, whereas COMP1,2,4 and 5 are filled with sterilized outer substrate. Roots are contained in COMP3 by 30µm meshes (white dashed lines), whereas extraradical AMF hyphae are restricted from COMP1 by a 1 µm filter (green dashed line). **(b)** All compartments were filled with 30% non-sterilized OS, mixed with Oil-Dri and sand. Roots are contained in COMP3 by 30µm meshes (white dashed lines), whereas extraradical AMF hyphae are restricted from COMP1 and 2 by a 1 µm filter (green dashed line).

Experiment II

To investigate the effect of an actively growing AMF mycelium on the indigenous soil microbiome, we filled each of the compartments of the microcosm described above with 750 g of a mixture of 30% non-autoclaved OS, 4% autoclaved Oil-Dri (Damolin GmbH, Oberhausen, Germany) and 66% autoclaved sand. In this experiment, COMP1 and COMP2, and COMP2 and COMP3 were separated by 1µm nylon filters to generate two AMF-free compartments. COMP3 and COMP4, and COMP4 and COMP5 were separated by 30µm nylon filters to create 2 compartments that could be colonized by extraradical AMF hyphae (Fig. 1b). We set up 11 biological replicates with Prunella plants in the center compartment (as described above) and 5 biological replicates of unplanted control. The plant growth conditions were similar to those described above for Experiment I, but the experiment was executed in a greenhouse at botanical gardens of Utrecht university.

Harvest and mycorrhizal root colonization analysis

In both experiments, the shoots of 3-month-old plants were cut at the soil surface, dried at 70℃ for 48 h, and weighed. The microcosm soil was sampled by deconstructing the microcosm compartment by compartment, homogenizing the soil of each compartment, and collecting approximately 500 mg of soil in 2 ml tubes. For sampling of AMF hyphae, 30 g of soil substrate

was collected from COMP5 and stored in a 50ml tube at -20℃. The plant roots in COMP3 were collected by carefully removing soil from the roots and rinsing them under the running tap. For each microcosm, a 1 cm fragment of the rinsed root was cut weighed and stored in 50% ethanol for mycorrhizal root colonization analysis. Another 1 cm fragment of roots was cut, weighed, and stored at -80℃ for root microbiome analysis. The rest of the roots were weighed, dried at 70℃ for 48hr and weighed again. From this root water content was determined and the total root dry weight was calculated based on the combined fresh weight of all three root samples.

To check the mycorrhizal colonization of roots, the root fragments stored in 50% ethanol were cleared in 10% KOH and stained with 5% ink-vinegar following a protocol described by Vierheilig *et al*. (1998). The percentage of total mycorrhiza colonization and frequency of hyphae, arbuscules and vesicles were scored following the line-intersection method by checking 100 intercepts per sample (McGonigle *et al*., 1990).

Sampling of fungal hyphae from soil substrate

To sample fungal hyphae, we modified a wet sieving protocol typically used to collect mycorrhiza spores (Pacioni, 1992). The schematic graph of the fungal hyphae extraction procedure is shown in Fig. S1. Briefly, 500µm, 250µm, and 36µm sieves were surface sterilized to minimize irrelevant environmental microbes present in a hyphal sample by submersing in 0.5% sodium hypochlorite for 20 mins, then submersed in 70% Ethanol for 10 mins (Wagg *et al*., 2014). The sieves were stacked together with the biggest filter size on top and the smallest filter size at the bottom. Twenty-five g of soil substrate from COMP5 was placed on the top sieve. The small particles were washed down, and soil aggregates were broken down with sterilized water. The leftovers on all sieves were washed off into Petri dishes. Then, approximately 0.1 ml hyphae were picked from the samples in the Petri dishes using a set of flame-sterilized tweezers under a binocular microscope. We concentrated the hyphae in a single 1.5 ml tube filled with 0.2 ml 30% glycerin per compartment. This was then considered a hyphal sample (supplementary Fig. S2). The hyphal samples were stored at -80℃ until DNA extraction.

Soil, root, and hyphal microbiome profiling

For experiment I, the soil and root samples from COMP3 and concentrated hyphae samples from COMP5 were characterized by conducting 16S, ITS and 18S amplicon sequencing. For experiment II, the soil samples (both planted and unplanted soil) from COMP1, 2, 3, 4, 5, root samples from COMP3 and concentrated hyphae samples from COMP5 were characterized by conducting 16S and ITS amplicon sequencing. DNA extraction from soil, root and hyphal samples was performed using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany). The root and soil samples were homogenized in PowerBead solution for 10 mins at 30 m/s twice by Tissuelyser II. The hyphal samples were homogenized in PowerBead solution for 2 mins at 30 m/s 4 times by Tissuelyser II. The rest DNA extraction steps of the aforementioned samples were following the manufacturer's instructions. Extracted DNA was quantified using

Qubit dsDNA BR Assay Kit and Qubit Flex Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

DNA was amplified following a two-step PCR protocol. In the first step, we amplified bacterial 16S rRNA gene V3-V4 region (341F and 806R; Herlemann *et al*., 2011), fungal ITS2 (5.8SFun and ITS4Fun; Gao *et al*., 2019) or protistan 18S rRNA gene V4 region (V4_1f and TAReukREV3) (Xiong *et al*., 2020) using primers described in Supplementary Table S1. The microbial communities were amplified in 24 µl reaction volume containing 7.5 ng DNA template, 12 µl KAPA HiFi HotStart ReadyMix (F. Hoffmann-La Roche AG, Basel, Switzerland), 2.5 µl 2 µM (bacterial and fungal)/0.8 µl 10 µM (protistan) forward and reverse primers and the rest volume were supplemented by MilliQ-purified water. The resulting PCR products were purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK) according to the manufacturer's instructions. The purified PCR products were then used as template DNA in the second PCR. The second PCR was performed similar as abovementioned but using primers from the Illumina Nextera Index Kit v2 which contain an error-tolerant 6-mer barcode to allow multiplexed library sequencing. The resulting PCR products were then cleaned-up again using AMPure XP beads. The two step PCR were processed on a thermocycler (Hybaid, Ashford, UK) with cycling conditions as described in supplementary Table S2. The cleaned-up PCR products were quantified using Qubit dsDNA BR Assay Kit and Qubit Flex Fluorometer. Equal amounts of PCR product (2 µl 4nM) were pooled and sequenced on an Illumina MiSeq Sequencer (Illumina, San Diego, USA) using a paired-end 300bp V3 kit at Utrecht Sequencing Facility (www.useq.nl).

Bioinformatics

Sequence reads were processed in the Qiime2 environment (version 2019.07, https://qiime2.org/) (Bolyen *et al*., 2019). We used the Demux plugin to assess paired-end sequence quality. The imported primer sequences were removed using Cutadapt (Martin, 2011). The paired-end sequences were dereplicated and chimeras were filtered using the Dada2 denoise-paired script (Callahan *et al*., 2016), which resulted in the identification of amplicon sequence variants (ASVs) and a count table thereof. Fungal ITS2 sequences were further processed by filtering nonfungal sequences using ITSx (Bengtsson-Palme *et al*., 2013). 16S, ITS2 and 18S ASVs were taxonomically annotated employing a pre-trained naive Bayes classifier (Werner *et al*., 2011) against, respectively, the SILVA (v132) (Quast *et al*., 2013), UNITE (v8) (Kõljalg *et al*., 2013) and PR2 databases (v4.12) (Guillou *et al*., 2013). From this taxonomic annotation, 16S ASVs assigned as mitochondria and chloroplast were removed, 18S ASVs assigned as Rhodophyta, Streptophyta, Metazoa, Fungi and *Embryophyceae* were removed. The raw sequencing data were deposited at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) by the study PRJEB59555.

Statistics

All statistical analyses were conducted in R version 4.0.2 (For, R Foundation Statistical Computing, Vienna, 2020). All bioinformatic files generated by Qiime2 were imported to R

with Qiime2R (Jordan E Bisanz, 2018). Bray-Curtis distances were calculated by and visualized in principal coordinate analysis (PCoA) using the *Phyloseq* package (McMurdie & Holmes, 2013). Pairwise permutational analysis of variance (PERMANOVA) was performed using Adonis function in the Vegan package with 9999 permutations (Oksanen *et al*., 2013). *Indicspecies* was used for correlation-based indicator species analysis (De Cáceres & Legendre, 2009). The visualization of microbial taxonomy and differentially abundant ASVs between sample types used ggplot2 (Wickham, 2008) and Complex Heatmap package (Gu *et al*., 2016).

Results

Experiment I: AMF-associated microbes on extraradical hyphae in a sterilized soil substrate

To understand the role of mycorrhizal hyphae in shaping the soil microbiome, we grew Prunella plants in the middle compartment of a 5-compartment microcosm. Prunella plants in the middle compartment (COMP3) grew in either organic or conventional soil (OS or CS) substrate, whereas the other compartments were filled with soil substrate that was sterilized to reduce soil fungistasis (Garbeva *et al*., 2011) and promote colonization of these compartments by extraradical AMF hyphae. The soil in this experiment was taken from the Agroscope long term farming system and tillage (FAST) experiment at Reckenholz, Switzerland, and was harvested from plots that had been managed with either organic or conventional cultivation practices since the summer of 2009. Compartments were separated by a 30µm nylon filter that restrained the growth of roots to COMP3 but allowed extraradical hyphae to pass through to compartments 4 and 5 (COMP4 and COMP5).

We cultivated the Prunella plants for 3 months, and subsequently sampled the roots and soil in COMP3 and hyphae in COMP5, in which extraradical AMF hyphae could be found and that was arguably shielded from direct influence of root exudates by buffer COMP4. We isolated DNA from these samples and subsequently analyzed the composition of fungal, bacterial and protistan communities by sequencing ITS, 16S and 18S amplicons, respectively.

After sequence denoising and filtering of non-fungal reads, we obtained 981,508 reads from fungal communities from all 36 samples together. As root samples produced relatively high numbers of non-fungal ITS sequences and low numbers of fungal reads, all ITS data were rarefied to a sequencing depth of 500 reads. This sequencing depth was sufficient for community composition analysis as confirmed by the rarefaction curve (Fig. S3a). Similarly, we obtained 1,816,422 16S rRNA gene sequences bacterial community that were rarefied to sequence depth of 3800 before further analysis (Fig. S3b). We also analyzed the extracted DNA for protistan communities associated with plant roots, fungal hyphae and soil samples. For protistan community analysis, on average 99.13% of the reads in the root samples were plant reads and these root samples were not considered for further analysis. After denoising and filtering of Rhodophyta, Streptophyta, Metazoa, Fungi, and *Embryophyceae* sequences,

347,684 18S sequences remained from the soil and hyphal samples and these data was rarefied to a sequence depth of 970 for further analysis (Fig. S3c).

Glomeromycota abundantly present in hyphal and root samples

Glomeromycota, the fungal phylum to which all AMF belong, were detected at 71% average relative abundance (RA) of the root fungal community whereas average 51% of the fungal reads in the hyphal samples of COMP 4 and 5 were annotated as *Glomeromycota,* making this taxon the dominant fungal phylum in both the root and hyphal samples. In soil samples from COMP3, however, this phylum was below 1% in 12 out of 14 samples **(**Fig. 2a). This shows that AMF, although lowly abundant in the FAST soil, are strong colonizers of plant roots and over the course of the experiment had become the dominant fungi living on the root. Moreover, AMF hyphae had grown and extended from the roots in COMP3 to COMP5, where we were able to sample these hyphae. Within the *Glomeromycota*, we found sequences belonging to two prevalent AMF species. *Rhizophagus irregularis* (average RA: 42% in root and 36% in hyphal samples, respectively) and *Septoglomus viscosum* (average RA: 25 % in root and 14% in hyphal samples, respectively) were the most abundant species in the fungal community. In addition to *Glomeromycota, Chytridiomycota* also take up a considerable percentage of the reads in some of our hyphal and soil samples but were hardly detected on the roots. It is hard to morphologically distinguish hyphae of *Glomeromycota* from those of other fungi, and that part of the picked hyphae belonged to non-mycorrhizal species that colonized the sterilized substrate in COMP4 and COMP5 from the soil in comp3.

Hyphae harbor a distinct microbial community

We hypothesized that the hyphal samples include the microbes that live around and attached to the mycorrhizal fungi, whereas the root samples additionally include those microbes that are promoted by the roots themselves. The fungal PCoA plot shows a clear separation between the soil, root and hyphal sample types (Fig. 2c). Sample types significantly explained 42.9% of the variation within the fungal community (PERMANOVA, R^2=0.429, F = 12.416, $p < 0.001$) and each of the sample types was significantly distinct from the two other sample types (Table S3). This shows that there is a significant rhizosphere effect shaping the fungal community on the root, but also that the hyphal samples consist of a fungal community that is different from the root samples. In the 16S amplicon data, we also observed a clear separation of bacterial communities between all sample types in the PCoA plot (Fig. 2d). Almost half (49.6%) of the variation is explained by sample type (PERMANOVA, R^2=0.496, F = 18.751, $p < 0.001$) and a pairwise PERMANOVA test shows that all sample types (root, soil and hyphal) are significantly different from each other (Table S3). This shows that indeed the hyphae picked from COMP5 harbor a bacterial community distinct from those in the root and soil samples. Also, the protistan communities are significantly different in the hyphal and soil samples. Here, sample type significantly explained 30.9% of the observed variation (PERMANOVA, R^2 = 0.309, F = 9.883, $p < 0.001$). Protistan communities of root samples were not considered in this analysis as the 18S data of root samples were dominated by plant reads.

Together our data show that the fungal, bacterial and protistan communities in our hyphal samples were distinct from both the root and soils samples. This suggests that the hyphae assemble a specific microbiome separate from the roots. However, for the fungal community analysis of the hyphal samples, it is difficult to disentangle the fungal reads that belong to the picked hyphae from those representing fungal species associated with these hyphae.

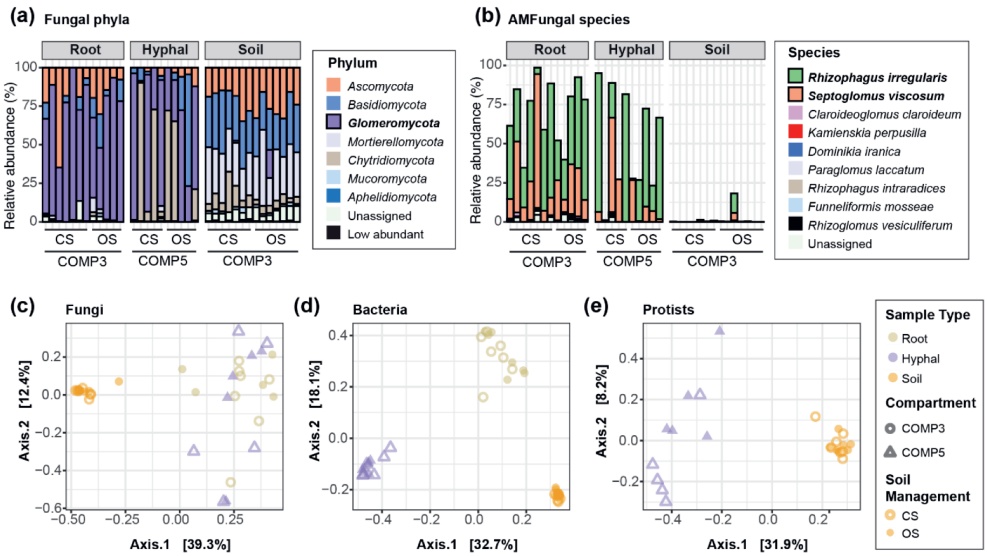

Fig. 2. Hyphal microbial communities differentiate from root and soil microbial communities in Experiment I.

(a) Relative abundance of fungal phyla in root, soil and hyphal samples in Experiment I. Colors represent the distinct phyla. Phyla with relative abundance below 1% were aggregated and categorized as low abundant. **(b)** Relative abundance of *Glomeromycota* spp. in root, soil and hyphal samples in Experiment I. Colors represent the distinct AM fungal species. **(c)** PCoA of fungal communities using Bray-Curtis distances in root, soil and hyphal samples of plants growing in either CS or OS. **(d)** PCoA of bacterial communities in root, soil and hyphal samples of plants growing in either CS or OS. **(e)** PCoA of protist communities in soil and hyphal samples of plants growing in either CS or OS. Colors in c-e indicate different sample types. Shapes depicts the compartments. Open circle stands for microcosms containing CS, closed circles stands microcosms with OS.

Field management type shapes the microbiome of soil, but not of hyphae and roots

Previous work demonstrated that the soil microbiome is affected by soil management practices (Hartman *et al*., 2018). The long-term FAST experiment contains plots that have been managed using either conventional or organic cultivation practices for over a decade. In our experiment, we filled microcosm with either OS or CS from this experiment, to study the influence of management practices on rhizosphere and hyphosphere microbiome composition. At the end of our experiment and following 3 months of Prunella cultivation in the greenhouse that the soil in COMP3 is still significantly influenced by preceding management practices in the FAST experiment. This is evidenced by a significant difference in the fungal, bacterial as well as protistan community composition between OS and CS samples (Fig. 3a, 3c, 3e; Table S4). In the fungal soil community, we found that the fungal genera *Absidia, Cladorrhinum, Cunninghamella* and *Paramyrothecium* are enriched in OS, whereas *Ganoderma, Myxarium, Psathyrella, Rhizopus, Solicoccozyma, Trichoderma* are more abundant in CS (Fig. 3b). For bacterial soil community, we found bacterial classes of *ABY1, Acidimicrobiia, Alphaproteobacteria, Clostridia, Erysipelotrichia* are enriched in OS whereas *Parcubacteria* and *WWE3* are enriched in CS (Fig. 3d; Table S4). For protistan communities, we find *Archaeplastida* enriched in CS and *Stramenopiles* enriched OS (Fig. 3f; Table S4). Remarkably, we did not find significant effects on soil management in the root and hyphal samples (Table S4). One explanation is that the signature of soil management type on soil microbiome disappears while root and hyphae selectively assemble their microbiomes, even though the distinction between OS and CS can still be observed in the soil in between roots in COMP3 (Fig. S4). Alternatively, microbes on the hyphae that differentiate between OS and CS do not disperse quickly or are not well adapted to the new soil conditions in COMP5 and as such differences are confined to COMP3 with the original soil inoculum. Moreover, the microbial difference between OS and CS soil affected neither mycorrhizal colonization nor plant performance (Fig. S5).

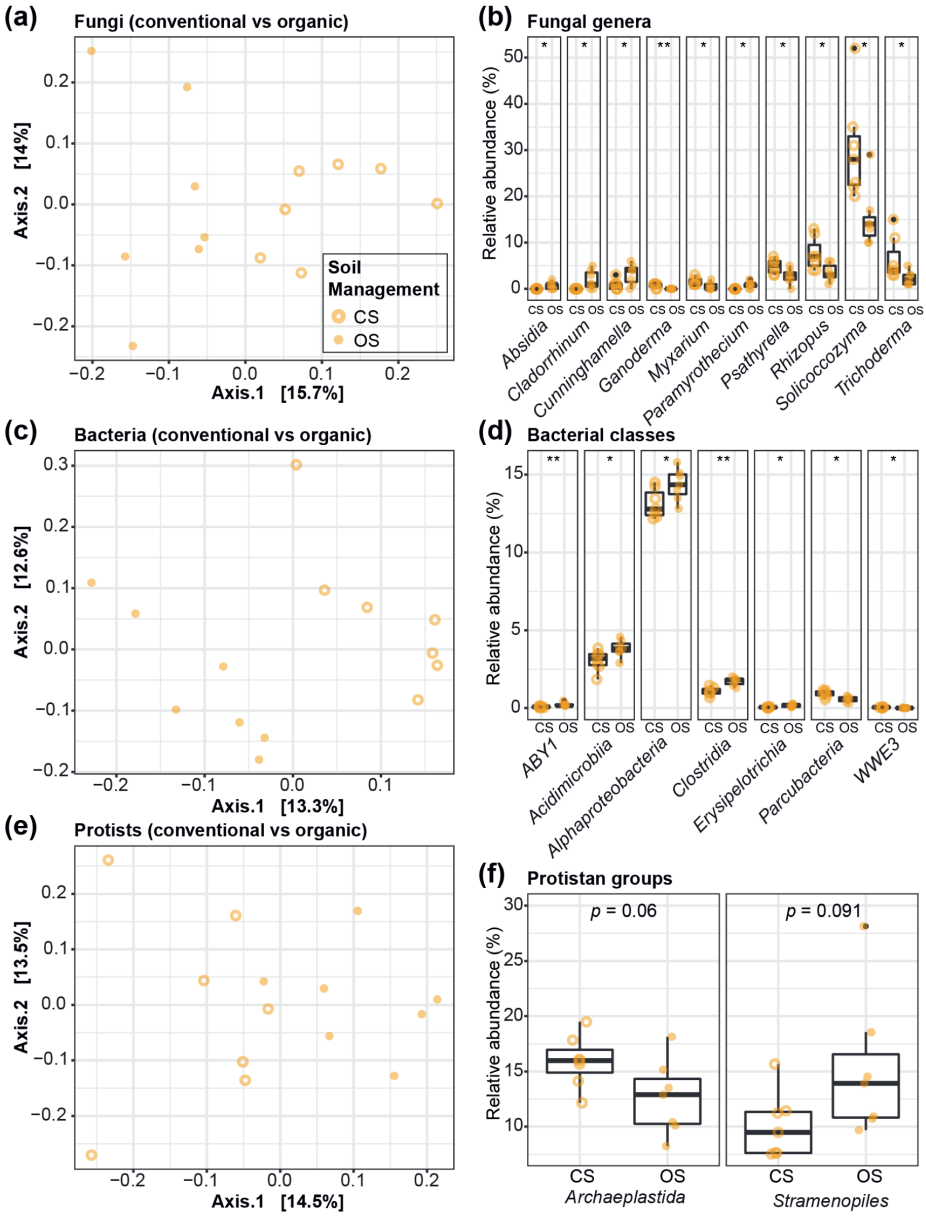

Fig. 3. The soil management effects on soil microbial communities in Experiment I.

(a) PCoA of fungal communities using Bray-Curtis distances in CS and OS. **(b)** Relative abundance of fungal genera that are differentially abundant between CS and OS. **(c)** PCoA of bacterial communities using Bray-Curtis distances in CS and OS **(d)** Soil bacterial community differential abundant classes in CS and OS. **(e)** PCoA of protistan communities using Bray-Curtis distances in CS and OS. **(f)** Soil protistan community differential abundant groups in CS and OS. Open orange circle stands for samples planted in CS, closed orange circles stands for samples planted in OS. The number of y-axes in **(b), (d), (f)** show the percentage of relative abundance (%). The asterisk representing *p*-value in **(b), (d), (f)** are determined by Wilcoxon text (p* < 0.05, p** < 0.01).

Specific protist groups are enriched on hyphae

As a critical component of most soil microbial communities, the protists are often overlooked. Here, we investigated the hyphal protist community and found that there are 7 protist groups that together comprise 99.3% of the protistan RA in hyphal samples. These top-seven most-abundant groups are *Rhizaria, Alveolata, Stramenopiles, Archaeplastida*, *Amoebozoa, Hacrobia* and *Opisthokonta* (Fig. S6a). Of these seven taxa, only the *Hacrobia* shows significantly higher abundance in hyphal than soil samples (t-test, $p < 0.05$; Fig. S6c). At the highest taxonomic resolution, we found 210 protistan ASVs in the hyphal samples of which 80 ASVs are also detected in soil samples (Fig. S6d). These 80 shared ASVs represent 64.7% relative abundance (RA) on hyphal samples and 31.9% RA on soil samples (Fig S6e), which implies that the majority of the hyphal protistan community in COMP5 are derived from the soil samples in COMP3. Subsequently, we used *Indicspecies* to identify protistan ASVs that are positively correlated with either hyphal or soil samples. In this way we found that 201 of the total number of 1168 ASVs had a significantly different abundance between hyphal and soil samples. Of those 201 ASVs, 16 ASVs were significantly enriched on the hyphae. These ASVs belonged mostly to the protistan groups *Rhizaria, Alveolata, Stramenopiles* and *Archaeplastida* respectively (Fig. S6b).

Experiment II: Hyphae-associated microbes on extraradical hyphae in non-sterilized soil substrate

In the experiment described above, we found that fungal hyphae that grew from Prunella roots into a sterilized soil substrate harbor a microbial community that is distinct from the soil community that held the Prunella roots and from the community on the roots themselves. We followed up on this experiment to assess how the soil surrounding roots and hyphae is affected by the plant-fungus symbiont. To this end, we again planted 2-weeks-old Prunella seedlings in the middle compartment (COMP3) of 5-compartment microcosms, but now filled all compartments with the same non-sterilized OS substrate. Again, the roots were restrained to COMP3 by filters with 30µm pore size that did allow extraradical growth of fungal hyphae to COMP4 and 5. Moreover, filters with 1 µm pore size prevented the growth of hyphae into COMPs 1 and 2 (Fig. 2b). We thus hoped to create compartments in each microcosm in which the soil microbiome was shaped by the combination of root, hyphae and their combined exudates (COMP3), by plant-associated hyphae alone (COMP5), or by neither (COMP1). We hypothesized that only buffer COMPs 2 and 4 would be affected by root exudates, of which COMP4 would additionally be shaped by the plant-associated hyphae that pass through them. We left 5 replicates unplanted as a control. After 3 months of Prunella cultivation, we isolated DNA from soil samples of each compartment and in addition from COMP3 root samples and COMP5 hyphal samples. As we were unable to pick hyphae from unplanted microcosms, we were unable to obtain hyphal samples from unplanted microcosms and we assume that most picked hyphae in the microcosms with Prunella plants belong to plant-associated fungi. The ITS and 16S amplicon were sequenced. After filtering, we obtained 5,639,844 fungal and 1,256,644 bacterial sequences, that were rarified to 6400 and 3800, respectively (Fig. S3 b, d).

Glomeromycota again dominated the fungal community of both root and hyphal samples (RA of 61% and 40%, respectively; Fig. 4a). In addition to the *Glomeromycota* spp. that were also found in our first experiment, *Rhizophagus irregularis* and *Septoglomus viscosum,* also *Funneliformis mosseae* was abundantly present in the root and hyphal samples of experiment II (Fig. 4b). Again, the hyphal samples consisted of fungal and bacterial communities that were not only significantly different from root samples, but also from the soil microbial communities in the compartment from which they were acquired (Fig. 4c, 4d, Table S5).

In contrast to our expectations, we did not find a strong influence of plant growth on the soil microbial communities. Whereas sample type (root, hyphal, or soil) explained 40,8 % of the variation in fungal communities and 18% of the bacterial community over all compartments, the presence of Prunella roots explained only 2% of the difference between unplanted and planted microcosms for fungal reads and 1.7% of the difference for bacterial reads (Table S6). Moreover, the fungal and bacterial communities of the 5 distinct compartments in the microcosms with plants were not significantly different from each other (PERMANOVA; Fungi, $R^2 = 0.077$, $F = 1.052$, $p = 0.257$; Bacteria, $R^2 = 0.087$, $F = 1.095$, $p = 0.101$), whereas all soil samples group together and away from the root and hyphal samples in PCoA (Fig. 4a, 4b). Nonetheless, both the bacterial and fungal community in the root containing COMP3 (Fig. S4) differed significantly from COMP3 soil communities of unplanted microcosms (Table S6). Moreover, the fungal community of COMP4 and the bacterial community in COMP2 were significantly affected by the presence of Prunella roots in the adjacent COMP3 and differed significantly from the same compartments in the unplanted microcosms (Table S6). This shows that roots do affect the soil microbial community of COMP3 and that root exudates can, to a lesser extent, also reach and affect the microbial communities of the adjacent COMPs 2 and 4. The roots however do not affect the outer COMPs 1 and 5. However, we were able to isolate hyphae from COMP5 and these hyphae harbor a microbial community that is distinct from the surrounding soil (Fig. 4d).

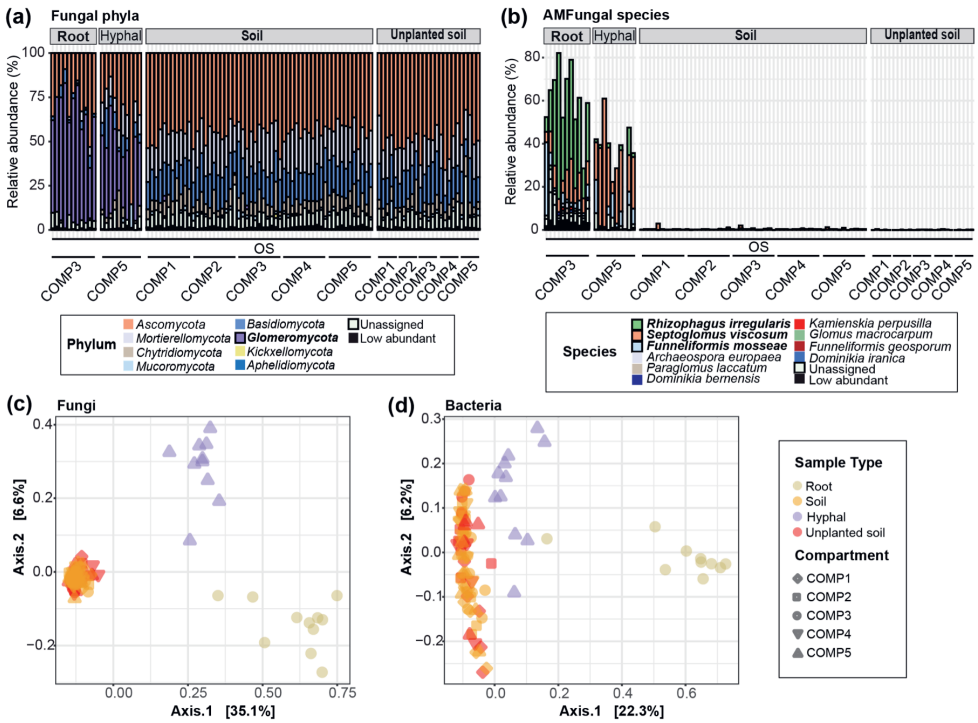

Fig. 4. Microbial communities of root, hyphal and soil samples in Experiment II.

(a) Relative abundance of fungal phyla in root, soil and hyphal samples in Experiment II. Colors represent the distinct phyla. Phyla with relative abundance below 1% were aggregated and categorized as low abundant. **(b)** Relative abundance of *Glomeromycota* spp. in root, soil and hyphal samples in Experiment II. Colors represents the distinct AM fungal species. **(c)** PCoA of fungal communities using Bray-Curtis distances in root, soil and hyphal samples of plants growing in OS. **(d)** PCoA of bacterial communities in root, soil and hyphal samples of plants growing in OS. Colors in **(c)** and **(d)** indicate different sample types. Shapes in **(c)** and **(d)** depict different compartments.

Soil and root bacterial ASVs predominantly colonize on the hyphae

We subsequently focused on the bacterial communities to better understand hyphal microbiome assembly. In both experiment I and II, we observed that the bacterial community occurring on hyphae is different from those on soil and root samples. In experiment I, we detected a total of 5139 bacterial ASVs of which 289 ASVs occurred in root, soil as well as hyphal samples (Fig. 5a). These shared ASVs account for 33.1 % RA in hyphal samples, and 35.1% RA in root samples, but make up only 10% RA in soil samples. Root and soil samples each uniquely share an additional 241 and 186 bacterial ASVs with hyphal samples. The 241 ASVs shared between roots and hyphae account for 28.6% RA in hyphal samples, whereas they represent only 5.6% RA in root samples. Similarly, the 186 ASVs uniquely shared between soil and hyphae represent 11.2% RA in hyphal samples, but only 2.2% RA in soil samples. In total, more than 70% RA in hyphal samples are taken up by the shared ASVs either from soil or root or both (Fig. 5b). This suggests that most bacteria on hyphae, that were isolated from sterilized

substrate in COMP5 in experiment I, originated from root and soil in COMP3 and travelled over the hyphae to COMP5.

In experiment II, however, all compartments were filled with the same soil substrate. Here, 492 bacterial ASVs were found to occur in root, hyphal as well as soil samples. These ASVs account for averagely 64.2% RA in hyphal samples and 67.1% RA in soil samples, but only 35.3% RA in root samples. The hyphal samples also uniquely share 75 ASV with root and 784 ASVs with soil samples. The 75 ASVs account for 2.7% RA in hyphal samples and 11.1% RA in root samples. The 784 ASVs account for 26.4% RA in hyphal samples and 20.7% RA in soil samples. In total, ASVs that represent more than 90% RA in hyphal samples are also detected root but mostly soil samples. (Fig. 5d). Thus, in this experiment the majority of the bacteria detected on hyphae likely originated from the surrounding soil, but a small minority might have travelled from the root compartment.

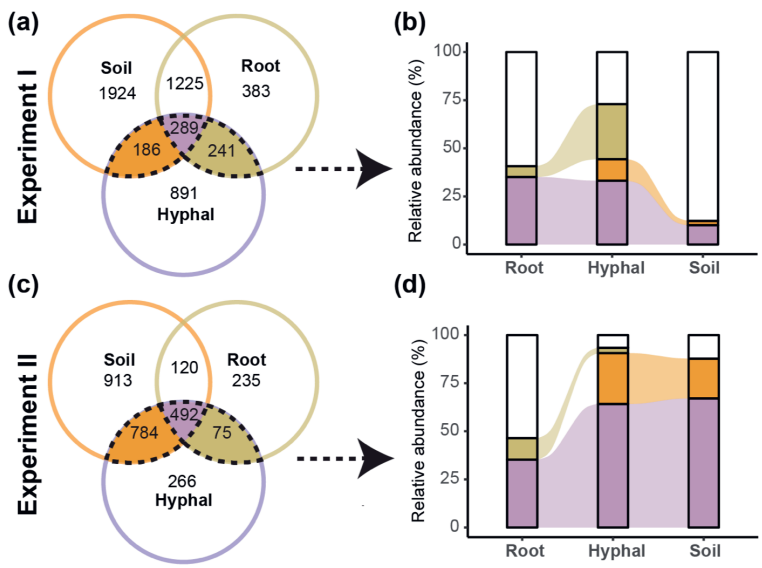

Fig. 5 The hyphal samples shared bacterial ASVs among soil and root samples.

(a) Venn diagram of unique and shared bacterial ASVs in root, hyphal and soil samples of experiment I. The numbers represent ASVs numbers. The colors of rings indicate sample types. The colors framed in dash lines indicates the hyphae bacterial ASVs either shared with soil or root or both. **(b)** Sankey plot of hyphal samples shared ASVs' RA in each sample types. The colors depict the hyphal ASVs either shared with soil or root or both. **(c)** Venn diagram of unique and shared ASVs in root, hyphal and soil samples of experiment II. **(d)** Sankey plot of hyphal samples shared ASVs' RA in each sample types. Only ASVs minimum present in 3 samples are considered here.

Overlap in hyphae associated bacteria between experiments I and II

We subsequently focused on the bacterial communities to identify bacterial taxa that associate with fungal hyphae. We therefore identified 81 bacterial genera that occur in hyphal samples of both experiments (Fig. 6a). In experiment I, these shared hyphal genera together comprised 42.9% RA in hyphal and 19.9% in soil samples, whereas they represent 27.6% of the bacterial RA in hyphal and 16.2% RA in soil samples of Experiment II. Of those 81 genera, the genera *Halangium, Massillia, Pseudomonas, Devosia, SWB02, Cellvibrio,* possible genus 04*, Noviherbaspirillum, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Ohteakwangia, Pseudoxanthomonas, Sulfurifustis* and *Pseudoduganella* were significantly more abundant in hyphal samples than in soil samples in both experiments (Fig. 6b). These 13 consistently enriched hyphal genera represented 24.6% and 5.8% RA in the hyphal samples of, respectively, Experiment I and II, whereas they comprise 1.5% and 0.3% RA in the soil samples of those respective experiments. Interestingly, *Halangium* is consistently the most abundant bacterial genus on hyphae taking up averagely 6.4% and 3% RA in experiment I and II, respectively.

To identify specific bacterial strains associated with fungal hyphae at the highest resolution, we used *Indicspecies* to identify bacterial ASVs that are positively correlated with either hyphal or soil samples. In this way, we found 452 out of a total of 1607 bacterial ASVs to be enriched in the hyphal samples of experiment I and 109 ASVs of 1617 ASVs enriched in experiment II (Fig. 6c, 6d). These enriched ASVs account for an average relative abundance of 80.1% and 20.4% in the hyphal samples of experiment I and II, respectively. Approximately half of the ASVs enriched on hyphae belong to *Proteobacteria* (234 of the 452 in Exp. I and 54 out of 109 in Exp. II). 234 and 54 ASVs of the hyphal enriched ASVs in experiment I and II respectively belong to *Proteobacteria,* but also *Actinobacteria, Planctomycetes* and *Acidobacteria* are well represented among the hyphal enriched ASVs (Fig. 6c, 6d). Only six bacterial ASVs are enriched in the hyphal samples of both experiments. These ASVs are all *Proteobacteria* and belong to the genera *Pesudomonas*, *Devosia*, *Sulfurifustis*, *Phenylobacterium* and uncultured *Myxococcales.*

In summary, certain bacterial genera appear to be consistently enriched in our hyphal samples and they comprise a considerable part of bacterial abundance. The genus of *Halangium* represents the most strongly enriched genus and dominates hyphal samples of two independent experiments. Moreover, the genus *Pseudomonas* and *Devosia* standout as not only these genera are consistently enriched on hyphal samples of both experiment but each of them also comprise a specific ASV that is consistently associated with AMF hyphae.

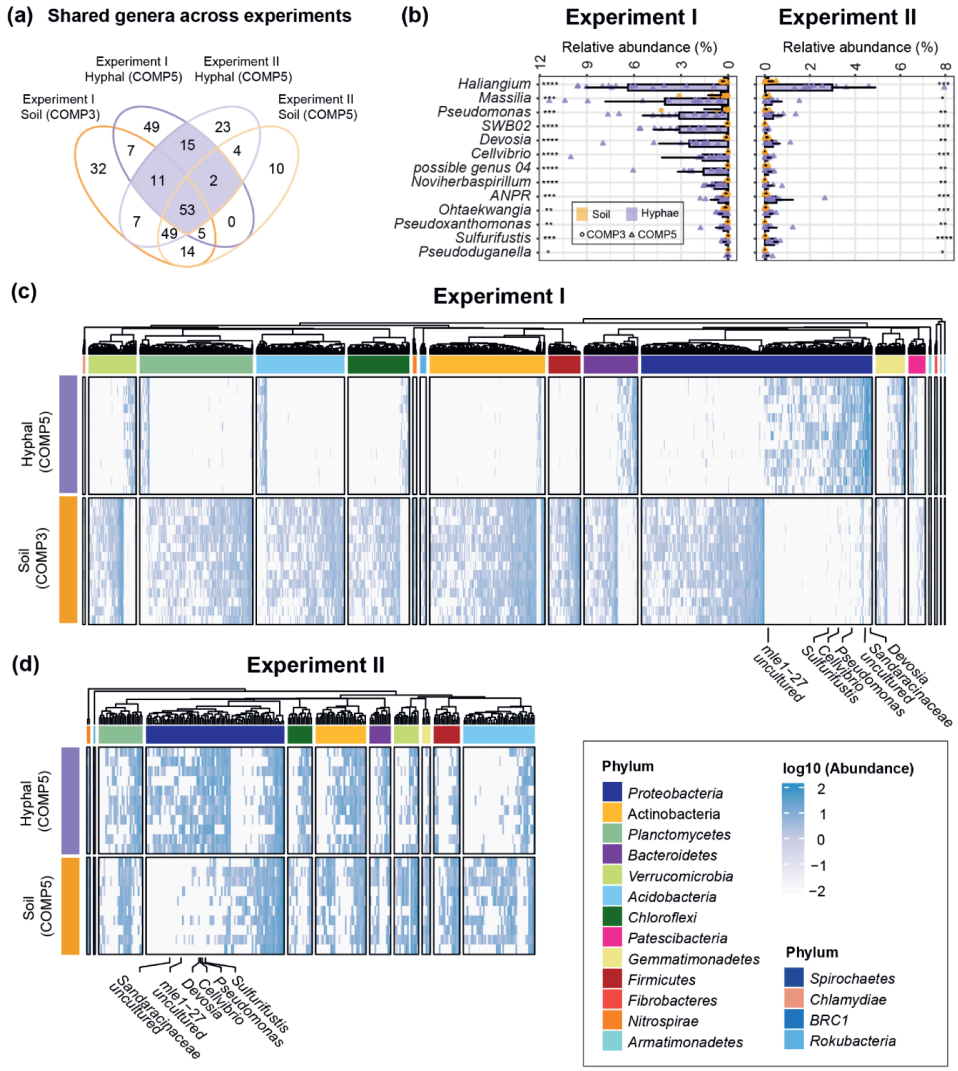

Fig. 6. Specific bacterial genera and ASVs are consistently enriched on hyphae in both experiments.

(a) Venn diagram showing the occurrence of bacterial genera on hyphal and soil samples across 2 experiments. The colors of the rings depict the sample type either from experiment I or experiment II. Genera with relative abundance below 0.1% were aggregated and categorized as lowly abundant. **(b)** Relative abundance of genera that are consistently enriched in hyphal samples across the 2 experiments (wilcox-test, $p^{*} < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$, $p^{****} < 0.0001$, $p^{ns} > 0.05$). Colors indicate sample types; shapes of symbols indicate the microcosms of samples from which they are derived. ANPR*: *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*. Bacterial ASVs that are differentially abundant between hyphal and soil samples in Experiment I **(c)** and II **(d)**. Heatmap shows log-transformed relative abundance of ASVs that significantly associate with either hyphal or soil samples. ASVs are ordered by phylogenetic distance and distinct phyla are indicated by vertical color bars on the left of the heatmap. Six consistently enriched bacterial ASVs are marked with their genus names or higher taxonomic rank when genus could not be identified. Bacterial phyla lower than 1% RA is not considered in the heatmap.

Discussion

In this study, we characterized the fungal, bacterial and protist communities associated with hyphae to understand the role of AMF in microbiome assembly. Moreover, we used soil from a long-term field experiment to understand the effect of organic and conventional management practices on the hyphosphere microbiome. To this end, we performed two separate experiments with 5-compartment microcosms in which plants were growing in-, and restricted to, middle compartment. Extraradical hyphae were allowed to pass through the root-restricting filters in these mesocosms and extend from the middle compartment to the outer compartments from which they were sampled.

We observed that fungal hyphae are enriched with a specific community of bacteria and protists. Although the functionality of protists in terrestrial ecosystem is largely unrevealed (Gao *et al*., 2019) and protists are difficult to extract and cultivate (Geisen & Bonkowski, 2018), we did identify 16 protistan ASVs that were enriched in hyphal samples and these ASV included *Hartmannella* and *Chlorella* spp. Amoebae of the genus *Hartmannella* were previously found to produce ammonium when fed with Enterobacteriaceae, and production of ammonium could thus theoretically improve plant growth (Weekers *et al*., 1993). *Chlorella* green algae can accumulate phosphorus and promote plant growth in cocultivation with rhizobacteria (De Jesus Raposo & De Morais, 2011; Schreiber *et al*., 2018). Although difficult, it would be interesting to investigate whether such severely underexplored microorganisms as protists play a role in the functioning of the plant-AMF symbiosis. Previous studies did also find that the protist community is influenced by AMF (De Gruyter *et al*., 2021), and moreover that vice versa the AMF utilization of organic nitrogen is also influenced by protist (Rozmoš *et al*., 2021b). Such emergent evidence reminds us of the potential importance of protists in plant microbiome functioning.

In contrast to protists, much is known about the interactions of plants with bacteria (Hayat *et al*., 2010) and bacteria have been found to affect AMF and the plant AMF symbiosis (Frey-Klett *et al*., 2007; Zhang *et al*., 2021). We found that the bacterial communities in our hyphal samples are distinct from the surrounding soil and that there thus is selection of specific bacteria on fungal hyphae. The bacterial genus *Haliangium* consistently show up in all our hyphal samples and it was the most abundant bacteria genus of all the genera in hyphal samples in both of our experiments. Although for now only two *Halangium* isolates exist and both are derived from the marine environment (Fudou *et al*., 2002), sequences derived from this genus have frequently been detected in soil samples (Lévesque *et al*., 2020; Qiu *et al*., 2012; Uddin *et al*., 2019). Interestingly, Petters *et al*. (2021) characterized micropredators in European mineral and organic soils using metatranscriptomic identified *Haliangiaceae* and *Polyangiaceae* as potential bacterivores groups in most soils. Moreover, a marine isolate of *Haliangium ochraceum* was found able to prey on 9 out of 11 tested bacterial species. This suggest that *Halangium* spp. could play an important predatory role in shaping soil microbiome and their abundant presence on AMF (Emmett*et al*., 2021) suggest that they particularly do so

on AMF hyphae. Future studies could manipulate presence of these potential bacterial predators on AMF to investigate their effects on plants and their symbionts.

In addition to *Halangium*, also the genera *Pseudomonas* and *Devosia* were constantly enriched in hyphal samples in our experiments. Intriguingly, we could pinpoint a single *Pseudomonas* ASV as well as a *Devosia* ASV that was consistently enriched in our experiments on AMF hyphae. The *Pseudomonas* strains have been identified as mycorrhiza helper bacteria that promote the colonization of ectomycorrhizas and arbuscular mycorrhizae in multiple research (Frey-Klett *et al*., 2007). For instance, when *Pseudomonas putida* was coinoculated with *Glomus fistulosum*, the bacteria promoted hyphal growth of this AMF (Vosátka & Gryndler, 1999). Moreover, *Pseudomonas fluorescens* BBc6R8 was found to promote AMF biomass likely by reducing toxic metabolites in autoclaved soil (Brulé *et al*., 2001). Of the 34 described *Devosia* species (Talwar *et al*., 2020), one was reported to form nodules and promote growth of an aquatic legume plant (Rivas *et al*., 2002), whereas four other isolates were found to reduce nitrate to nitrite (Chen *et al*., 2019; Jia *et al*., 2014; Liu *et al*., 2020; Zhang *et al*., 2012). Both findings suggests that *Devosia* spp. could play a role in the uptake of nitrogen by plant-AMF symbiont.

In both experiments, we investigated microbial communities on roots in the middle compartment of the microcosms. First, we analyzed the fungal communities and found that roots harbor a fungal microbiome that is distinct from that of the surrounding the soil. Moreover, the root fungal microbiomes in both experiments are strongly enriched for and dominated by *Glomeromycota*., the monophyletic fungal taxon that includes all AMF (Taylor*et al*., 2015). This shows that Prunella roots selectively assemble a fungal microbiome from the surrounding soil and that this microbiome is dominated by AMF. Also, the bacterial community on these roots is distinct from the bacterial community of the surrounding soil and the assembly of this community is likely driven by the combined actions of roots and the AMF that dominate these roots.

In both experiments, we also sampled hyphae that grew in the outer compartment (COMP5) of the microcosms. The hyphae sampled were strongly enriched in *Glomeromycota* demonstrating that a large part of these hyphae is likely extraradical hyphae that extend from the roots prunella roots in the microcosms. However, other fungal hyphae (e.g., from the phylum Chytridiomycota (Exp. I) and Ascomycota (Exp. II)) were also detected in the hyphal compartment and as such we cannot conclude whether the detected microbes are specific for AMF or fungi in general.

Root exudates are thought to play a crucial role in shaping the rhizosphere environment (Sasse *et al*., 2018). We confirmed this and found that soil in compartments that contained roots (COMP3) had significantly different microbial communities compared to microbial communities in unplanted compartments. We did not detect the significant difference between fungal compartments and control compartments in EII. This shows that the influence of the hyphae growing into the outer compartment does not extend far from the hyphal surface. In

line with this, previous studies showed that soil microbiome of hyphae-enriched compartments is similar to bulk soil microbiomes (Petters *et al*., 2021; Zhang *et al*., 2018). In contrast, other studies using leguminous plants observed that the microbiomes of plants where AMF had established differed from mutant plants impaired in AMF symbiosis (Thiergart *et al*., 2019; Wang *et al*., 2021). However, legumes can live in symbiosis with both AMF and *Lotus japonica* mutants plants that were specifically impaired in the fungal symbiosis harbored a bacterial root microbiome that was not distinct from that of wild-type plants.

Still, in analogy to the rhizosphere where rhizosphere effect size varies with the plant species (Sasse *et al*., 2018), also the AMF hyphosphere effect size may also vary with the AMF species. The assembly of hyphosphere microbiomes has been investigated on only a few AMF species and a broader range of AMF species needs to be characterized in future (Emmett *et al*., 2021; Rillig *et al*., 2006). Moreover, although the hyphosphere effect was limited to microbes in the immediate proximity of hyphae, we did find that fungal, bacterial and protist communities in the hyphal samples were different from the communities on roots and soil. For the fungal communities, this is a bit difficult to interpret. Although the hyphal samples are clearly enriched for AMF hyphae compared to the soil samples, it is not unlikely that these samples also contain hyphae of saprophytic fungi and of fungi that live in association with either AMF hyphae. It is difficult to distinguish fungal ASV produced by the hyphae from the fungal ASVs associated with the hyphae.

In the first experiment, we used soil from the FAST experiment (Wittwer *et al*., 2021)that was either managed by conventional or organic agricultural practices to fill the middle compartments of the mesocosms. Even after 3 months of *P.vulgaris* growth in this compartment, the microbial community of the soil in the middle compartment was still significantly different as a result of those two management practices. However, these management practices did not have an apparent influence on communities found on roots growing in those soils, nor on the communities on hyphae extending from that compartment. Apparently, both plants and AMF select for microbes that occur in both soil types and this selection negates the difference between the soil communities. It is thus unlikely that these management practices affect functions of the microbiome in the rhizo- or hyphospheres of this experiment. We therefore followed up on this first experiment with a second experiment that only used organically-managed soil.

For now, however, speculations on multipartite interaction between plants, AMF and associated microbes lack experimental evidence, but our findings do show that there are specific microbes that associate with AMF hyphae. To investigate their role in AMF functioning, in chapter 4 we will attempt to isolate AMF-associated microbes and test the effect of these microbes on the plant-AMF symbiosis.

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Authors' contributions

C.Z., C.E.S., R.L.B. and M.G.A.v.d.H. conceived and designed the experiments. C.Z. and B.N.T. performed the experiments. C.Z. analyzed the data. C.Z., M.G.A.v.d.H., and R.L.B. wrote the manuscript.

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Supplementary information

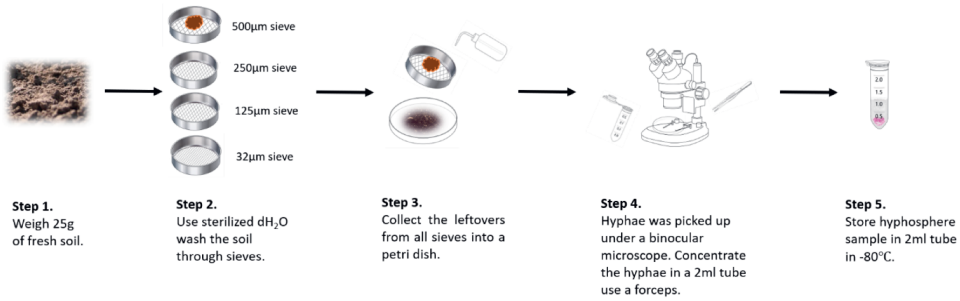

Fig. S1. Schematic flow chart of hyphae sample extraction protocol.

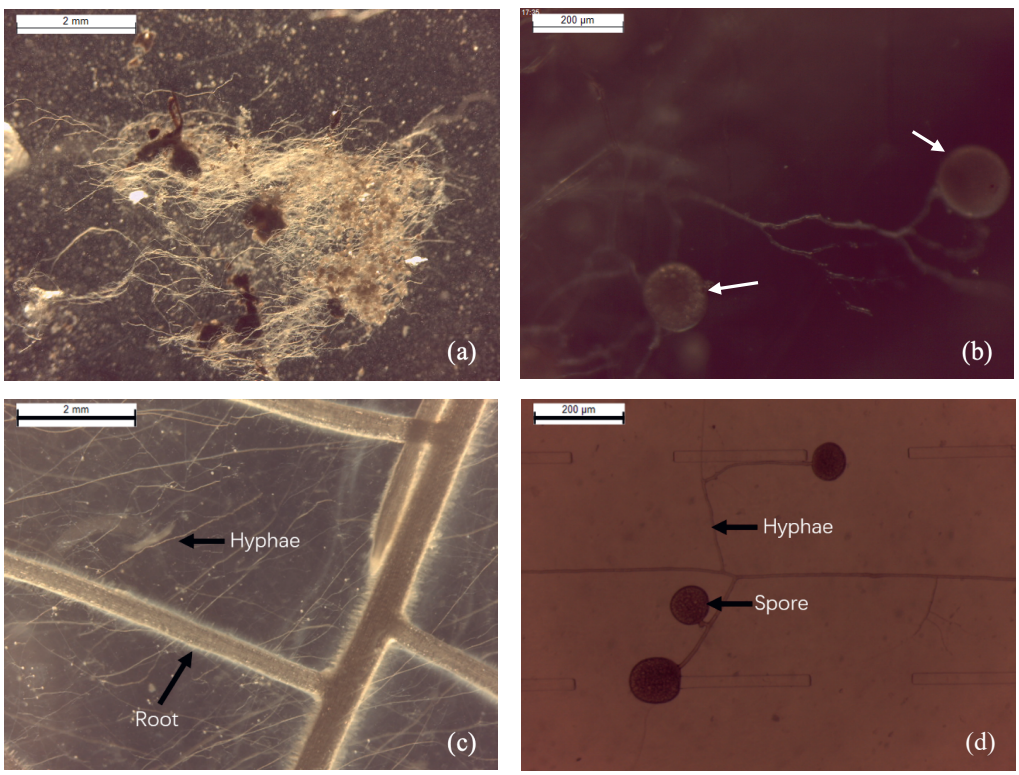

Fig. S2. AMF hyphal sample concentrated under binocular microscope.

(a) Concentrated hyphal sample from COMP5. **(b)** AMF spores on concentrated hyphal sample indicated by arrow. **(c)** and **(d)** are reference AMF morphology cultured on agar medium.

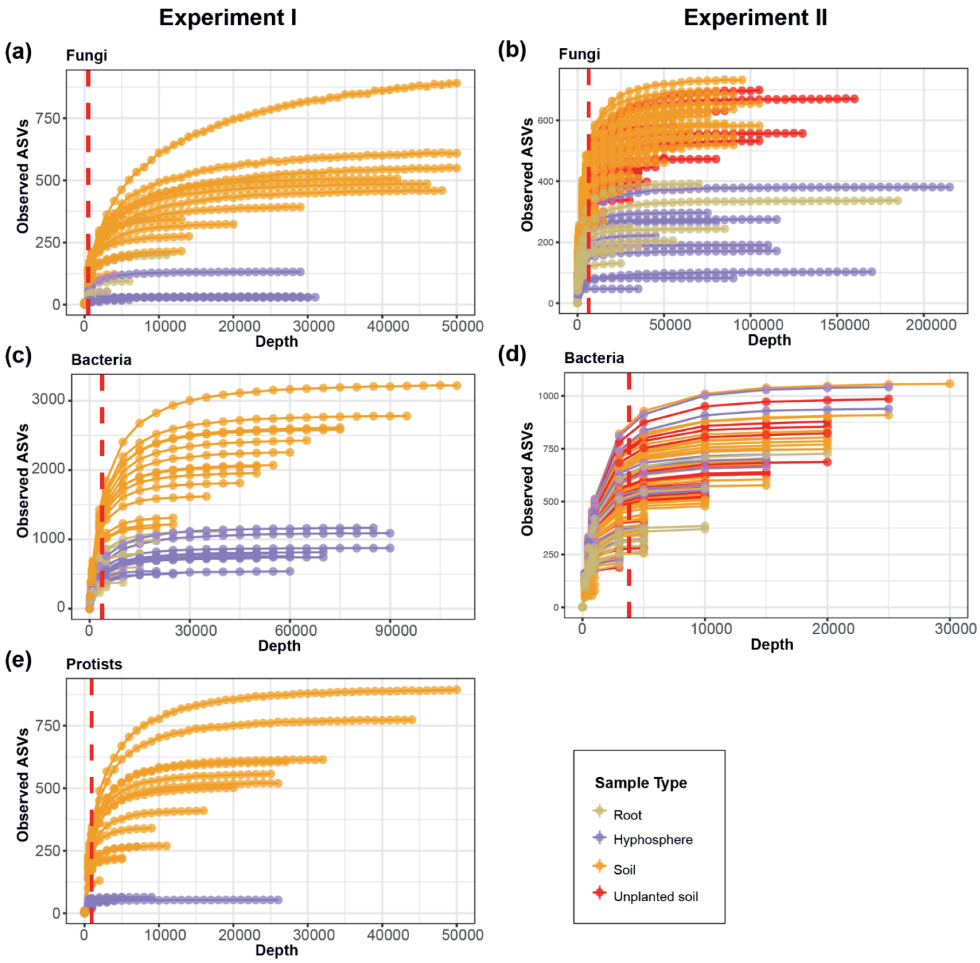

Fig. S3. Rarefaction cure of fungal and bacterial communities.

(a) Experiment I fungal community rarefaction curve. The sample types were colored by four colors. The red dash lines indicate the selected rarefaction depth. **(b)** Experiment II fungal community rarefaction curve. **(c)** Experiment I bacterial community rarefaction curve. **(d)** Experiment II bacterial community rarefaction curve. **(e)** Experiment I protistan community rarefaction curve. The colors represent the sample types.

Fig. S4. Plant roots predominant COMP3.

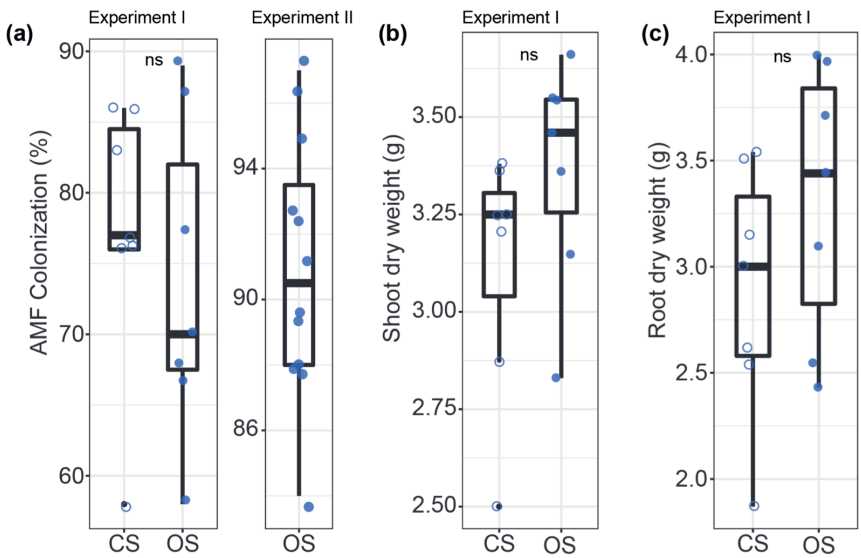

Fig. S5. Effect on soil management practices on mycorrhizal colonization and plant growth compared

(a) AMF colonization in CS and OS in experiment I and II, respectively. **(b)** Shoot and **(c)** root dry weight of 3-months-old Prunella plants growing on CS or OS in experiment I. Box plots of 7 and 12 replicate samples in experiment I and II respectively.

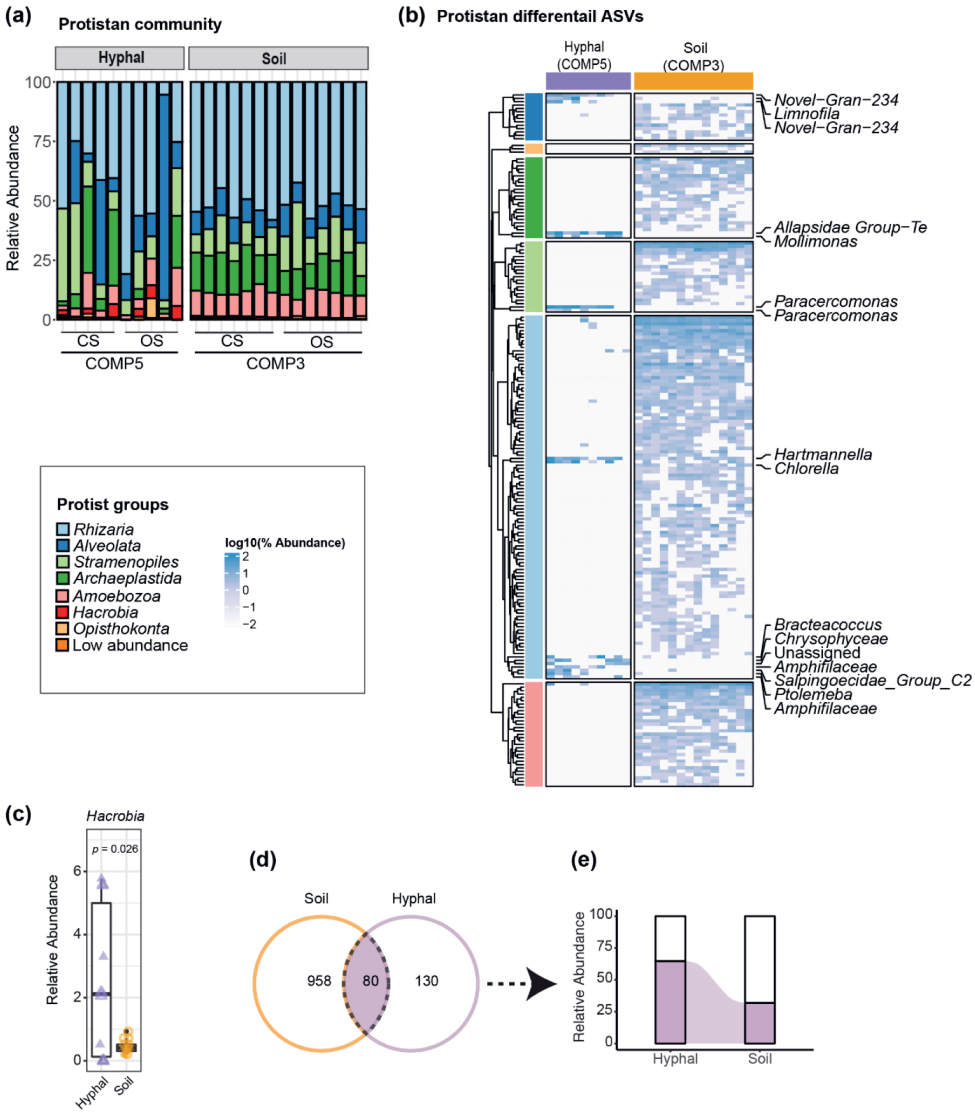

Fig. S6. Differential abundant protist groups between hyphosphere and soil.

(a) Protist groups composition of hyphal and soil samples. **(b)** Differential abundant protistan ASVs between hyphal and soil samples. Color bars on the bar plot and heatmap indicates protists groups. Log^{10} transformed abundance used to presents ASVs' abundance. **(c)** Hacrobia differential abundance in hyphal and soil samples. The colors indicate the sample types. **(d)** Venn diagram of hyphal and soil protistan ASVs. **(e)** Sanky plot of shared ASVs RA in the hyphal and soil samples.

Table S1. Primer used for amplifying ITS, 16S and 18S

Target gene	Primer pairs	Sequence
ITS	5.8S Fun	AACTTTYRRCAAYGGATCWCT
	ITS4 Fun	AGCCTCCGCTTATTGATATGCTTAART
16S	341F	CCTACGGGNGGCWGCAG
	805R	GACTACHVGGGTATCTAATCC
18S	V4_1f	CCAGCASCYGCGGTAATWCC
	TAReukREV3	ACTTTCGTTCTTGATYRA

Table S2. Two step PCR cycling conditions for amplifying ITS, 16S and 18S

First step

ITS			16S			18S		
Temperature	Time	Cycles	Temperature	Time	Cycles	Temperature	Time	Cycles
96℃	2min	1×	95℃	3min	1×	95℃	5min	1×
94℃	30sec		95℃	30sec		95℃	30sec	
58℃	40sec	25×	55℃	30sec	25×	55℃	30sec	25×
72℃	2min		72℃	30sec		72℃	45sec	
72℃	10min	1×	72℃	5min	1×	72℃	10min	1×
15℃	Hold	-	15℃	Hold	-	15℃	Hold	-

Second step

ITS			16S			18S		
Temperature	Time	Cycles	Temperature	Time	Cycles	Temperature	Time	Cycles
95℃	3min	1×	95℃	3min	1×	95℃	3min	1×
95℃	30sec		95℃	30sec		95℃	30sec	
55℃	30sec	10×	55℃	30sec	10×	55℃	30sec	10×
72℃	30sec		72℃	30sec		72℃	30sec	
72℃	5min	1×	72℃	5min	1×	72℃	5min	1×
15℃	Hold	-	15℃	Hold	-	15℃	Hold	-

Table S3. The effects of sample types on microbial communities of experiment I determined by pairwise PERMANOVA on Bray-Curtis distance with 9999 permutations.

Sample type	Fungi			Bacteria			Protist		
	F	R^2	*p*-value	F	R^2	*p*-value			
Root & Hyphal	**2.750**	**0.121**	**0.003*** **	**16.06**	**0.391**	**<0.001*** **	**-**	**-**	**-**
Root & Soil	**22.563**	**0.485**	**<0.001*** **	**16.018**	**0.381**	**<0.001*** **	**-**	**-**	**-**
Soil & Hyphal	**15.584**	**0.415**	**<0.001*** **	**24.456**	**0.495**	**<0.001*** **	**9.9**	**0.309**	**<0.001*** **

Table S4. Soil management effects on microbial communities of experiment I determined by pairwise PERMANOVA on Bray-Curtis distance with 9999 permutations.

CS vs OS	Fungi			Bacteria			Protist		
	F	R^2	*p*-value	F	R^2	*p*-value	F	R^2	*p*-value
Root	1.058	0.096	0.360	1.227	0.093	0.077.	-	-	-
Hyphal	0.523	0.061	0.876	1.019	0.084	0.394	1.325	0.142	0.092
Soil	**1.926**	**0.138**	**<0.001*** **	**1.767**	**0.128**	**<0.001*** **	**1.583**	**0.117**	**0.001****

Table S5. Sample type effects on microbial communities of experiment II determined by pairwise PERMANOVA on Bray-Curtis distance with 9999 permutations.

	Fungal			Bacteria		
Sample type	**F**	**R^2**	***p*-value**	**F**	**R^2**	***p*-value**
Root & Hyphal	**7.182**	**0.274**	**<0.001*** **	**11.389**	**0.375**	**<0.001*** **
Root & Soil	**53.223**	**0.454**	**<0.001*** **	**25.496**	**0.302**	**<0.001*** **
Soil & Hyphal	**22.012**	**0.259**	**<0.001*** **	**7.293**	**0.108**	**<0.001*** **

Table S6. Plant effects on soil microbial communities of experiment II determined by pairwise PERMANOVA on Bray-Curtis distance with 9999 permutations.

Soil vs Unplanted soil	Fungi			Bacteria		
	F	R^2	*p*-value	F	R^2	*p*-value
COMP1	0.093	0.074	0.217	1.155	0.081	0.156
COMP2	**1.387**	**0.090**	**0.032***	0.948	0.068	0.637
COMP3	**1.406**	**0.091**	**0.056.**	**1.537**	**0.099**	**0.003****
COMP4	1.278	0.084	0.114	**1.242**	**0.087**	**0.039***
COMP5	0.989	0.066	0.459	0.975	0.070	0.465

Chapter 4

A bacterial member of the mycorrhizal microbiome promotes plant mycorrhization, nitrogen uptake, and growth

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Abstract

Arbuscular mycorrhiza fungi (AMF) are obligate-biotrophic mutualists that colonize plants and help with nutrient uptake. Previously, we showed AMF hyphae harbor a microbiome that is distinct from that on roots and in the bulk soil. Yet, the functions of this AMF microbiome are still unclear. Here, we isolated 143 bacteria from AMF hyphae. We characterized the microbial collection by sequencing 16S rRNA genes and matched these sequences to previously-generated amplicon data. In this way, we identified 5 isolates that represented bacteria taxa that are consistently enriched on AMF hyphae. Subsequently, we selected and tested these 5 mycorrhiza-associated bacterial isolates and examined their effect on the plant and AMF. Here, in three complementary experiments, we found that one of the AMF hyphae associated bacterial strains, *Devosia* sp. ZB163, synergistically interacts with mycorrhiza to promote plant nutrient uptake and growth. Our results underline that plants and their associated AMF both foster a microbiome that in turn provides important functions for the plant-fungus symbiont. In future, deciphering the functioning of the plant and hyphosphere microbiome will help develop more sustainable agricultural practices.

Keywords: arbuscular mycorrhiza fungi, bacteria, mycorrhization, nitrogen uptake, plant growth

Introduction

There is a large variety of microorganisms present in most soils. Bacteria and fungi dominate these soils and their numbers often exceed respectively 10^9 and 10^6 colony-forming-units per gram of soil (Bhattarai, 2015). Plants intimately interact with the soil microbiome which plays a key role in the performance and fitness of plants (Banerjee & van der Heijden, 2022; Berendsen *et al*., 2012; Liu *et al*., 2020). The plant microbiome can sustain multiple ecosystem functions and play important roles in nutrient cycling and acquisition, but also in the protection of plants against pests and pathogens (Berendsen *et al*., 2012; Pineda *et al*., 2017).

Arbuscular mycorrhiza fungi (AMF) are among the best-studied plant symbionts and their beneficial functioning has been broadly acknowledged (Jacott *et al*., 2017). AMF are known to increase plant water and nutrient uptake (Kakouridis *et al*., 2022; Cooper, 1978; Dierks *et al*., 2022; Frey & Schüepp, 1993; George *e t al*., 1994; Govindarajulu *et al*., 2005a), increase drought and salinity tolerance (Begum *et al*., 2019; Del Val *et al*., 1999; Hajiboland *et al*., 2010; Liu *et al*., 2020; Mathur *et al*., 2019; Ouziad *et al*., 2005; Talaat & Shawky, 2014) and increase resistance to root and foliar pathogens (Campos-Soriano *et al*., 2012; Fritz *et al*., 2006; Norman *et al*., 1996; Song *et al*., 2015; Vigo *et al*., 2000). However, the role of other microbes in the functioning of AMF has often been overlooked. Nonetheless, it is known that other plant symbionts can affect AMF functioning. For instance, the sorghum-associated endophyte, *Azospirillum brasilense,* increased plant dry weight, shoot-to-root ratios, and the nitrogen (N) content when co-inoculated with AMF (Pacovsky *et al*., 1985). Moreover, in model grassland communities, the combination of N-fixing rhizobia and AMF resulted in increased plant

diversity and improved nutrient uptake, suggesting that these two types of plant symbionts function in a complementary manner (van der Heijden *et al*., 2016).

Like plant roots, also AMF themselves harbor specific microbiomes. The AMF-associated microbes can live either intracellularly or on the outside of their hyphae. Two groups of intracellular endobacteria have been discovered on AM fungal hosts and both groups are obligate endosymbionts. *Burkholderia*-related endobacteria have been found only on members of the family *Gigasporaceae* (Bonfante *et al*., 1994; Mondo *et al*., 2012). *Mycoplasma*-related endobacteria on the other hand are more widely distributed among AM fungal species (Macdonald., 1982). The presence of *Burkholderia*-related endobacteria enhances the growth of the host fungal hyphae, whereas *Mycoplasma*-related endobacteria seems to inhibit the growth of these hyphae (Desirò *et al*., 2018; Lumini *et al*., 2007; Torres-Cortés *et al*., 2015). In addition, external mycorrhizal fungal hyphae are colonized by specific hyphal microbiomes (Emmett *et al*., 2021; Nuccio *et al*., 2022; Scheublin *et al*., 2010; Toljander *et al*., 2006; Zhang *et al*., 2021).This showcases the divergent relationship of AMF with their endobacteria. Extracellularly, phosphate-solubilizing bacteria (PSB) have been shown to mineralize organic phosphorus (P) so that inorganic P can subsequently be absorbed by the AM fungal mycelium (Toro *et al*., 1997; Zhang *et al*., 2016). Moreover, several so-called mycorrhization helper bacteria have been identified that can assist and promote the establishment of mycorrhizal symbionts on the plant root. Several bacteria from the genera *Pseudomonas, Bacillus, Paenibacillus, Rhodococcus,* and *Streptomyces* spp. have been found to increase AMF colonization of roots (Frey-Klett *et al*., 2007a; Zhang *et al*., 2022). These bacteria are mostly isolated from either soil, roots, or root nodules, but also bacteria on the outside of the AMF themselves appear to affect AMF functioning. One study observed that a *Bacillus pabuli* isolate LA3, which was isolated from AMF spores, was found to stimulate AM fungal spores' germination (Xavier & Germida, 2003). Moreover, scanning electron microscopy of *Glomus geosporum* suggests that the bacteria associated with these AMF spores are possibly feeding on the outer hyaline wall layer and it was suggested that this may also benefit the germination of these spores (Roesti *et al*., 2005).

However, as it is difficult to isolate fragile hyphae from most soils, it is also difficult to specifically isolate and identify bacteria that associate with AMF hyphae. Consequently, the functioning of the AMF microbiome has rarely been studied. Here, we used a sieving-based strategy to isolate AMF hyphae together with hyphae-adhering bacteria. We mapped the sequence of our isolates to previously-generated 16S amplicon sequence variants (ASVs) that were either enriched in samples of AMF hyphae or of soil (Chapter 3). Subsequently, we screened this collection of AMF-associated bacteria and tested the impact of various AMF associated bacteria on plant growth and mycorrhization. We observed that one AMF hyphal associated bacteria (*Devosia* sp.) stimulates AMF colonization of plant roots and promotes plant growth by stimulating plant nitrogen uptake.

Methods

Isolation of hyphae-adhering bacteria

In chapter 3, we sampled hyphae from microcosms with *Prunella vulgaris* (henceforth *Prunella*) plants. These hyphae were isolated from a hyphal compartment where root could not enter. Extraradical hyphae were isolated from this compartment using a sequential sieving method (Chapter 3; Method: Sampling of fungal hyphae from soil substrate). Here, we used two strategies to isolate AMF associated bacteria from those hyphal samples. The first strategy was to place hyphae on agar plates directly and let the bacteria attached to the hyphae grow. Briefly, concentrated hyphal samples stored in -80 ℃ were thawed at room temperature. In a sterile laminar flow cabinet, the hyphae were gently rinsed in a sterile 3.5% $Na_4P_2O_7$ solution to disaggregate small soil particles (Riding, 2004; Scheublin *et al*., 2010), then rinsed twice with sterile 0.9% saline water in a 2-ml tube and subsequently transferred to a sterile petri-dish with sterile saline water. From there, single hyphal strands were picked from the saline water onto an agar plate using sterile tweezers. A maximum of eight hyphae were placed evenly distributed on a single agar plate (Fig. S1 a, b, c, d).

The second strategy was to suspend hypha-adhering bacteria in solutions and culture serial diluted solutions on agar plates. Briefly, the hyphae were concentrated, gently rinsed by a sterile 3.5% $Na_4P_2O_7$ solution and saline water as described above. Rinsed hyphae sample were transferred to 900µl sterile 0.9% saline water, followed by rigorous shaking for 40s at 5.5 m/s in a Tissuelyser II (Qiagen, Hilden, Germany). Serial dilutions of these samples were then plated on agar-solidified culture media (Fig. S1 e, f). In both of the above strategies, seven distinct agar-solidified media were used to culture hyphae-adhering bacteria (Table S1). Single bacterial colonies were picked after 3-21 days of incubation at 28 ℃ and streaked on ISP2 agar medium (Table S7). After 3-7 days of incubation at 28℃, isolates were examined for purity and overnight cultures of single colonies in 28℃, medium at were stored with 25% glycerol at -80℃ for future use.

Characterization of bacterial isolates and mapping to ASVs

To characterize the bacterial isolates, we used a pipette tip to transfer a single colony growing on ISP2 medium to 50 µl of sterile water. The bacterial suspension was then incubated at 95℃ for 15mins and immediately cooled on ice. Subsequently the bacterial lysate was centrifuged at 10,000×g for 1min to remove cell debris. Two microliter of supernatant was taken as DNA template to amplify the 16S rRNA gene using 2.5µl 27F and 2.5µl 1492R primers (Frank *et al*., 2008), complemented with 1µl dNTP, 1µl Dreamtap polymerase (Thermo Scientific), 5µl 10×Dreamtap buffer (Thermo Scientific) and 36µl H_2O. The PCR reaction was processed on a thermocycler (Hybaid, Ashford, UK) with the cycling conditions in Table S2. PCR products were sequenced at Macrogen Europe (Amsterdam, the Netherlands). The 16S rRNA sequence were processed with MEGA 10.2.0 (Kumar *et al*., 2018) and submitted to EzBioCloud 16S database (Yoon *et al*., 2017) for taxonomy identification. We then mapped the 16S rRNA

sequence of the isolates hyphosphere and bulk soil bacterial ASVs (identified in Chapter 3 of this thesis) using VSEARCH (Rognes *et al*., 2016) at 99% sequence similarity.

Screening of AMF enriched bacteria for impact on plant growth

Prunella seeds were vapor-phase sterilized by exposure to chlorine gas for 4 hr (Chapter 3). The seeds were sown on agar-solidified half-strength Murashige and Skoog basal medium (Sigma Aldrich, St. Louis, MO, USA), with maximally 10 seeds per square Petri Dish (120x120mm, Greiner). Seeds were allowed to germinate and develop in a climate chamber under controlled conditions (short-day: 10h light/14h dark, 22°C). Two-week-old seedlings with roots of approximately ~ 0.5 cm in length that were free of visible contaminations were used in our experiment.

River sand was autoclaved twice at 121℃ for 45mins and mixed thoroughly with organic soil from the FAST experiment at Reckenholz (Switzerland; Wittwer *et al*., 2021) in a ratio of 4:1 (w/w). Five hyposphere-enriched bacteria (*Devosia* sp. ZB163 [HB1], *Bosea* sp. ZB026 [HB2], *Sphingopyxis* sp. ZB004 [HB3], *Achromobacter* sp. ZB019 [HB4], and *Microbacterium* ZB113 [HB5]) and three soil bacteria (*Arthobacter* sp. ZB074 [SB1], *Streptomyces* sp. ZB117 [SB2], *Pseudomonas* sp. ZB042 [SB3]) were cultured on ISP2 media at 28℃ for three days. A single bacterial colony was then suspended with a loop in 50 µl 10mM $MgSO_4$, spread over a Petri-dish with ISP2 agar-solidified medium and incubated at 28℃ until the bacterial colonies covered the full plate. Subsequently, 10 ml 10mM $MgSO_4$ was added to the plates and the bacteria were suspended with a sterile spatula. The suspension was then collected in a 15-ml Greiner tube followed by a double round of centrifugation and resuspension of the pellet in 10 ml 10mM $MgSO_4$. Finally, the suspensions of bacterial isolates were mixed through the sand/soil mixture to a final density of 3×10^7 CFU/g. Soil for the control treatments received an equal amount of sterile 10 mM $MgSO_4$. We conducted a total of 10 treatments, including a control treatment inoculated with 10mM MgSO4, 5 hyphal bacterial treatments (HB1, HB2, HB3, HB4, HB5), 3 soil bacterial treatments (SB1, SB2, SB3), a combined treatment of the 5 hyphal bacteria (HB SynCom), and a combined treatment of the 3 soil bacteria (SB SynCom). For each treatment, we filled 11 replicate 60-ml pots, resulting in a total of 110 pots (10 treatments x 11 replicates). One *P. vulgaris* seedling was sown in each pot and plants were grown in a greenhouse for 9 weeks with 16h light/8h dark at 22°C. Each pot received 10-15ml of water three times a week. The last three weeks, each plant was supplied with 15ml ½ Hoagland (Table S3) solution once a week.

Shoots were cut at the soil surface, lyophilized and weighted. Plant roots were removed from the soil and rinsed in sterile water. A 1-cm fragment of rinsed root was cut, weighted and stored in 50% ethanol for mycorrhizal root colonization analysis. The remaining roots were lyophilized, weighted and stored at -80℃. To check the mycorrhizal colonization on roots, root fragments stored in 50% ethanol were cleared in 10% KOH and stained with 5% ink-vinegar following a protocol described by Vierheilig *et al.*(1998). The percentage of mycorrhiza

colonization was scored following the line-intersection method (Mcgonigle *et al*., 1990) by checking 100 intercepts per sample.

AMF propagation

We cultured Ri T-DNA-transformed carrot root organs on one side of a two-compartment petri dish at 26°C for 2 weeks and then inoculated the organs with spores of *Rhizophagus irregularis* MUCL43194 (Fortin *et al*., 2002). The root compartments were filled with modified Strullu and Romand (MSR; Duchefa Biochemie, NL) medium supplemented with 1% sucrose and the hyphal compartment were filled with MSR medium (Table S4). *R. irregularis* then was left to colonize the root organs for 3 months during which *R. irregularis* mycelium colonized the hyphal compartment of the Petri-dish and formed spores. *R. irregularis* spores were harvested by chopping the agar-solidified medium of the hyphal compartment into small pieces using a sterile scalpel and subsequently dissolving the medium in a sterile citrate buffer (Table S5). Thousands of *R. irregularis* spores in citrate buffer were then transferred to sterile 1.5 ml-Eppendorf tubes in 500-µl aliquots and stored at 4°C.

Impact of Devosia sp. ZB163 and AMF on plant growth

Organic soil-sand mixture was autoclaved twice to remove the indigenous microbiota. In this experiment, we prepared four treatments: a control treatment receiving 10mM $MgSO_4$, a *Devosia* treatment receiving a suspension of *Devosia* sp. ZB163 mixed thoroughly into the organic soil-sand mixture to a final density of 3×10^7 CFU/g, an AMF treatment receiving 100 mycorrhiza spores (*R. irregularis*), and a combined treatment of *Devosia* and AMF receiving both a density of 3×10^7 CFU/g of the *Devosia* suspension in the organic soil-sand mixture and 100 mycorrhiza spores (*R. irregularis*). Two-week-old Prunella seedlings were prepared as described above and transplanted into 60-ml pots filled with the organic soil-sand mixture. AMF-treated pots received mycorrhiza spores immediately prior to seedling transplantation. Eleven replicate pots were prepared per treatment resulting in a total of 44 pots (4 treatments x 11 replicates). Plants were allowed to grow under climate-controlled conditions at a light intensity of 200 µE/m^2/s with a 16 h photoperiod for 8 weeks at 22°C. Each pot received 10-15ml of water three times a week. To determine the effect of N and P availability on plant growth, we conducted a complementary experiment with the same four treatments and 20 biological replicates, resulting in a total of 80 pots. Moreover, the plants were supplied with 5ml modified Hoagland solution without N, P (Table S6) once per week from week 6 onwards. Shoot weight, root weight and mycorrhization were assessed as described above.

Nitrogen and phosphate accumulation in plant leaves

Lyophilized Prunella leaves were first ground to powder. To determine P content, approximately 50 mg of powdered leaves were digested in 1 ml HCl/HNO_3 mixture (4:1, v/v) in a closed Teflon cylinder for 6 hr at 140℃. The P concentrations were determined colorimetrically using a Shimadzu UV-1601PC spectrophotometer (Murphy, 1962). The N concentrations were determined by dry combustion of a 3-4 mg sample with a Flash EA1112 elemental analyzer (Thermo Scientific, Rodano, Italy).

Absolute quantification of *Devosia* sp. ZB163 on plant roots

To quantify the absolute abundance of the *Devosia* strain on plant roots, we spiked root samples with 14ng DNA of *Salinibacter ruber*, an extremely halophilic bacterium that exists in hypersaline environments, (Stämmler *et al*., 2016), but does not occur in our soil samples. Subsequently, the DNA of the root samples was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The 16S rRNA gene V3-V4 region was amplified following a two-step PCR using the primers 341F and 806R (Herlemann *et al*., 2011)and barcoding primers (Baym *et al*., 2015). The amplified DNA was cleaned-up, quantified, normalized, pooled and subsequently sequenced on the Novaseq 6000 SP platform (2 × 250 bp) by Genome Quebec (Montreal, Canada). The raw sequencing data were demultiplexed, trimmed, dereplicated, and filtered for chimeras by DADA2 (Callahan *et al*., 2016) in the QIIME2 environment (version 2019.07, https://qiime2.org/; Bolyen *et al*., 2019). Amplicon sequence variants (ASVs) were generated and annotated against the SILVA reference database (v132; Quast *et al*., 2013). ASVs assigned to mitochondria and chloroplast were removed. Since ASVs that are present in only a few samples may represent PCR or sequencing errors, we removed the ASVs that were present in ≤4 samples. Filtered ASV counts were constructed into an ASV table. The absolute abundance amount of detected *Devosia* sp. ZB163 DNA using the following formula.

$$Estimated\ Devosia\ DNA\ (ng) = Salinibacter\ DNA\ (ng) \times \frac{Devosia\ relative\ abundance}{Salinibacter\ relative\ abundance}$$

The raw sequencing data were deposited at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) by the study PRJEB59555.

Devosia genome sequencing

Devosia sp. ZB163 was cultured on ISP2 medium for 7 days at 28 ℃. DNA was extracted from a loop of bacterial cells using the MagAttract Microbial DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was amplified following the Hackflex protocol (Gaio *et al*., 2022) followed by DNA purification using the AMPure XP clean-up (Beckman Coulter, High Wycombe, UK). The purified DNA was sequenced with Novaseq 6000 SP platform (2 × 250 bp) by Genome Quebec (Montreal, Canada). The raw sequencing data were trimmed with Cutadapt. Quality checked and assembly was performed using the A5-miseq pipeline (Coil *et al*., 2015). The raw sequencing data were deposited at the National Center for Biotechnology Information, GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) by the accession PRJNA931835.

Genome analysis

Devosia sp. ZB163's genome was annotated using prokka (Seemann, 2014) and RAST (Aziz *et al*., 2008). Mining for orthologs of genes in the genomes of *Devosia* was performed using reciprocal BLASTp analysis. Genes were considered orthologs when the e-value < 10^{-5}. Moreover, the whole *Devosia* genome was blast against a nifH database formatted for the

dada2 pipeline (Moynihan, 2020). This *nifH* database was based on the *nifH* ARB database from the Zehr lab (Heller *et al*., 2014).

Statistical analysis

All statistical analyses were conducted in R version 4.0.2 (For, R Foundation Statistical Computing, Vienna, 2020). ASVs that are positively associated with hyphosphere, or soil microbiome were identified by R package *indicspecies* (de Cáceres & Legendre, 2009) and considered robustly enriched if their abundance was significantly higher in hyphal samples than both roots and soil samples as determined by one-way analysis of variance (ANOVA). The effect of microbial treatments on plant weight, AMF colonization rate, and plant nutrient uptake was assessed by one-way ANOVA and followed by the Tukey HSD test. All bioinformatic files generated by qiime2 were imported to R by qiime2R (Jordan E Bisanz, 2018). Absolute abundance of *Devosia* sp. ZB163 was assessed for variation among treatments by ANOVA and followed by a Tukey HSD test. The correlation between *Devosia* sp. ZB163 absolute abundance and plant weight, AMF colonization, and plant nutrient uptake were assessed by simple linear regression.

Results

Bacterial isolates of the hyphosphere microbiome

We isolated bacteria from AMF hyphae collected from hyphal compartments in microcosms with *Prunella vulgaris* (henceforth: Prunella) plants. These hyphae were gathered by sequential sieving of the soil substrate of a compartment of the microcosm that was colonized by extraradical hyphae but free of Prunella roots (Chapter 3). We either placed single hyphal strands on an agar-solidified growth medium and streaked individual bacterial colonies that appeared alongside these hyphae (Fig. S1). Alternatively, we washed hyphal samples in sterile 0.9% saline water and isolated bacteria through dilution plating.

In total, we isolated 143 bacteria (Table S7) and characterized the isolates by 16S rRNA gene sequencing. The 143 isolates belong to 3 bacterial phyla and mainly represent *Actinobacteria* (72.7%), *Proteobacteria* (17.5%), and *Firmicutes* (9.8%; Fig. 1). Within the *Actinobacteria,* 4 genera were presented. *Microbacterium* (70 isolates) accounted for almost half of the isolates, whereas also *Arthrobacter* (16 isolates), *Pseudonocardia* (10 isolates), and *Agromyces* (8 isolates) were identified. Within the *Proteobacteria,* 10 genera were identified, represented by 10 *Achromobacter* isolates, 5 *Devosia* isolates, 2 *Bosea and* 2 *Ensifer* isolates, and single isolates of *Kaistia*, *Lysobacter*, *Noviherbaspirillum*, *Pseudomonas*, *Roseomonas* and *Sphingopyxis*. Moreover, six *Firmicutes* genera were represented in the isolate collection representing the genera *Brevibacillus* (6 isolates), *Paenibacillus* (3 isolates), *Gottfriedia* (2 isolates), *Fictibacillus* (1 isolate), *Lysinibacillus* (1 isolate) and *Ureibacillus* (1 isolate).

Subsequently we identified bacterial isolates that were selectively enriched on AMF. To this end, we made use of the data set in chapter 3, where we profiled hyphal, root, and soil microbial communities by amplicon sequencing. In total, we found that the sequences of 120 out of 143

bacterial isolates shared at least 99% NI with 48 of the total number of 596 ASVs that were detected to be associated with hyphal samples. Of the 48 ASVs that were detected in hyphal samples and that were represented in our isolate collection, 7 ASVs were robustly and significantly more abundant in hyphal samples than in either soil or root samples (Fig. 1b). These 7 hyphal ASVs were represented by 67 bacterial isolates and comprised single *Bosea, Achromobacter*, *Microbacterium, Arthobacter*, *Streptomyces*, and *Pseudomonas* ASVs, and 2 *Sphingopyxis* and 2 *Devosia* ASVs (Fig. 1b). The 2 *Devosia* ASVs respectively matched with 99.5% and 99,0% NI with three indistinguishable *Devosia* isolates. Likewise, the 2 *Sphingopyxis* ASVs respectively matched with 99.75% and 100% NI with the only *Sphingopyxis* isolate in the collection. Ultimately, we selected 5 isolates to represent the 7 hyphal ASV and these hyphosphere bacteria (HB) were subsequently used to examine their influence on the AMF symbiosis. In addition, we select 2 bacterial isolates that matched with ASVs that were enriched in soil (Fig. 1b) and an additional bacterial strain that did not match with any of the ASVs in our data set. These soil bacteria (SB) were used as additional control treatments.

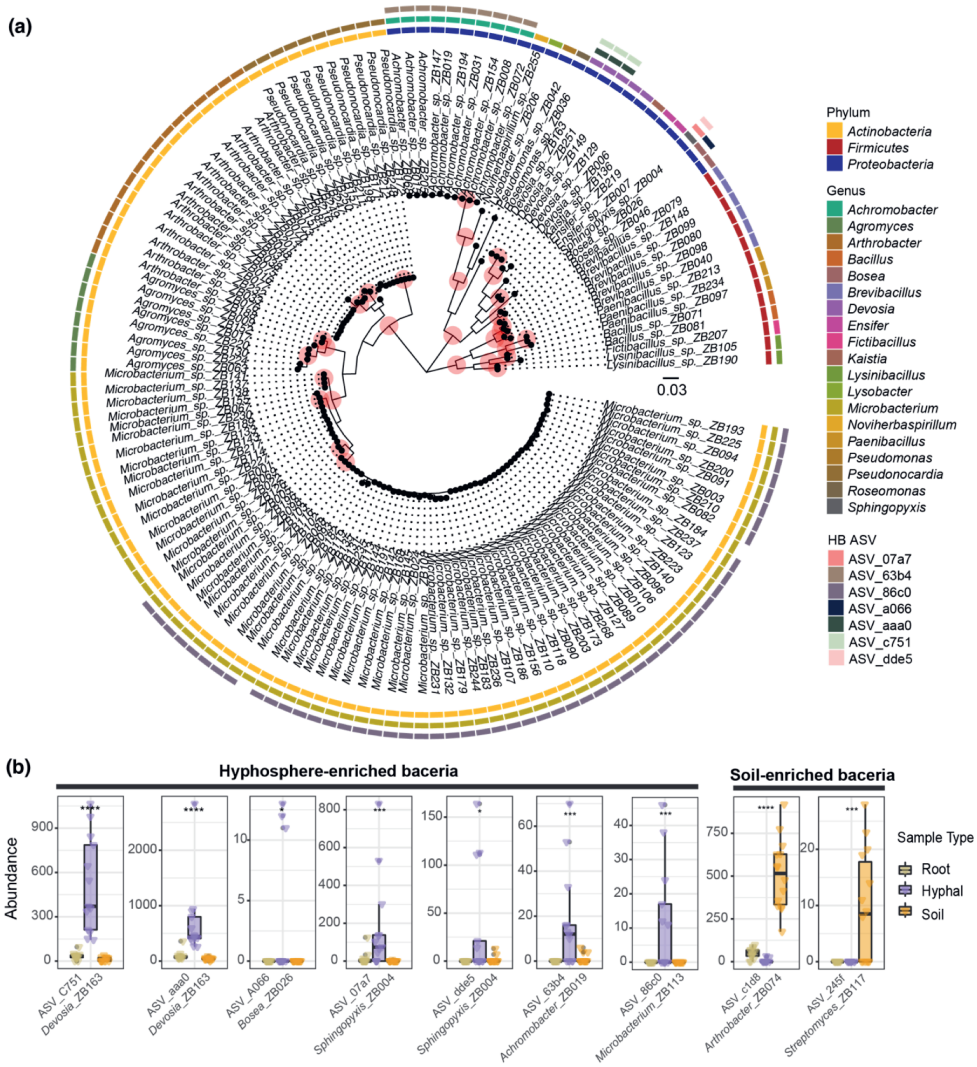

Fig. 1. Taxonomic diversity of the isolated bacteria from hyphal samples and matching with hyphosphere ASVs. (a) 16S rRNA gene-based maximum-likelihood tree showing the phylogenetic relationships between bacterial isolates. The scale bar represents a 3% difference in nucleotide identity. Bootstrap values > 90 % (based on 1000 resampled datasets) are indicated at branches by red nodes. The colored rings from inside to out respectively represent 1st the phyla of isolates, 2nd the genera of the isolates, and 3rd and 4th the ASVs that match with the isolate 16S sequence at >99%NI. **(b)** Average relative abundance of the selected ASVs in the root, hyphal, and soil samples in Experiment I of chapter 3 of this thesis. Sample types were indicated by color. Each selected ASVs ID was labeled together with a selected corresponding bacterial isolate with matching sequence. Significance differences are indicated with asterisk (one-way ANOVA, *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001).

Devosia sp. ZB163 promotes plant growth in organic soil

We tested whether the selected bacterial isolates affected the symbiosis between *P. vulgaris* plants and AMF. To this end, we inoculated soil-sand mixture with each of the 5 hyphosphere bacteria (HB: *Devosia* sp. ZB163, *Bosea* sp. ZB026, *Sphingopyxis* sp. ZB004, *Achromobacter* sp. ZB019, and *Microbacterium* ZB113) or the 3 soil bacteria (SB; *Arthobacter* sp. ZB074, *Streptomyces* sp. ZB117, *Pseudomonas* sp. ZB042) at an initial density of 3×10^7 CFU/g. In addition, two treatments either combined 5 HBs or 3 SBs as two separate synthetic communities (HB/SB SynCom) were applied to the soil-sand mixture with a cumulative initial abundance of 3×10^7 CFU/g. Finally, we transplanted 2-week-old prunella plants to the inoculated pots. After 9 weeks of growth in a greenhouse, we harvested the shoots and roots of these plants and found that only plants inoculated with either *Devosia* sp. ZB163 (hereafter: *Devosia*) or the HB SynCom had significantly higher shoot dry weight than control plants (Fig. 2a). This indicates that *Devosia* can promote plant growth. The average root weight of all plants with bacterial treatments was higher than not-inoculated control plants, but this difference was not significant in ANOVA. All control and treatment plants in this experiment were colonized by AMF and the mycorrhization at the end of the experiment was not significantly affected by the distinct bacterial treatments in this experiment (Fig. 2c).

Chapter 4

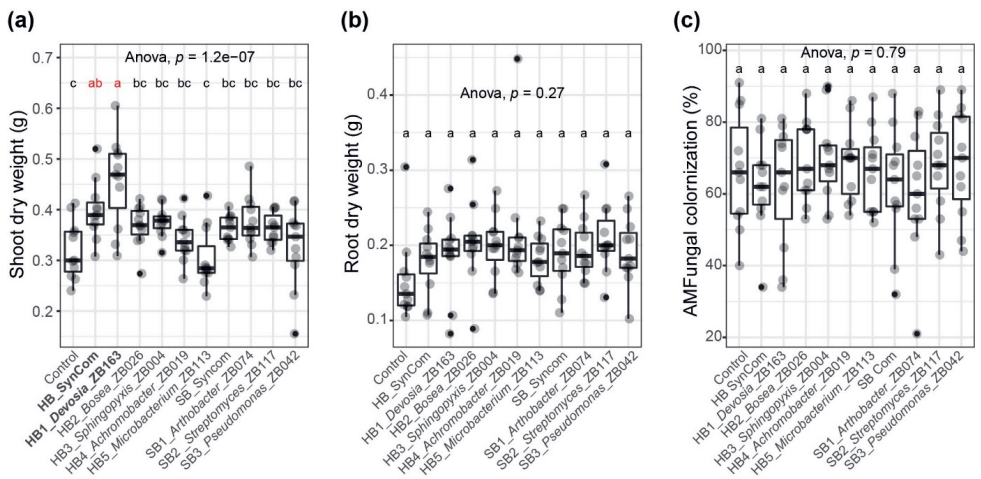

Fig. 2. Screening hyphal bacterial effects on plant growth. (a) Shoot dry weight of 9-week-old Prunella plants **(b)** Root dry weight comparison between bacterial treatments. **(c)** AMF colonization percentage comparison between bacterial treatments. Significance differences are indicated with letters (ANOVA and Tukey's Honest HSD test).

Devosia sp. ZB163 promotes plant growth and mycorrhization

To explore whether plant growth promotion by *Devosia* sp. ZB163 relies on the presence of AMF, we depleted the indigenous microbiome by autoclaving the soil-sand mixture and again inoculated *Devosia* at an initial density of 3×10^7 CFU/g soil prior to transplantation of *Prunella* seedlings (hereafter: *Devosia* treatment). Subsequently, 100 monoxenic *R. irregularis* spores were injected near the seedling's roots (hereafter: AMF treatment). To ensure nutrient poor conditions and stimulate AMF colonization, the plants in this experiment were not provided with nutrients in addition to what was present in the soil-sand mixture.

After 8 weeks of growth under controlled conditions in a climate chamber, plants inoculated with *Devosia* had a significantly higher shoot and root weight, indicating that, even without AMF, *Devosia* sp. ZB163 can promote plant growth. Four out of the eleven plants that were inoculated with AMF (but without *Devosia*) were bigger than control plants and the leaves of these plants were more bright green (Fig. 3f). These four plants were the only plants in which mycorrhiza had colonized the roots and, likely as a result, the average weight of roots and shoots was not affected by the AMF treatment. However, plants that had been inoculated with the combination of AMF and *Devosia* did have significantly higher shoot and root weights. Remarkably, 10 out of 11 plants that had received the combination of *Devosia* and AMF were bright green and colonized by mycorrhiza. This suggests that *Devosia* sp. ZB163 not only promoted plant growth directly but also improved AMF establishment in this experiment. As one of the *Devosia* species, *Devosia neptuniae*, has been reported to fix N (Rivas *et al*., 2002) and AMF are known to provide plants with both N and P (George *et al*., 1995), we measured leaf N and P content. We found that the leaves of all plants that were colonized by AMF contained more P, whereas the plants that were inoculated with *Devosia* had higher N content (Fig. 3d). This suggests that *Devosia* and AMF promote plant growth by stimulating the uptake of respectively N and P in a complementary manner. We hypothesized that this did not result in even higher plant growth in the combination treatment (Fig. 3a, 3b) as other mineral components of poor soil/sand mixture also constrained the growth of plants in these experiments.

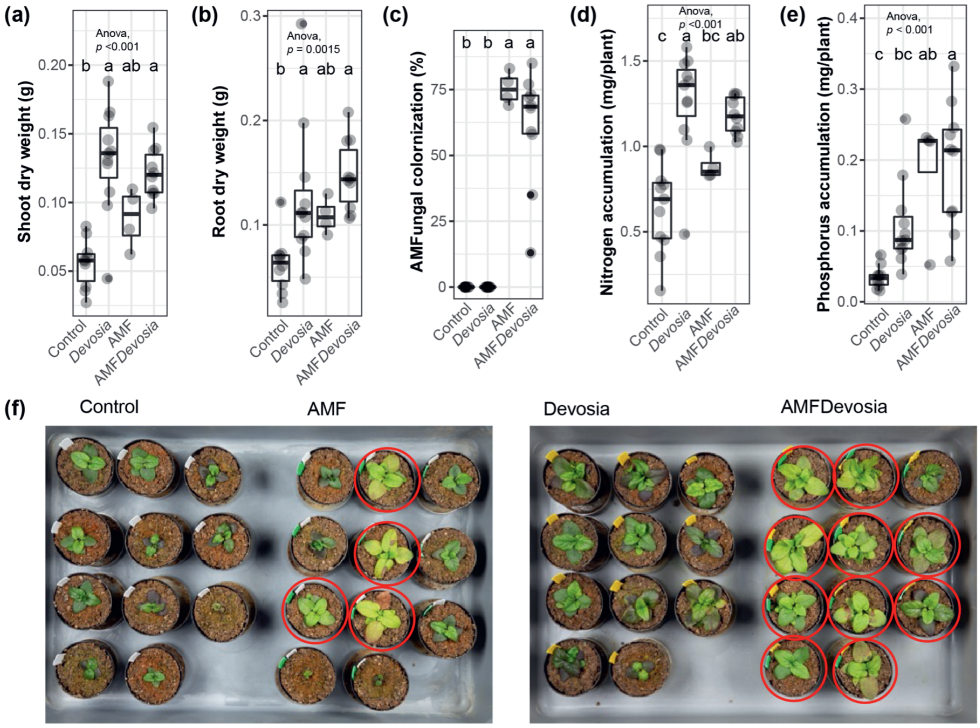

Fig. 3. *Devosia* promotes plant growth, mycorrhization, and N accumulation.

Boxplots show **(a)** shoot dry weight, **(b)** root dry weight, **(c)** percentage of each root system colonized by AMF, **(d)** shoot nitrogen accumulation, or **(e)** shoot P accumulation of 8-week-old Prunella plants cultivated in autoclaved soil (Control) or inoculated with *Devosia* sp. ZB163 (Devosia), *R. irregularis* (AMF), or both symbionts. In the 6th, 7th and 8th week, plants were watered with modified Hoagland solution without N and P. Significance differences are indicated with letters (ANOVA and Tukey's Honest HSD test). **(f)** Photographs of the Prunella plants immediately before harvest. Red circles indicate plants that were later found to be colonized by AMF.

Synergy of *Devosia* sp. ZB163 and AMF synergistically promote plant growth

We subsequently repeated this experiment but now provided the plants with a modified Hoagland solution that included most micronutrients but was deficient in N and P (Table S6). Again, *Devosia* promoted plant growth, but in this experiment also AMF led to a significantly higher dry weight of both shoots and roots (Fig. 4a, 4b). In this experiment, AMF established successfully in the roots of all plants to which they were inoculated, but the mycorrhizal colonization was higher on plants that were also inoculated with *Devosia* (Fig. 4c). Notably, this combination treatment of AMF and *Devosia* resulted in the significantly highest plant shoot weight among all treatments showing that AMF and the *Devosia* ZB163 can synergistically promote plant growth (Fig. 4a). In line with this, we found that accumulation of N was significantly increased in plants inoculated with *Devosia* and accumulation of P increased in plant inoculated with AMF, but the plants inoculated with both AMF and *Devosia* accumulated most N and P.

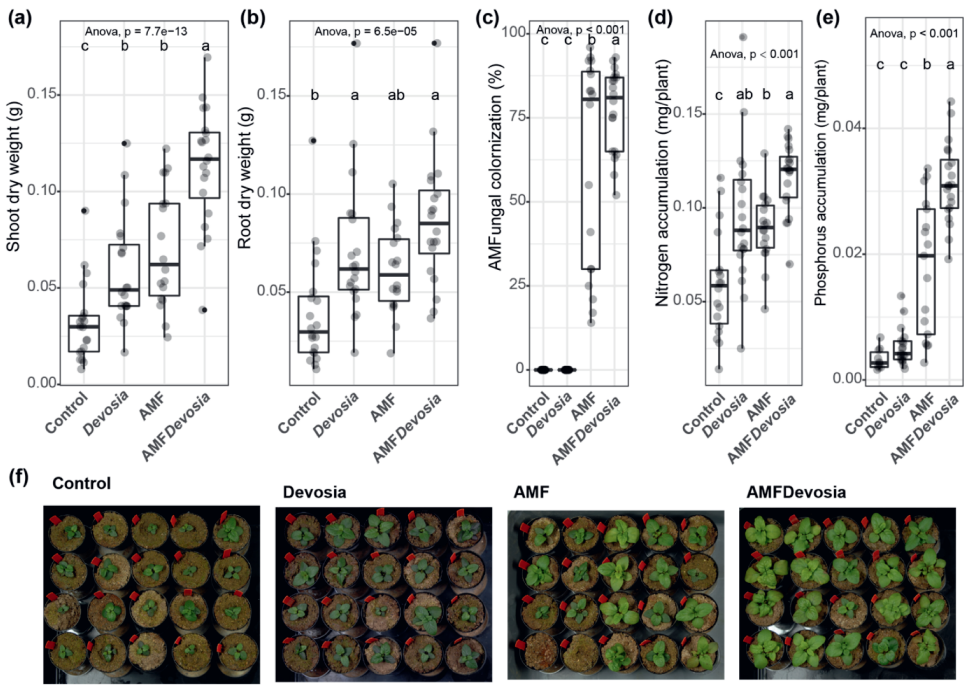

Figure 4. *Devosia* sp. ZB163 and AMF can synergistically promote plant growth and plant N and P accumulation.

Boxplots show **(a)** shoot dry weight, **(b)** root dry weight, **(c)** percentage of each root system colonized by AMF, **(d)** shoot nitrogen accumulation, or **(e)** shoot P accumulation of 8-week-old Prunella plants cultivated in autoclaved soil (Control) or inoculated with *Devosia* sp. ZB163 (Devosia), *R. irregularis* (AMF), or both symbionts. Plants were regularly watered with modified Hoagland solution deficient in a source of N and P. Significance differences are indicated with letters (ANOVA and Tukey's Honest HSD test). **(f)** Photographs of the Prunella plants immediately before harvest. Two AMF-treated plants died shortly after transplantation and were not considered in panels **a**-**e**.

We subsequently quantified the absolute abundance of *Devosia* by sequencing 16S rRNA gene amplicons of DNA isolated from the roots of plants used in this experiment and spiked with a known amount of 14ng DNA (Stämmler *et al*., 2016). We detected low amounts of *Devosia* on the roots of plants that were not inoculated with *Devosia*, indicating that some level of cross contamination occurred in our experiment (Fig. 5a). Nonetheless the numbers of *Devosia* were significantly higher (average 3.6-fold) on roots that were inoculated with *Devosia*.

We subsequently analyzed the correlation between absolute *Devosia* abundance and several parameters. We observed that, independent of AMF presence, *Devosia* abundance positively correlates with plant nitrogen accumulation (Fig. 5b), but also with shoot and root dry weight (Fig. 5c, 5d). This shows that *Devosia* sp. ZB163 can directly stimulate plant growth and nitrogen uptake. Moreover, the absolute abundance of *Devosia* significantly correlates with the percentage of AMF colonization (Fig. 5e), suggesting that *Devosia* indeed accelerates the colonization of plant roots by AMF. In line with this, we observed that *Devosia* abundance correlates significantly with increased phosphorus accumulation, but only in presence of AMF (Fig. 5f) and that the hyphal colonization percentage correlates with phosphorus accumulation (Fig. S2). Together these data show that Devosia can stimulate plant growth directly, likely by increasing nitrogen uptake, as well as indirectly by promoting AMF colonization and corresponding phosphorus uptake.

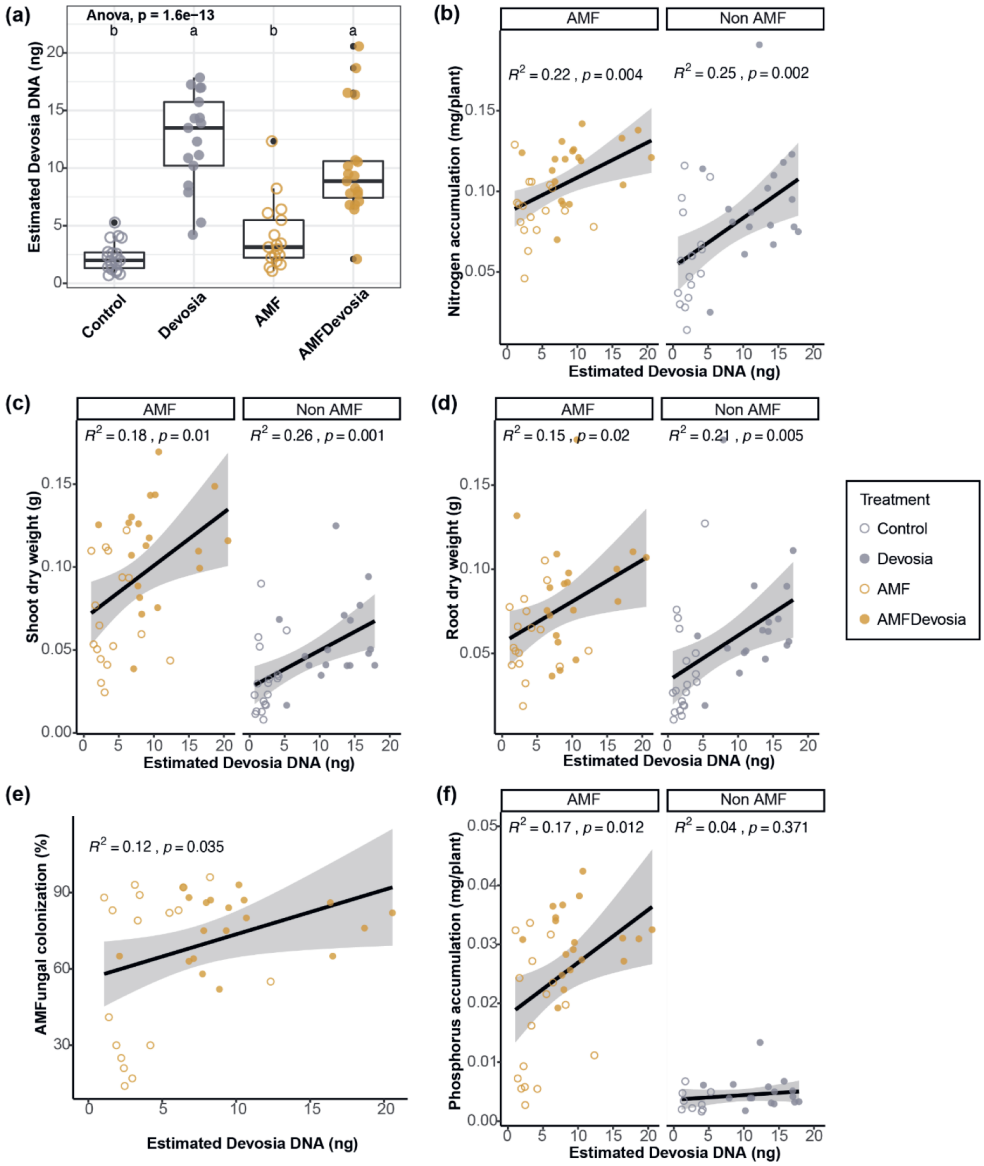

Fig. 5. Absolute abundance of *Devosia* sp. ZB163 significantly correlates with plant weight, mycorrhization, and nutrient content.

(a) Boxplot of the absolute abundance of *Devosia* DNA on roots of plants in sterilized soil inoculated with a mock solution (Control), *Devosia* sp. ZB163 (*Devosia*), *R. irregularis* (AMF), or both symbionts. Letters indicate significant differences as determined by ANOVA with Tukey's HSD test. **(b-e)** Scatter plots of the correlation between the absolute abundance of *Devosia* DNA and **(b)** total plant nitrogen accumulation, **(c)** shoot dry weight, **(d)** root dry weight **(e)** hyphal colonization, and **(f)** total plant phosphorus accumulation. Correlations and probabilities thereof are determined using linear regression.

Devosia sp. ZB163 lacks genes required for atmospheric nitrogen fixation

The genome of Devosia sp. ZB163 was subsequently sequenced using the Illumina Novoseq platform (Génome Québec, Canada) resulting in a sequenced genome of approximately 4.6 Mb that was predicted to have 4486 coding sequences (CDSs) and a GC content of 65.7%. As we found that *Devosia* sp. ZB163 promotes plant nitrogen uptake, we subsequently performed a reciprocal BLASTp to search for orthologues of known nitrogen-related genes (Table 1). We first explored the *Devosia* genome for genes that are required for atmospheric nitrogen fixation. Some orthologues to known genes involved in the regulation of nitrogen were detected. We found orthologues to *fixL, fixJ*, and *fixK.* The two-compartment regulatory system, FixL/FixJ activates the transcription of *fixK gene* which is reported to control the expression of N_2 fixation genes and genes required for the sensing of microaerobic conditions (Dixon & Kahn, 2004). Moreover, we found an orthologue of *nifU* that putatively encodes a protein critical for the maturation of nitrogenase catalytic components (Zhao *et al*., 2007). However, these regulatory genes are not enough to encode an active nitrogenase. In this respect, we found orthologues of neither *nifA, nifD, nifH* nor *nifK* in the genome of ZB163 using translated amino acid sequence of these genes from *Devosia neptuniae*, *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, *Klebsiella pneumoniae* (Masterson *et al*., 1985; Rivas *et al*., 2002; Roberts *et al*., 1978; Schlüter *et al*., 2010). The *nifADHK* gene cluster typically encodes the molybdenum nitrogenase complex that is most commonly found in diazotrophs (Dixon & Kahn, 2004). Next, we blasted the *Devosia* sp. ZB163 genome to a *nifH* database that contains 34 420 *nifH* sequences, but again did find a hit for *nifH* in the genome of ZB163. Finally, also the gene clusters *vnfHDGK* and *anfHDGK* encoding the less common nitrogenase complexes were not detected in the *Devosia* sp. ZB163 genome (dos Santos *et al*., 2012). This strongly suggests that unlike other *Devosia* isolates, *Devosia* sp. ZB163 is not able to fixate atmospheric nitrogen.

However, bacteria can also increase the amount of nitrogen that is available to plants through the mineralization of organic nitrogen. The ammonification process in the soil mineralizes organic nitrogen to ammonia. The organic soil was reported to slowly-release urea (Lin *et al*., 2021). Then, the urea, as an organic nitrogen source, is catalyzed by urease to ammonia that can be subsequently supplied to plants. Using protein sequence from *Devosia rhizoryzae*, *Devosia oryziradicis* (Chhetri *et al*., 2022), we detected the presence of the geneclusters *UreDFG* and *UrtABCDE* that are required to catalyze the hydrolysis of urea, forming ammonia and carbon dioxide. By genome annotation of prokka and RAST (Aziz *et al*., 2008; Seemann, 2014), we detected *UreE* (Table 1). Besides, plants can also take up nitrate. The nitrification bacteria catalyze ammonium to nitrate with amoA gene. Again, we did not detect any amoA orthologs in the *Decosia* genome using the translated amino acid sequences of these genes from *Nitrosomonas europaea* (Amoo & Babalola, 2017).

Table 1. Nitrogen metabolism related pathways genes annotated in *Devosia* genome.

Gene	Location	Strand	Hits
nifU	scaffold_0_406316_406873	+	NifU family protein [*Devosia* sp.]
fixK	scaffold_0_564243_563566	-	CRP/FNR family nitrogen fixation transcriptional regulator
fixL	scaffold_1_81963_81343	-	response regulator FixJ [*Devosia* sp. Root413D1]
fixJ	scaffold_1_83308_81950	-	putative FixL oxygen regulated histidine kinase [uncultured bacterium 1062]
ureG	scaffold_3_209051_208419/ scaffold_3_594828_594178/ scaffold_0_761242_761880	-/-/+	urease accessory protein UreG [*Devosia rhizoryzae, Devosia oryziradicis*]
ureF	scaffold_3_210273_209473/ scaffold_3_597921_597244/ scaffold_0_760487_761230	-/-/+	urease accessory protein UreF [*Devosia rhizoryzae, Devosia oryziradicis*]
ureE	scaffold_3_210743_210270/ scaffold_3_598363_597914/ scaffold_0_759916_760527	-/-/+	urease accessory protein UreE [*Devosia* sp.]
ureD	scaffold_3_219092_218151/ scaffold_0_761880_762788	-/+	urease accessory protein UreD [*Devosia rhizoryzae, Devosia oryziradicis*]
Urease alpha subunit	scaffold_3_215267_213558/ scaffold_3_596522_594825/ scaffold_0_758160_759881	-/-/+	urease subunit alpha [*Devosia rhizoryzae, Devosia oryziradicis*]
Urease beta subunit	scaffold_3_217611_217306/ scaffold_3_597223_596528/ scaffold_0_757645_758115	-/-/+	urease subunit beta [*Devosia rhizoryzae, Devosia oryziradicis*]
Urease gamma subunit	scaffold_3_218142_217840/ scaffold_0_757305_757607	-/+	urease subunit gamma [*Devosia rhizoryzae, Devosia oryziradicis*]
UrtE	scaffold_3_219871_219095	-	urea ABC transporter ATP-binding subunit UrtE [*Devosia rhizoryzae, Devosia oryziradicis*]
UrtD	scaffold_3_220730_219975	-	urea ABC transporter ATP-binding protein UrtD [*Devosia rhizoryzae, Devosia oryziradicis*]
UrtC	scaffold_3_221902_220727	-	urea ABC transporter permease subunit UrtC [*Devosia rhizoryzae, Devosia oryziradicis*]
UrtB	scaffold_3_223973_222219	-	urea ABC transporter permease urea ABC transporter permease subunit UrtB [*Devosia rhizoryzae, Devosia oryziradicis*]
UrtA	scaffold_3_225350_224043	-	branched-chain amino acid ABC transporter substrate-binding protein [*Devosia rhizoryzae, Devosia oryziradicis*]

Discussion

In this study, we isolated bacteria from AMF-rich hyphal samples to investigate the influence of these bacteria on the plant-AMF symbiosis. The majority of 143 AMF-associated bacteria that were isolated in this study could be matched to 48 ASVs generated from samples of the same experiment. We subsequently selected 5 bacterial isolates that matched to ASVs that were robustly enriched on hyphal samples and examined their effect on Prunella plants in an agricultural soil that was naturally rich in AMF inoculum. Here, we identified *Devosia* isolate ZB163 as an AMF-associated bacterium that can strongly promote growth of Prunella plants. We showed that the addition of this bacteria by itself promoted the growth of Prunella plants

and that the abundance of this bacterium on the root was positively correlated with plant weight and plant total nitrogen.

Besides the direct effect of *Devosia* isolate ZB163 on plant growth, the bacterium also appears to stimulate the mycorrhization of Prunella plants. In two of the three experiments, we found that plants with *Devosia* were either more often colonized by AMF or colonized to higher extent. This suggest that *Devosia* isolate ZB163 accelerates the mycorrhiza colonization process, and functions as a mycorrhization helper bacteria (Frey-Klett *et al*., 2007). Only in the first experiment did we not find increased mycorrhization of plants that were inoculated with *Devosia* isolate ZB163, but in this experiment also the control plants were colonized to a high level, perhaps as a result of endogenous microflora. Mycorrhizae are well known for their ability to scavenge phosphorus and provide the plants with this macronutrient (Bolan, 1991), and in our experiment mycorrhized Prunella plants grew bigger and accumulated more phosphorus. *Devosia* isolate ZB163 by itself did not affect plant phosphorus content, but in presence of the mycorrhiza the abundance of isolate ZB163 was significantly correlated with increased phosphorus accumulation. This shows that, although *Devosia* isolate ZB163 does not itself provide phosphorus to the plant, it can indirectly provide extra phosphorus by stimulating mycorrhization. In line with this, we found that the combined treatment of AMF and *Devosia* isolate ZB163 can lead to more growth promotion than either microbe alone.

The increased amount of total nitrogen in Prunella plants that were inoculated with *Devosia* isolate ZB163, suggests that the bacterium mediates the uptake of nitrogen by the plant. Free-living plant-associated bacteria have been found to provide plants with nitrogen through either the assimilation of atmospheric dinitrogen into ammonium or the ammonification of organic nitrogen from the soil to ammonia (Islam *et al*., 2009; Li *et al*., 2014). Previously a *Bacillus thuringiensis* strain, capable of nitrogen assimilation, was isolated from AMF spores and was found to promote hyphal growth (Cruz & Ishii, 2012). Although the effect of that *Bacillus thuringiensis* on the mycorrhizal colonization of plants was not tested, this example shows that nitrogen assimilators can stimulate mycorrhiza. Moreover, it is acknowledged that the mycorrhiza can absorb N and supply it to plants (Govindarajulu *et al*., 2005; Johansen *et al*., 1993).

To unearth how *Devosia* isolate ZB163 promotes Prunella nitrogen uptake, we sequenced the genome of *Devosia* isolate ZB163 and searched for genes involved in the conversion of nitrogen. *Devosia* sp. J1 and J2 have been identified that can form nodules and fix atmospheric nitrogen in symbiosis with the aquatic legume *Neptunia natans (L.f.)* Druce (Rivas *et al*., 2002). In the same study, the *Devosia* sp. J1 and J2 were found to possess a *nifH* gene similar to *nifH* of *Rhizobium tropici*, suggesting the *Devosia* sp. J1 and J2 may have acquired the *nifH* gene by horizontal transfer. However, the genome of *Devosia* sp. ZB163 did not contain orthologs for any of the known nitrogenase genes. This indicates that *Devosia* sp. ZB163 cannot assimilate atmospheric nitrogen. Besides ammonium, plants can also take up and assimilate nitrate. The rate-limiting step is the nitrification of ammonium to nitrite and the step is catalyzed by the enzyme amoA (Amoo & Babalola, 2017). Again, however, the genome of

Devosia isolate ZB163 did not contain any orthologs for amoA, indicating *Devosia* sp. ZB163 does not contribute to the nitrification of ammonium, and this is unlikely the cause of plant growth promotion by this bacterium.

We then looked for genes involved in ammonification of organic nitrogen. Microbes can catalyze the ammonification of organic nitrogen to ammonia which can be directly taken up by plants (Liou & Madsen, 2008). In *Devosia* sp. ZB163, we detected gene clusters putatively encoding enzyme complexes that are involved in the decomposition of urea. The urease complex catalyzes the hydrolysis of urea to ammonia, and us such ZB163's urease activity might result in increased nitrogen availability to the prunella plants in our experiments. Future experiments should determine whether *Devosia* sp. ZB163 can really speed up the ammonification process through the production of urease and whether increased availability of ammonium is the cause of the plant growth promotion.

Alternatively, *Devosia* sp ZB163 might induce a response in the plant that enhances nitrogen uptake. For example, an *Achromobacter* sp. in the root of oilseed was found to stimulate the uptake rate of nitrate by stimulating the plant's ionic transport system while simultaneously promoting the formation and length of root hairs (Bertrand *et al*., 2000). Also, the plant-beneficial *Pseudomonas simiae* WCS417 was shown to stimulate nutrient uptake and root architecture. Upon colonization by this bacterium, *Arabidopsis thaliana* plants formed more lateral roots with more and longer root hairs (Zamioudis *et al*., 2013). Moreover, *P. simiae* WCS417 hijacked the plant's iron deficiency response and induced the secretion of iron-mobilizing coumarins that are thought to benefit both plants and bacteria (Stringlis *et al*., 2018). It will be intriguing to find out whether *Devosia* sp. ZB163 similarly promotes the formation of an extensive root system in Prunella plants, as extensive root branching likely also affects the rate of mycorrhization (Frey-Klett *et al*., 2007). In line with this hypothesis, we did see a significant correlation of root dry weight and the abundance of *Devosia* sp. ZB163 on the roots in our experiments.

In summary, we isolated bacteria from AMF hyphae and identified *Devosia* sp. ZB163 stimulates plant mycorrhization, plant nitrogen accumulation, and plant growth. Future research should elucidate the mechanisms by which the bacterium has these beneficial effects. Interestingly, *Devosia* is enriched on AMF hyphae compared to the surrounding soil, suggesting that some form of cooperation may exist. Although the plant-mycorrhiza symbiosis is most often considered a bilateral relationship, it is clear that both plant and mycorrhiza foster a microbiome that can influence and strengthen the plant holobiont. Our study improves our fundamental understanding of plant and mycorrhiza functioning, but also has practical value. Mycorrhizae are a long-standing promise for sustainable agriculture by reducing the requirements of crop fertilizers. Our study suggests the performance of mycorrhiza and crops in the agricultural field might benefit considerably from the application of mycorrhiza helper bacteria, such as *Devosia* sp. ZB163.

Acknowledgements

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Authors' contributions

C.Z., M.G.A.v.d.H. and R.L.B. conceived and designed the experiments. C.Z., B.K.D., J.S., A.H., B.N.T., G.S., and M.R.C. performed the experiments. C.Z. analyzed the data. C.Z., M.G.A.v.d.H., and R.L.B. wrote the manuscript.

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Supplementary information

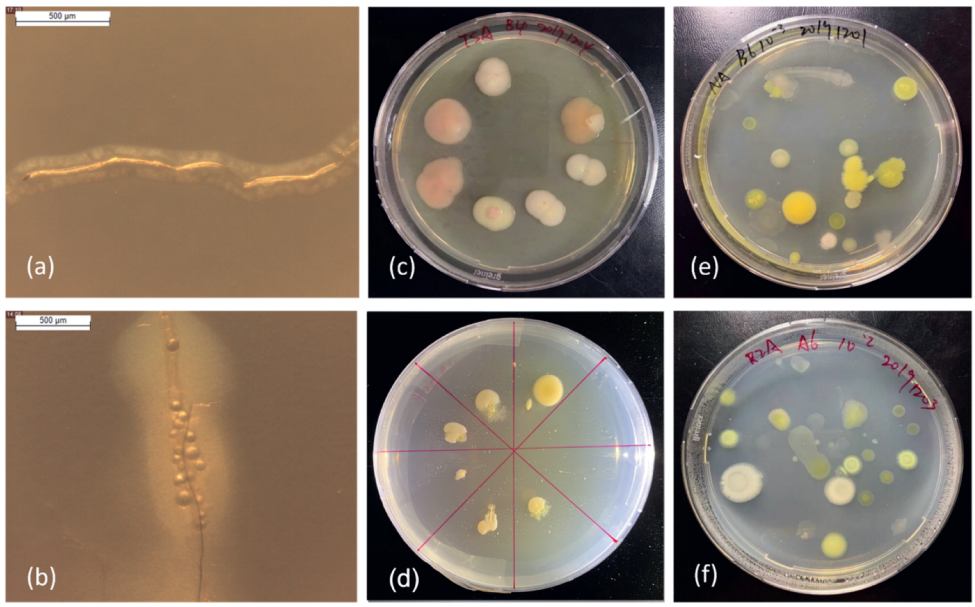

Fig. S1. Isolation AMF associated microbes using two strategies.

Stereo microscope images (a) and (b) show bacteria growing from mycorrhiza hyphae after 3 days of incubation. (c) and (d) shows the microbes growing from mycorrhiza hyphae on agar plates after 20 days of incubation. (e) and (f) show bacterial forming units grown from serial diluted hyphosphere samples after 20 days incubation.

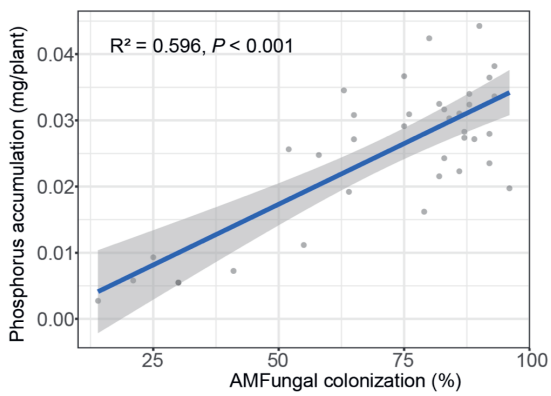

Fig. S2. Relationship between AMF root colonization (%) and plant phosphorus accumulation.

Table S1. AMF associated bacteria isolation media.

Media	Compound	Amount/L
Trypic Soy Broth Medium	Casein	17 g
	Soya peptone	3 g
	NaCl	5 g
	K_2HPO_4	2.5 g
	Dextrose	2.5 g
	(Agar)	20 g
	pH: 7.2	
1/10 TSA	Casein	1.7 g
	Soya peptone	0.3 g
	NaCl	0.5 g
	K_2HPO_4	0.25 g
	Dextrose	0.25 g
	(Agar)	20 g
	pH: 7.2	
Yeast Extract Manitol Medium	Yeast extract	0.5 g
	Mannitol	5 g
	K_2HPO_4	0.5 g
	$MgSO_4 \cdot 7H_2O$	0.2 g
	NaCl	0.1 g
	(Agar)	20 g
	pH: 7.0	
Tap Water Yeast Extract Medium	Yest extract	0.25 g
	K_2HPO_4	0.5 g
	(Agar)	18 g
	Tap water to 1L	
	pH: 7.2	
R2A medium	Casein acid hydrolysate	0.5 g
	Yeast extract	0.5 g
	Proteose peptone	0.5 g
	Dextrose	0.5 g
	Starch	0.5 g
	Dipotassium phosphate	0.3 g
	Magnesium sulfate	0.024 g
	Sodium pyruvate	0.3 g
	(Agar)	15 g
	pH: 7.2	
1/5 R2A	Casein acid hydrolysate	0.1 g
	Yeast extract	0.1 g
	Proteose peptone	0.1 g
	Dextrose	0.1 g
	Starch	0.1 g
	Dipotassium phosphate	0.06 g
	Magnesium sulfate	0.005 g
	Sodium pyruvate	0.06 g
	(Agar)	15 g
	pH: 7.2	
Nutrient Agar	Peptone	5g
	yeast extract	3g
	NaCl	5g
	Agar	15g
	pH: 7.4	

Table S2. PCR cycling conditions for amplifying 16S.

Step	Temperature	Time	Cycles
1	94℃	5min	1×
2	94℃	1min	
3	55℃	1min	30×
4	72℃	1min	
5	72℃	10min	1×
6	12℃	Hold	

Table S3. ½ Hoagland solution ingredients.

Macronutrients	Concentration (mM)
KNO_3	3
$(NH_4)H_2PO_4$	1
Ca(NO3)2•4H2O	2
$MgSO_4•7H_2O$	0.5
Micronutrients	**Concentration (μM)**
KCl	25
H_3BO_3	12.5
$MnSO_4•H_2O$	1
$ZnSO_4•7H_2O$	1
$CuSO_4•5H_2O$	0.25
$(NH_4)_6Mo_7O_{24}•4H_2O$	0.25
$C_{10}H_{12}FeN_2NaO_8$	10

Table S4. Modified Strullu and Romand (MSR) medium supplemented with 1% sucrose.

Component	Company	Amount/L
Strullu-Romand powder	Duchefa Biochemie (Haarlem, The Netherlands)	0.594g
Sucrose	Sigma (St. Louis, Missouri, United States)	10g
0.152M $Ca(NO_3)_2$	Merck (Darmstadt, Germany)	10ml
Phytagel	Sigma (St. Louis, Missouri, United States)	3g
dH_2O	-	976ml

Table S5. Citrate buffer for dissolving MSR medium.

Component	Amount/L
Citric acid (192.13M)	0.3456g
Sodium citrate (294.10M)	2.4108g

Table S6. ½ Hoagland solution without N, P.

Macronutrients	Concentration (mM)
K_2SO_4	3
$CaSO_4 \bullet 2H_2O$	2
$MgSO_4 \bullet 7H_2O$	0.5
Micronutrients	**Concentration (μM)**
KCl	25
H_3BO_3	12.5
$MnSO_4 \bullet H_2O$	1
$ZnSO_4 \bullet 7H_2O$	1
$CuSO_4 \bullet 5H_2O$	0.25
$(NH_4)_6Mo_7O_{24} \bullet 4H_2O$	0.25
$C_{10}H_{12}FeN_2NaO_8$	10

Table S7. Hyphsophere bacterial isolates.

Isolates.ID	Kingdom	Phylum	Class	Order	Family	Genus	Species	Similarity (%)
Agromyces_sp._ZB009	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_laixinhei	97.28
Agromyces_sp._ZB017	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_neolithicus	96.71
Agromyces_sp._ZB063	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_indicus	97.29
Agromyces_sp._ZB130	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_neolithicus	96.85
Agromyces_sp._ZB152	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_subbeticus	97.14
Agromyces_sp._ZB188	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_italicus	97.71
Agromyces_sp._ZB220	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_neolithicus	96.71
Agromyces_sp._ZB224	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Agromyces	Agromyces_mediolanus	96.42
Arthrobacter_sp._ZB016	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	99.82
Arthrobacter_sp._ZB033	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	99.86
Arthrobacter_sp._ZB037	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Arthrobacter_sp._ZB038	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Arthrobacter_sp._ZB048	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	99.86
Arthrobacter_sp._ZB070	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Arthrobacter_sp._ZB073	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Arthrobacter_sp._ZB074	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_globiformis	99.57
Arthrobacter_sp._ZB075	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Arthrobacter_sp._ZB076	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Arthrobacter_sp._ZB077	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Arthrobacter_sp._ZB084	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_ginkgonis	99.71
Arthrobacter_sp._ZB151	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_ginkgonis	99.28
Pseudarthrobacter_sp._ZB201	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Pseudarthrobacter_sulfonivorans	98.73
Arthrobacter_sp._ZB216	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_crystallopoietes	98.86

Arthrobacter_sp._ZB232	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Arthrobacter	Arthrobacter_QXT-31	100
Microbacterium_sp._ZB001	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100
Microbacterium_sp._ZB003	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.28
Microbacterium_sp._ZB005	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_hibisci	98.43
Microbacterium_sp._ZB010	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.28
Microbacterium_sp._ZB022	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	99.71
Microbacterium_sp._ZB024	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	98.86
Microbacterium_sp._ZB067	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	99.71
Microbacterium_sp._ZB069	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	99.71
Microbacterium_sp._ZB082	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	98.86
Microbacterium_sp._ZB088	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.71
Microbacterium_sp._ZB089	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB090	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB091	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.43
Microbacterium_sp._ZB093	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB094	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.16
Microbacterium_sp._ZB096	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.28
Microbacterium_sp._ZB098	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_deserti	99.8
Microbacterium_sp._ZB102	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB103	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB106	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB107	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB108	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.08
Microbacterium_sp._ZB110	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.43
Microbacterium_sp._ZB113	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.43
Microbacterium_sp._ZB114	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100
Microbacterium_sp._ZB116	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.26
Microbacterium_sp._ZB118	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB119	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium pumilum	98.3
Microbacterium_sp._ZB123	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	98.86
Microbacterium_sp._ZB126	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100
Microbacterium_sp._ZB127	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB131	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB132	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99
Microbacterium_sp._ZB133	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100
Microbacterium_sp._ZB135	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB137	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_esteraromaticum	99.71
Microbacterium_sp._ZB138	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_esteraromaticum	99.71
Microbacterium_sp._ZB140	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.28
Microbacterium_sp._ZB141	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_esteraromaticum	99.71
Microbacterium_sp._ZB142	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.28
Microbacterium_sp._ZB143	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100

Microbacterium_sp._ZB144	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.43
Microbacterium_sp._ZB145	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.14
Microbacterium_sp._ZB155	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_esteraromaticum	99.71
Microbacterium_sp._ZB156	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB168	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99
Microbacterium_sp._ZB173	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB179	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.06
Microbacterium_sp._ZB182	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_aoyamense	99.57
Microbacterium_sp._ZB183	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB184	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	98.68
Microbacterium_sp._ZB186	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.14
Microbacterium_sp._ZB189	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100
Microbacterium_sp._ZB191	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.14
Microbacterium_sp._ZB193	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.14
Microbacterium_sp._ZB196	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_hibisci	98.43
Microbacterium_sp._ZB200	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.31
Microbacterium_sp._ZB203	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB210	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.28
Microbacterium_sp._ZB217	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100
Microbacterium_sp._ZB223	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	98.71
Microbacterium_sp._ZB225	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.43
Microbacterium_sp._ZB227	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_algeriense	100
Microbacterium_sp._ZB230	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.29
Microbacterium_sp._ZB231	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.29
Microbacterium_sp._ZB235	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	98.82
Microbacterium_sp._ZB236	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_saccharophilum	99.57
Microbacterium_sp._ZB237	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	98.85
Microbacterium_sp._ZB244	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	98.61
Microbacterium_sp._ZB268	Bacteria	Actinobacteria	Actinomycetia	Microbacteriales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum	99.28
Pseudonocardia_sp._ZB157	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB171	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB172	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB198	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB211	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB212	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB247	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB248	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB249	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Pseudonocardia_sp._ZB250	Bacteria	Actinobacteria	Actinomycetia	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Pseudonocardia_carboxydivorans	100
Gottfriedia_sp._ZB071	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Gottfriedia	Gottfriedia_OV186	99.71
Gottfriedia_sp._ZB081	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Gottfriedia	Gottfriedia_OV186	99.86
Brevibacillus_sp._ZB040	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	Brevibacillus_centrosporus	98.84

Brevibacillus_sp._ZB079	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	Brevibacillus_choshinensis	100
Brevibacillus_sp._ZB080	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	Brevibacillus_nitrificans	99.43
Brevibacillus_sp._ZB098	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	Brevibacillus_centrosporus	100
Brevibacillus_sp._ZB099	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	Brevibacillus_choshinensis	100
Brevibacillus_sp._ZB148	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	Brevibacillus_choshinensis	99.41
Fictibacillus_sp._ZB207	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Fictibacillus	Fictibacillus_halophilus	99.57
Lysinibacillus_sp._ZB105	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	Ureibacillus_chungkukjangi	99.86
Ureibacillus_sp._ZB190	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Ureibacillus	Ureibacillus_chungkukjangi	99.86
Paenibacillus_sp._ZB097	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	Paenibacillus_qinlingensis	98.71
Paenibacillus_sp._ZB213	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	Paenibacillus_xylanisolvens	97.98
Paenibacillus_sp._ZB234	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	Paenibacillus_xylanisolvens	97.86
Achromobacter_sp._ZB008	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_deleyi	100
Achromobacter_sp._ZB015	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_kerstersii	99.57
Achromobacter_sp._ZB019	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_deleyi	99.43
Achromobacter_sp._ZB031	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_deleyi	100
Achromobacter_sp._ZB068	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_kerstersii	99.71
Achromobacter_sp._ZB072	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_deleyi	100
Achromobacter_sp._ZB147	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_deleyi	100
Achromobacter_sp._ZB154	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_deleyi	100
Achromobacter_sp._ZB194	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_piechaudii	100
Achromobacter_sp._ZB205	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	Achromobacter_deleyi	100
Bosea_sp._ZB026	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Boseaceae	Bosea	Bosea_thiooxidans	100
Bosea_sp._ZB046	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Boseaceae	Bosea	Bosea_robiniae	99.47
Devosia_sp._ZB006	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia	Devosia_riboflavina	99.28
Devosia_sp._ZB129	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia	Devosia_riboflavina	100
Devosia_sp._ZB149	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia	Devosia_Root413D1	99.86
Devosia_sp._ZB163	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia	Devosia_Root413D1	99.71
Devosia_sp._ZB251	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia	Devosia_Root413D1	99.86
Ensifer_sp._ZB007	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ensifer	Ensifer_adhaerens	100
Ensifer_sp._ZB219	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ensifer	Ensifer_adhaerens	100
Kaistia_sp._ZB136	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Kaistiaceae	Kaistia	Kaistia_defluvii	100
Lysobacter_sp._ZB206	Bacteria	Proteobacteria	Gammaproteobacteria	Lysobacterales	Lysobacteraceae	Lysobacter	Lysobacter_soli	99.29
Noviherbaspirillum_sp._ZB255	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Noviherbaspirillum	Noviherbaspirillum_autotrophicum	97.57
Pseudomonas_sp._ZB042	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_migulae	99.48
Roseomonas_sp._ZB036	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Roseomonas	Roseomonas_aerophila	99.57
Sphingopyxis_sp._ZB004	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis	Sphingopyxis_italica	99.28

Chapter 5

Summarizing discussion

Identifying microbial taxa and interactions to assist plant growth.

In order to feed the growing global population, synthetic fertilizers, herbicides, and pesticides have been widely used in conventional agriculture systems. While these chemicals have increased crop yields, they have also led to pollution of soil with chemical residues and a reduction in soil biodiversity (Riah *et al.*, 2014; Walder *et al.*, 2022; Wittwer *et al.*, 2021). Organic agriculture, on the other hand, seeks to lower environmental impact and maintain soil biodiversity by rejecting the use of synthetic compounds (Edlinger *et al.*, 2022; Hole *et al.*, 2005; Wittwer *et al.*, 2021). However, organic farming also results in lower crop yields than conventional farming (Jouzi *et al.*, 2017). To narrow the yield gap between organic and conventional farming, the use of biofertilizers in organic agriculture is a promising way to increase crop productivity in an environmentally friendly manner.

Biofertilizers are living microorganisms that are applied to seed, plant surfaces, or soil and promote plant growth through various mechanisms, including improving nutrient availability, boosting root biomass or root area, and enhancing the plant's ability to absorb nutrients (Vessey, 2003). A number of biofertilizers are derived from microbes that live in close association with plants. These microbes play vital roles in plant growth and survival, but also in plant decay. For example, nitrogen fixers such as *Rhizobium* can help legumes acquire nitrogen from the atmosphere, reducing the need for synthetic fertilizers (Somasegaran & Hoben, 2012). Other organisms, such as the nitrogen-fixing cyanobacterium *Anabaena azollae*, can also provide this function and are often applied to wetland rice in Southeast Asia to increase nitrogen availability (Mandal *et al.*, 1999). Arbuscular mycorrhizal fungi (AMF) are among the best studied plant-beneficial microbes that symbiotically colonize plant roots and help access remote nutrient pools. Commercialized mycorrhiza inoculants have been applied to maize to improve growth (Faye *et al.*, 2013). However, even the microbes that colonize decaying plant material can be important for plant growth. Microbial decomposers break down dead plant material and transform it into plant-accessible nutrients that can sustain a successive plant community. Using biofertilizers that can break down organic matter into inorganic nutrients can improve soil fertility and benefit crop growth (Rajendran & Devaraj, 2004). Therefore, it is important to explore natural microbial communities to find more biofertilizers that can increase crop productivity. It is also important to understand the interactions between microbial groups, which may guide us in improving the use efficiency of biofertilizers. In this thesis, we were interested in identifying the microbial taxa colonized in different ecological niches, as well as understanding the interactions between microbial groups and how to select potentially beneficial microbes from complex microbial communities.

Diverse interactions between microbial alliances and saprotrophic fungi or arbuscular mycorrhiza fungi

Saprotrophic fungi live together with bacteria in plant litter. The composition of saprotrophic fungi and bacteria can vary depending on environmental conditions (Hättenschwiler, Tiunov,

& Scheu, 2005). In Chapter 2, we manipulated the composition of saprotrophic fungi (35 strains) and bacteria (41 strains) in experimental microcosms, which allowed the synthetic communities to colonize plant litter and roots. By amplicon sequencing, we characterized the fungal and bacterial communities on plant litter and roots. The results show that over 60% of the bacterial and fungal isolates we introduced into the microcosms were present in the microbiome profiles of the litter and root samples. However, some of the inoculated isolates were not detected, which may be due to their inability to grow or survive in the microcosm conditions. The calcined clay used in the microcosm probably did not prove to be a microbe-friendly environment for their survival, and the physicochemical differences between this substrate and natural soil may have exerted a selective pressure on the inoculated microbes, favoring those that could adapt quickly to the new growth conditions. The microbes that we used in our synthetic communities originated from plant roots and also colonized the plant roots to a higher density than the litter. Therefore, it is important for future studies to focus on the role of microbes that originate from plant litter in litter decomposition. In particular, it would be interesting to isolate bacteria that attach to or live within fungal hyphae and examine the specific interactions between these bacteria and fungi and their impact on litter decomposition. Additionally, the broader interactions between microbial groups such as protists and arbuscular mycorrhiza fungi should also be considered, as these groups are widely distributed in soil microbiome and may have significant impacts on the litter decomposition process.

In addition to saprotrophic fungi and bacteria, which co-occur in plant litter and can be a potential source of biofertilizers, arbuscular mycorrhiza fungi that live in symbiosis with diverse microbes may also be a resource for finding biofertilizers (Emmett *et al.*, 2021; Zhang *et al.*, 2021). Unlike the plant root microbiome, which has been considerably profiled (Bakker *et al.*, 2012; Chaparro *et al.*, 2014; Quiza *et al.*, 2015), the mycorrhiza hyphal microbiome, has not been widely characterized.

In chapter 3, we aimed to investigate the fungal, bacterial, and protistan communities associated with the hyphae of Prunella vulgaris plants grown in microcosms with soil that had undergone either conventional or organic management practices for the past decade. We found that root, hyphal and soil samples comprised distinct microbial communities. Moreover, we found that the microbial communities in the soil were influenced by the management practices, but the microbial communities that formed on the roots and hyphae after three months of AMF-plant development were not affected by these practices. This suggests that both the plant and fungus select specific microbes, which diminishes the difference between the organic and conventional soil microbiomes.

For the protistan community, we identified 16 protists ASVs that were enriched in hyphal samples. The functional roles of these protists in the mycorrhiza hyphosphere are largely unknown, but previous studies have suggested that some protists may have the potential to promote plant growth. Weekers *et al.* (1993) found that when they fed *Enterobacteriaceae* to Amoebae of the genus *Hartmannella,* the Amoebae produced ammonium that could promote plant growth. Also, the co-cultivation of green algae of *Chlorella* with rhizobacteria enhanced

phosphorus availability in soil and promoted plant growth (de Jesus Raposo & de Morais, 2011; Schreiber *et al.*, 2018). Moreover, Rozmoš *et al.* (2021) recently found a protist that enhances the utilization of organic nitrogen by AMF. These examples highlight the largely untapped potential of protists in promoting plant growth and warrant a much broader effort for protists to unravel their roles in the plant's performance. Our result shows for the first time that specific protistan groups are associated with fungal hyphae. However, whether these protists feed on the hyphal exudates, or they are predators which target fungal hyphae or other microbes in the mycorrhiza hyphosphere, is still unclear to us.

The bacterial community associated with the hyphae was also different from those in the roots and soil, and the bacterial genus *Haliangium* was the most abundant on hyphal samples. While little is known about *Haliangium*, one isolate identified as *Haliangium ochraceum* has been suggested to feed on other bacteria and could have a predatory role in shaping the hyphal microbiome (Petters *et al.*, 2021). In addition, the genera *Pseudomonas* and *Devosia* as well as specific *Pseudomonas* and *Devosia* ASVs were consistently enriched in our experiments on AMF hyphae. Many *Pseudomonas* strains have been identified as mycorrhiza helper bacteria (MHB) that promote the colonization of ectomycorrhizas and arbuscular mycorrhizae (Frey-Klett *et al.*, 2007). We summarize the AM fungal hyphae enriched prokaryotic organisms in this study and compared it with other studies (Table 1). Further research is needed to fully understand the functional roles of these microbes and their potential to promote plant growth.

Table 1. Mycorrhiza enriched prokaryotic organisms on hyphosphere. Only the studies that sampled fungal hyphae are considered in this comparison.

AMF	AMF enriched bacteria	Conditions	Reference
***Glomus* sp. MUCL 43205; *Glomus intraradices* MUCL 43194**	**Species:** *Paenibacillus brasilensis* PB177	*In vitro*	Toljander *et al.*, 2006
Glomus intraradices*; *Glomus proliferum	**Family:** *Oxalobacteraceae* **Genus:** *Duganella, GeJanthinobacterium, Massilia*†*, Streptomyces*†	*In vitro*	Scheublin *et al.*, 2010
Dominant by *Funneliformis, Rhizophagus*	**Phylum:** *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes* **Genus:** *Rhizobacter, Sinorhizobium, Pseudomonas*†*, Streptomyces*†*, Variovorax, Labrenzia, Bradyrhizobium, Burkholderia, Ralstonia, Gemmatirosa*	*In situ*	Zhang *et al.*, 2018
Glomus versiforme, Rhizophagus irregularis	**Order:** *Betaproteobacteriales, Myxococcales, Fibrobacterales, Cytophagales, Chloroflexales, Cellvibrionales*	*In situ*	Emmett *et al.*, 2021
Rhizophagus intraradices	**Phylum:** *Myxococcota*, *Fibrobacterota*, *Verrucomicrobiota*, *Nitrososphaera* (Archaea)	*In situ*	Nuccio *et al.*, 2022
Dominant by *Rhizophagus irregularis*, *Septoglomus viscosum*, *Funneliformis mosseae*	**Genus:** *Halangium, Massillia*†*, Pseudomonas*†*, Devosia, SWB02, Cellvibrio,* possible genus 04*, Noviherbaspirillum, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Ohteakwangia, Pseudoxanthomonas, Sulfurifustis, and Pseudoduganella*	*In situ*	This study (Chapter 3)

† The bacterial taxa that have been enriched in more than one study.

Functional roles of the bacteria interacting with saprotrophic fungi and arbuscular mycorrhiza fungi

There is a wealth of knowledge about microbial taxa inhabiting diverse ecological niches (Konopka, 2006). However, the functions of many microbes are still inadequately understood. In particular, the interactions and influences of the two most abundant microbial kingdoms, Eubacteria and Fungi, on ecosystem functioning are unclear. In this thesis of chapter 2, we found that fungi are the main decomposer of plant litter. Litter decomposition in the treatments inoculated with fungi was 47% higher compared to the treatments that were not inoculated with fungi. Previous studies have suggested that bacteria appear to strongly contribute to litter decomposition (Adhi *et al.*, 1989; Lin *et al.*, 2012). Although in our study litter loss in the control was not significantly different from the treatment inoculated with bacteria, suggesting that bacteria are not so important for litter decomposition, litter loss in the bacteria-inoculated treatments increased at the end of the experiment from 8 to 12 weeks. This suggests that bacteria do contribute to litter decomposition later on in the process and this needs to be elucidated in further studies.

So far, only a few studies have manipulated microbial communities to investigate their roles and interactions, and we lack direct experimental evidence of fungi as the main drivers of litter decomposition. Fungal OTUs of *Fusarium, Aspergillus*, and *Penicillium* enriched on plant litters were previously reported to degrade lignin and cellulose (Jiang *et al.*, 2014; Lozovaya *et al.*, 2006; Perestelo *et al.*, 1997; Song & Fan, 2010), suggesting that these taxa have pivotal roles in litter decomposition. Even though some litter enriched fungal OTUs, such as fOTU1 (*Fusarium solani*), fOTU2 (*Fusarium oxysporum*), and fOTU5 (*Fusarium proliferatum*) were studied for their plant pathogenic lifestyle (Dugan *et al.*, 2003; Ma *et al.*, 2013; Ohara *et al.*, 2003), plants that were inoculated with these fungal OTUs were not showing any disease symptoms and the growth of those plants increased within 8 weeks in this study.

During the decomposition of litter, the C: N ratio decreases, and inorganic nutrients are released into the surrounding environment (Crowther *et al.*, 2012). In Chapter 2, we demonstrated that the greatest loss of litter mass occurred in the fungi-inoculated treatments during the first 4 weeks of decomposition. The increased growth of plants in the microcosms with fungi after 4 and 8 weeks may be due to the release of nutrients from the decomposing material. After 16 weeks, we found that shoot biomass was highest in the treatment that was inoculated with both bacteria and fungi, suggesting that bacterial and fungal communities can synergistically promote plant growth. Other studies have also suggested that fungi and bacteria can complement each other in providing plant nutrients and promoting plant growth (van der Heijden *et al.*, 2016; Yu *et al.*, 2021). In the earlier work of our lab, Wagg *et al.* (2014; 2019) manipulated the richness of soil microbial communities and found that a reduction in microbial richness and certain microbial groups in soil can decrease ecosystem functioning and plant growth. Another experiment that manipulated the composition of bacteria, fungi, and oomycetes on Arabidopsis roots showed that a combination of microbial communities can

improve plant growth more than any individual microbial group (Durán *et al.*, 2018). In our study, the mix treatment had the highest microbial richness and diversity as we co-inoculating these microcosms with both bacteria and fungi. Together these microbial groups sustain the most diverse ecosystem functions of all treatments, and likely as a result, guarantee the best plant growth in that treatment.

In addition to saprotrophic fungi, which break down litter and recycle nutrients for plants, other groups of fungi and their associate microbiome have the potential to serve as biofertilizers in agriculture. The use of arbuscular mycorrhizal fungi (AMF) in sustainable agriculture practices has been shown to have beneficial interactions with plants (Bender *et al.*, 2019; Siddiqui & Futai, 2008) In chapter 4, we employed a sieving-based strategy to isolate AMF hyphae along with bacteria that adhere to the hyphae. We acquired 143 bacterial isolates belonging to *Actinobacteria*, *Proteobacteria*, and *Firmicutes* that we could map to 48 ASVs detected on the hyphal samples analyzed in chapter 3. We subsequently tested 5 bacterial isolates that matched to ASVs which were significantly enriched on hyphal samples for their effects on plant growth and mycorrhization. In this way we identified one AMF-enriched bacteria *Devosia* sp., isolate ZB163, that promoted the nitrogen uptake, mycorrhization, and growth of Prunella plants.

Devosia sp. ZB163 increased the amount of total nitrogen in Prunella plants. Bacteria can increase nitrogen uptake through different mechanisms. Firstly, diazotrophs can fix nitrogen from the atmosphere. There is evidence that mycorrhiza co-inoculation with diazotrophs (*Acetobacter diazotrophicus*) increased the productivity of the tuber of sweet potatoes (Paula *et al.*, 1992). Legume plants form nodules that generate anaerobic environments for diazotrophs. However, when inoculating *Devosia* sp. ZB163 to the legume *Glycine max* (soybean), we did not observe nodule formation on the plant roots (data not shown). In addition, the *Devosia* sp. ZB163 genome does not harbor the genes required for nitrogen fixation in association with plants and it is thus unlikely that this bacterium functions as a free-living diazotroph. Secondly, some bacteria can mineralize organic nitrogen (e.g., proteins, chitins, amino acids, urea, and nucleic acids) to ammonia that plants can use directly (Liou & Madsen, 2008). As an important organic nitrogen source in soil, urea can be catalyzed urea to ammonia. We detected the complete gene cluster that putatively encode the urease complex in the *Devosia* sp. ZB163. However, urease also exists in plants (Polacco *et al.*, 2013). Whether the urease in the *Devosia* can limit the ammonia availability in the soil needs to be further verified by urease-depleted plants. Thirdly, it is acknowledged that bacterial nitrification increases nitrate in soils. However, the hypothesis that *Devosia* is a nitrification bacterium was also denied since we did not detect *amoA* gene which is the key gene for the bacterial nitrification process in the genome. Last, researchers found that upon the inoculation of an *Achromobacter* sp., the uptake of NO_3^-, K^+, and H^+ were increased in a seminal root of oilseed rape (Bashan, 1990; Bashan *et al.*, 1989; Bertrand *et al.*, 2000). The results suggest that the *Archromobacter* strain might stimulate the uptake rate of nitrate by stimulating the plant's ionic transport system. It would be interesting to test if the inoculation of *Devosia* increases the Prunella nitrogen transporter gene expression by transcriptome analysis. Together, we must conclude that although *Devosia* sp. ZB163

improves the total nitrogen amount in Prunella leaves, it does so through neither nitrogen fixation nor nitrification. The other pathways such as the ammonification or the stimulation of the ability of nitrogen transport in plants still need further verification.

A demand for investigating a broad range of microbial interactions in litter decomposition and mycorrhization

While a wide range of studies assessed the microbiome associated with soils, plants, and plant roots, the ecological function of many microbial communities is still poorly understood. For instance, there is a need for further investigation into the diverse range of microbial interactions that play a role in litter decomposition and or mycorrhiza formation and functioning. These processes are essential for the proper functioning of ecosystems and have significant impacts on the cycling of nutrients. By better understanding the complex interactions between microorganisms and plant litter, we can improve our ability to predict and manage the impacts of these processes on agriculture. In chapter 2, I used dead *Lolium multiflorum* plants as litter with a conservative C: N ratio (Martínez *et al.*, 2010). The quality of litter can affect their decomposition by influencing the composition of their microbiome (García-Palacios *et al.*, 2013; Smith & Bradford, 2003). Since we did not analyze the litter composition in this experiment, we cannot discuss how it may have changed among the different treatments. The microbes that we used in our synthetic communities originated from plant roots and also colonized the plant roots to a higher density than the litter. Therefore, future studies should use microbes that originate from plant litter to investigate their contribution to litter decomposition. Additionally, our synthetic communities were constructed with 41 bacteria and 35 fungi, which is relatively small compared to natural microbial communities. It is possible that these relatively small microbial communities lack specific taxa that play important roles in ecosystem functioning (Wagg *et al.*, 2019). Further research with a wider range of microbes from different microbial kingdoms is necessary to better understand the interactions and influence of microbial communities on plant growth, litter decomposition, and ecosystem functioning.

Different plants have preferences for the AMF species that they are colonized by (Torrecillas *et al*., 2012). The diverse mycorrhiza fungal species may also perform a selection process on its microbiome (Bahram *et al.*, 2018). In this thesis, we used one plant species to examine tripartite interactions among plant-mycorrhiza-bacteria may be revealed the specific beneficial effects of this interaction. To find bacteria that benefit the crop-mycorrhiza symbiosis may demand specific efforts of isolating bacteria from these crops-favored mycorrhizae. In this thesis of chapter 3, we not only detected bacteria interacting with mycorrhiza, but also fungal communities entangled with mycorrhiza hyphae and protists that exist on the hyphae. Fig. 1 summarizes the members of the mycorrhizal hyphal microbiome and presents their potential roles in nutrient cycling in the hyphosphere. In a further study, it is necessary to consider the plant-microbe interactions in a context of a broader range of microbes from diverse microbial groups.

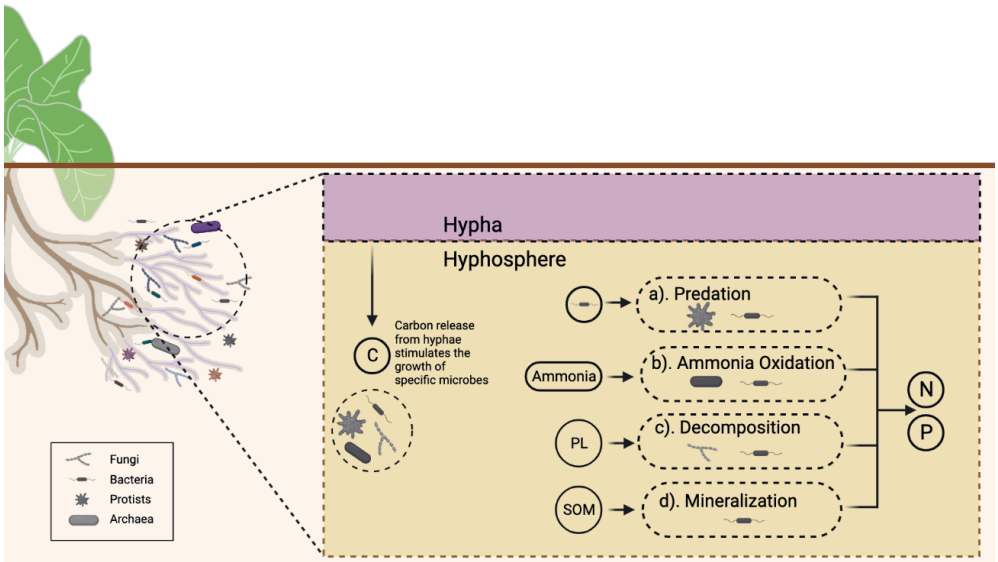

Fig. 1. Schematic graph of the mycorrhizal hyphal microbiome and its potential functions. **a)**. The presence of a specific protistan and bacterial community enriched on mycorrhizal hyphae suggests their potential to prey on bacteria and influence nutrient availability in soil (Emmett *et al.*, 2021; Nuccio *et al.*, 2022; Rozmoš *et al.*, 2021). **b).** Ammonia-oxidizing archaea and bacteria are enriched on mycorrhiza hyphae (Nuccio *et al.*, 2022; Teutscherova *et al.*, 2019). These microbes oxidize ammonia to nitrate, which is the first and rate-limiting step of nitrification. However, it is not yet clear whether this process contributes to plant growth and enhances N-acquisition or whether it leads to N_2O production and denitrification in the next steps of the nitrogen cycle. This warrants further investigation. **c).** Mycorrhizae are known to access plant litter (PL) patches and deliver mineral nutrients to their host plants (Herman, *et al.*, 2012; Nuccio *et al.*, 2013). Decomposers, such as saprotrophic fungi and certain bacterial groups, may assist in litter decomposition. **d)**. Specific bacterial groups can mineralize soil organic matter (SOM), making inorganic minerals available for plant nutrient uptake (Wang *et al.*, 2022; Zhang *et al.*, 2021). Fungi, bacteria, protists, and archaea are represented by the shapes shown in the legend frame. This figure was created with BioRender.com.

The challenge of developing gnotobiotic systems for plant and mycorrhiza growth

The study of gnotobiotic systems, or environments that are free of all microorganisms except for a specific set of known species, has long been of interest in the fields of biology, microbiology and agriculture. In the context of plant growth, gnotobiotic systems offer a unique opportunity to investigate the influence of specific microbial communities on plant development and productivity. However, the challenge of developing and maintaining gnotobiotic systems for plant growth is not a trivial one. In this thesis, we explored the various technical and logistical challenges that researchers face when attempting to create and maintain gnotobiotic systems for plant growth, as well as some of the strategies and approaches that have been developed to overcome these challenges.

In our Chapter 2 experiment, we detected bacteria in the control treatment, which should have been a microbe-free treatment according to our experimental design. We sterilized the substrate

and plant litter by autoclaving, and we confirmed the sterility by plating the substrate immediately following the autoclaving process. Additionally, we used sterilized Prunella seedlings that were free from visible microbial colonies in the microcosm. Therefore, it is most likely that the microbes in the control and fungi treatments were introduced after the preparation and assembly of the microcosms, or alternatively some bacteria act as endophytes in the seeds and were introduced to the microcosms by seed addition. This resulted in both the control and fungi treatments having a low abundance of bacteria strains. In the 8-week observation period, the plant growth responses to the distinct treatments were similar in the control and bacteria treatments and therefore the contamination did not affect our conclusion that fungi are the primary decomposers of the litter in this system. The percentage of litter decomposition was significantly reduced in the control and bacteria treatments in the absence of fungi, compared to the fungi-treated litter.

It would be beneficial to develop a gnotobiotic system for mycorrhiza development in order to investigate the mycorrhiza-microbe interactions in a simplified system and identify the underlying mechanisms. We attempted to create such a system but encountered several challenges. Specifically, we filled sterile Eco2boxes (Duchefa Biochemie, NL) with autoclaved soil and inoculated them with bacteria and monoxenic mycorrhizal spores. However, we were unable to maintain the sterility of the boxes, as unknown colonies formed and most of the boxes became contaminated. To overcome these challenges, we need to consider a few factors when developing a gnotobiotic system for investigating mycorrhiza-microbe interactions. Firstly, we need to find a substrate that is physiochemically similar to natural soil but can also support the survival and movement of the inoculated microbes. The use of calcined clay, for example, may not provide the necessary conditions for microbial growth. Secondly, AMF requires a certain level of CO_2 to colonize plant roots (Diop, 2003), so we need to find a way to maintain this concentration while also preventing contamination from the ambient environment. Finally, mycorrhiza colonization of plant roots takes several weeks, which increases the risk of microbial infection and complicates the microbial communities present in the system. In order to successfully establish a gnotobiotic system for studying mycorrhiza-microbe interactions, it is necessary to address the challenges previously mentioned.

Conclusion

In summary, this research has deepened our understanding of the interactions between different microbial groups and their roles in the processes of litter decomposition and mycorrhization. By studying these processes under controlled environmental conditions, we were able to identify key decomposers of plant litter and explore the interactions between mycorrhiza and the microbiome. This work has laid the foundation for further investigation into the potential of using microbial inoculum to improve nutrient cycling and plant growth in agricultural systems. In addition, we developed a new protocol for isolating and cultivating fungal hyphae-associated bacteria, which will be invaluable for future research on mycorrhiza-microbiome

interactions and the identification of mycorrhiza helper bacteria that could be developed into microbial inoculants.

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Summary

As the global population continues to grow, there is a need to find environmentally friendly ways to meet the increasing demand for food. One solution is to explore the use of microorganisms, such as bacteria, fungi, and protists, as biofertilizers in sustainable agricultural systems. Some of these microorganisms can improve soil nutrient availability and enhance plants' ability to absorb nutrients. However, identifying potential biofertilizers from the complex microbiomes from diverse ecological niches, such as decaying plant litter, plant roots, and plant symbiosis, is challenging. This is because microbiomes themselves are not only composed of millions of microbes but also because there are millions of interactions happening between these microbes that are influenced by both biotic and abiotic factors.

To dissect such complex microbiomes that may contain potential biofertilizers, we focused on two plant beneficial processes that enhance nutrient uptake. One of these plant beneficial processes is litter decomposition, which has the potential to improve soil nutrient availability (Chapter 2). While it has been suggested that bacteria and fungi may have a synergistic impact on litter decomposition, there is limited experimental evidence to support this idea. To address this gap in knowledge, I conducted a study under controlled environmental conditions to examine the effects of various microbial organisms on litter decomposition and plant growth. The microbial organisms used in the study included 41 bacteria and 35 saprotrophic fungi, which were isolated from plant roots grown in an agricultural field.

According to the results presented in **Chapter 2**, fungi are the main decomposers of plant litter and played a role in stimulating plant growth when microbial abundance was low. While there was no clear evidence of complementary effects of bacteria and fungi on litter decomposition, bacteria may have contributed to the process during a specific period of the experiment. When both bacteria and fungi were inoculated together, the highest plant biomass was observed among the microbial treatments at 16 weeks, indicating a synergistic effect of the two microbial groups on plant growth. Furthermore, the litter and root systems develop distinct microbiomes even when they were exposed to the same inoculum, suggesting that each ecological niche has its own preferred microbiome. This research experimentally demonstrates that fungi are the primary decomposers and highlights the importance of microbial interactions in plant growth.

The other beneficial process we studied in the thesis is plant mycorrhization which can enhance a plant's ability to absorb nutrients (Chapters 3 & 4). Arbuscular mycorrhiza fungi (AMF) are an important group of soil fungi that form symbiotic relationships with the roots of most land plants, helping them to absorb mineral nutrients and water. While the composition and functional roles of the microbial community surrounding AMF are not well understood, they are thought to play a key role in plant-mycorrhiza symbiosis. To better understand these roles, we developed a new protocol for isolating hyphae and studying fungal hyphae, roots, and soil from compartmentalized microcosms. Using amplicon sequencing techniques, I characterized the fungal, bacterial, and protistan communities associated with fungal hyphae and examined the impact of different agricultural soil management practices on AMF and their associated

microbiomes. I also attempted to cultivate the bacterial communities associated with fungal hyphae *in vivo.* Through characterization, we identified five bacteria that were significantly enriched in hyphal samples and matched the sequencing data of the hyphal microbiome described in Chapter 3. Subsequently, we studied the impact of these bacteria on plant-mycorrhiza symbiosis. We subsequently focused on one specific hyphal-enriched bacterium that increases nitrogen uptake, mycorrhization, and plant growth, and sequenced its genome to understand the mechanisms by which it benefits plant-mycorrhiza symbiosis.

In **Chapter 3** of this thesis, we analyzed the microbial communities of hyphae, roots, and soil in order to identify the AMF species colonizing the roots of *Prunella vulgaris*, as well as the bacteria and protists associated with the mycorrhizal hyphae. Our findings showed that the plants were predominantly colonized by specific AMF species, and certain bacterial genera were consistently enriched in the hyphosphere in two independent experiments. Additionally, specific protistan groups were found to colonize the fungal hyphae. After three months of symbiosis development, we observed that the microbial communities of the soil were still influenced by preceding management practices, but the microbial communities of the hyphae and roots were not. These results demonstrate the importance of AMF in shaping the microbial communities in the surrounding soil.

In **Chapter 4**, we examined the effects of 5 mycorrhiza-associated bacterial isolates on plants and arbuscular mycorrhiza fungi (AMF). Through three complementary experiments, we discovered that one of the AMF hyphae-associated bacterial strains, *Devosia* sp. ZB163 synergistically interacts with mycorrhiza to enhance plant nitrogen uptake and growth. Our results highlight the complexity and importance of the plant-AMF-microbiome interaction and its impact on plant growth.

Overall, these findings highlight the potential of microbes as biofertilizers and the importance of the plant-microbe interaction in shaping the microbial community and influencing plant growth. Additionally, microbes that are closely associated with a particular ecological niche are likely to play more important roles in maintaining the balance and functioning of that niche than those that are loosely associated with it. It is demonstrated that functioning of AMF also relies on interactions with specific bacteria colonizing the hyphosphere. Further research is needed to fully understand the mechanisms behind these interactions and to identify potential biofertilizers that can be used in sustainable agricultural systems.

Nederlandse samenvatting

Door de groeiende wereldbevolking is het noodzakelijk om milieuvriendelijkere manieren te vinden om aan de stijgende vraag naar voedsel te voldoen. Een mogelijke oplossing hiervoor is het gebruik van micro-organismen met nuttige eigenschappen, zoals specifieke bacteriën, schimmels en protisten. Deze organismen kunnen als microbiologische bemesters in duurzame landbouwsystemen ingezet worden. Sommige van deze micro-organismen kunnen namelijk de beschikbaarheid van bodemnutriënten verbeteren en de capaciteit van planten om nutriënten op te nemen versterken. Echter, het identificeren van potentiële microbiologische bemesters uit de complexe microbiomen die gevonden worden in de verschillende ecologische niches van de bodem, is zeer uitdagend. Dit komt omdat bodem microbiomen bestaan uit miljoenen micro-organismen, maar ook omdat er tussen die micro-organismen miljoenen interacties plaatsvinden die beïnvloed worden door zowel biotische als abiotische factoren.

Om dergelijke complexe microbiomen te ontleden en microbiologische bemesters te identificeren, richtten we ons in dit proefschrift op twee plantengroeibevorderende processen die de opname van nutriënten versterken. Eén van deze plantengroeibevorderende processen is afbraak van dood plantmateriaal, omdat het in potentie de beschikbaarheid van bodemnutriënten voor de plant verbetert. Diverse wetenschapper wijzen erop dat bacteriën en schimmels een synergetisch effect kunnen hebben op afbraak van plantenresten. Echter er beperkt experimenteel bewijs voor deze stelling. Om deze kennislacune aan te pakken, voerde ik een studie uit onder gecontroleerde omgevingscondities om de effecten van verschillende micro-organismen op afbraak van plantenresten en plantengroei te onderzoeken.

In **Hoofdstuk 2** creëerden we verschillende synthetische gemeenschappen van micro-organismen, die bestonden uit 41 bacteriën en/of 35 soorten (taxa) saprotrofe schimmels, die allen eerder waren geïsoleerd van plantenwortels uit landbouwgrond. Vervolgens keken we onder gecontroleerde omstandigheden in gesloten kleine microcosmen naar de effecten van deze microbiële gemeenschappen op zowel de afbraak van dood plantmateriaal als de groei van planten. Onze resultaten suggereren dat schimmels de belangrijkste afbrekers van plantenresten zijn en dat ze vooral effect hadden op plantengroei wanneer de microbiële rijkdom laag was. Er was geen duidelijk bewijs van complementaire effecten van bacteriën en schimmels op afbraak van plantenresten. Toen beide bacteriën en schimmels samen werden geïnoculeerd, werd na 16 weken echter wel de hoogste plantenbiomassa waargenomen, wat aangeeft dat er wel een synergetisch effect was van de twee microbiële groepen op de plantengroei. Bovendien waren de plantenresten en de zich ontwikkelende wortelsystemen in de microcosmen door verschillende microbiomen gekoloniseerd, zelfs wanneer ze hetzelfde inoculum kregen. Deze studie toont experimenteel aan dat schimmels de belangrijkste afbrekers zijn van strooisel en benadrukt het belang van microbiële interacties voor de plantengroei.

Het tweede plantengroeibevorderende proces dat we in deze thesis onderzochten, concentreert zich op Arbusculaire mycorrhiza-schimmels (AMF) (Hoofdstukken 3 & 4). AMF zijn een

belangrijke groep bodemschimmels die symbiotische relaties vormen met de wortels van de meeste landplanten, en de opname van mineralen en water kunnen verhogen. AMF vormen een uitgebreid netwerk van schimmeldraden (hyfen) in de bodem. Deze hypfen worden ook weer door micro-organismen gekoloniseerd. Hoewel de samenstelling en functionele rollen van de microbiële gemeenschap rond AMF nog niet goed begrepen zijn, wordt aangenomen dat ze een sleutelrol spelen in de plant-mycorrhiza symbiose.

In **Hoofdstuk 3** van dit proefschrift analyseerden we de microbiële gemeenschappen op wortels van *Prunella vulgaris,* op de AMF hyfen die de wortels hadden gekoloniseerd en in de daaromliggende bodem. Door gebruik te maken van amplicon-sequencingtechnieken, beschreven we de schimmel-, bacteriële- en protistengemeenschappen die geassocieerd zijn met schimmelhyfen en onderzochten we de impact van verschillende landbouwmanagementpraktijken op AMF en hun geassocieerde microbiomen. In twee onafhankelijke experimenten vonden we dat bepaalde bacteriële genera consistent verrijkt waren op de hyfen en veel algemener waren op de hyphen als in de omliggende bodem of op de plantenwortelsBovendien werden specifieke protistengroepen aangetroffen die de schimmelhyfen koloniseren. Na drie maanden symbiose-ontwikkeling waren de microbiële gemeenschappen in de bodem nog steeds beïnvloed door voorgaande managementpraktijken, maar de microbiële gemeenschappen van de hyfen en wortels niet. Deze resultaten tonen aan hoe belangrijk AMF zijn in het vormgeven van de microbiologische gemeenschappen in de omringende bodem.

In **Hoofdstuk 4** onderzochten we de effecten van 5 mycorrhiza-geassocieerde bacteriële isolaten op de plant mycorrhiza symbiose. Deze 5 bacterie isolaten hadden we geisoleerd van de AMF hyfen. Door middel van drie complementaire experimenten ontdekten we dat één van de AMF hyfen-geassocieerde bacteriële stammen, *Devosia* sp. ZB163, synergetisch interacteert met AMF en de stikstofopname en groei van de plantversterkt. Onze resultaten benadrukken de complexiteit en het belang van de plant-AMF-microbiome-interacties en de impact hiervan op de plantengroei.

Samenvattend benadrukken de bevindingen in dit proefschrift het potentieel van microben als microbiologische bemesters en grote invloed van microbiële gemeenschappen op plantengroei. Daarnaast zijn microben die nauw geassocieerd zijn met een specifieke ecologische niche waarschijnlijk belangrijker voor het behouden van de balans en het functioneren van die niche dan degenen die er losjes mee geassocieerd zijn. Onze resultaten duiden erop dat nog een derde partner, de bakterien erg belangrijk zijn voor een goede funktionerende symbiose tussen planten en AMF. Verder onderzoek is nodig om de mechanismen achter deze interacties volledig te begrijpen en potentiële microbiologische bemesters te identificeren die kunnen worden gebruikt in duurzame landbouwsystemen.

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Curriculum vitae

Changfeng Zhang was born on January 22, 1990 in Tang Shang, He Bei, China. He began his undergraduate studies in biotechnology at the Central South University, September 2009. During his third year, he interned in the lab of Prof. dr. Xueduan Liu, studying the growth of acidophilic sulfur-oxidizing bacteria in the presence of nickel ions. In his final year, he worked with Prof. dr. Zhiguo He to investigate the ability of acidophilic bacteria to absorb Cu^{2+} and its impact on their growth. After graduating in June 2013, he pursued a master's degree at the Chinese Academy of Medical Sciences and Peking Union Medical College's, under the guidance of Prof. dr. Yuqin Zhang. There, he examined the culturable bacterial diversity in the rhizosphere of medicinal plants and screened their antimicrobial abilities. He also identified several new taxa and used a polyphasic approach to classify them. In February 2018, he began his Ph.D. studies in the Plant-Microbe Interactions group at the Institute of Environmental Biology, Utrecht University, under the supervision of Prof. dr. Marcel van der Heijden and dr. Roeland Berendsen. He conducted research on the microbial interactions in soil and rhizosphere and their impact on litter decomposition, mycorrhizal associations, and plant growth at both Agroscope (Zürich, Switzerland) and Utrecht University. After defending his thesis in April 2023, he plans to pursue an academic position to further study plant-microbiome interactions.