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RESEARCH

Bacterial community in the hyphosphere of an arbuscular mycorrhizal fungus differs from that in the surrounding environment and is influenced by hyphal disruption

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Abstract
Bacterial composition and functions in the hyphosphere of arbuscular mycorrhizal (AM) fungi are complex because AM fungal hyphae transport carbon compounds from plant photosynthesis which feed bacteria and act as signaling molecules. This function is lost when hyphae separate from roots, a common occurrence in soil. However, the impact of such disturbances on hyphal surface bacteria remains unclear. We used in vitro bi-compartmented Petri plates with carrot roots and the AM fungus *Rhizophagus irregularis* MUCL 43194, separating root and hyphal compartments. Treatments included hyphae connected to roots (+AM), no hyphae (-AM), and hyphae cut at different times (C3D and C0D, where C3D indicates hyphae cut 3 days before inoculation and C0D indicates hyphae cut on the day of inoculation) subjected to a bacterial suspension extracted from a field soil. Thirteen bacterial phyla were identified, with *Streptomyces*, *Pseudomonas*, *Rhodococcus*, and *Cellulomonas* dominating. Hyphae increased bacterial ASV relative abundance, notably enriching Actinobacteria ASVs. After 14 days, α-diversity decreased from -AM to C3D, C0D, and +AM, with fewer Bacteroidetes species in +AM compared to -AM. Root-connected hyphae led to deterministic bacterial assembly, while cut hyphae resulted in stochastic assembly. Our findings show that physical disruption of hyphae significantly affects bacterial diversity and may influence ecological functions.

Keywords AM fungi · Bacteria community · Community assembly · Hyphal injury · Hyphosphere

Introduction

Plants transfer 10–50% of the carbon (C) produced during photosynthesis from aboveground to belowground, where it feeds roots and associated microorganisms forming a unique plant microbiome (Choi et al. 2021; Yue et al. 2023). More than two-thirds of plant species are associated with arbuscular mycorrhizal (AM) fungi, with which they form symbiotic relationships, transferring up to 20% of C compounds in the form of fatty acids and sugars to the hyphae to maintain the symbiosis (Jakobsen and Rosendahl 1990). While AM fungi use plant-derived C to grow in abundance in the soil, with lengths ranging from 2.7 to 20.5 m per g soil (Wipf et al. 2019), their extraradical hyphae extensively absorb soil nutrients, especially phosphorus (P), to meet the growth needs of plants. Similar to the rhizosphere, the hyphosphere (i.e., the narrow zone of soil surrounding the hyphae), is host to numerous bacteria, some of which have a major influence on plant nutrition and biogeochemical nutrient cycles (Cheng et al. 2012; Garcia et al. 2016; González-Guerrero et al. 2016). For example, *Paenibacillus* sp. can degrade chitin to provide nitrogen (N), and *Rahnella aquatilis* can degrade phytic acid to enhance P nutrition for AM fungi, ultimately improving plant P status (Zhang et al. 2016; Rozmoš et al. 2022; Duan et al. 2023). The mycorrhiza-associated *Devosia* sp. ZB163 works synergistically with AM fungi in plant roots, strongly promoting plant growth, nitrogen uptake, and mycorrhization (Zhang et al. 2024).

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Only a small proportion of soil bacteria remain active in the absence of readily available C, while the majority remain dormant (Blagodatskaya and Kuzyakov 2013). *Bacillus* species, for example, can form spores that can remain dormant for decades and rapidly resume growth upon exposure to C-rich nutrients (Gao et al. 2023). Therefore, the driving force for hyphosphere bacteria to grow and interact with AM fungi is largely related to the release of hyphal exudates (i.e., sugars, carboxylates and amino acids) (Zhang et al. 2018; Rozmoš et al. 2022; Sun et al. 2023) into the hyphosphere (Zhang et al. 2022). These substances are essential for bacteria to produce energy through glycolysis, the citric acid cycle and oxidative phosphorylation as well as for synthesizing various macromolecules (Toljander et al. 2007; Bharadwaj et al. 2012; Luthfiana et al. 2021; Gralka et al. 2023). However, as obligate biotrophs, the C required for the growth, development and metabolism of AM fungi comes from host plants, part of which is exuded by the hyphae, in exchange for mineral nutrients such as N and P, depending on the plant, AM fungal species and their growth environment (Jakobsen and Rosendahl 1990; Jiang et al. 2017; An et al. 2019; Hawkins et al. 2023). Recently, a study has shown that a hyphosphere bacterium induces the synthesis and transport of fatty acids in a host plant by supplying P to an AM fungus, thereby promoting the C metabolism of the AM fungus and increasing its biomass (Duan et al. 2023), which may result in changes in the composition and quantity of hyphal exudates. Most importantly, hyphal exudates can continue to promote bacterial growth and stimulate the ability of bacteria to mineralize organic P (Zhang et al. 2016). In other words, the amount of C provided by host plants to AM fungi affects the C metabolism of AM fungi, which in turn influences the release of hyphal exudates and the recruitment of bacteria.

In situ $^{13}CO_2$ pulse-labelling in grassland revealed a rapid pathway for the movement of carbon from AM hyphae to the soil, a mechanism that disappears rapidly (within 24 h) after the connections between hyphae and roots are broken (Johnson et al. 2002). The supply of C to hyphae can be impeded either directly by hyphal severing, e.g., by arthropods feeding, or indirectly, e.g., by mechanical or chemical defoliation of plants. While the impact of these disturbances on hyphal development and spore production is well documented (Mitchell and Parkinson 1976; Heděnec et al. 2013; van der Heyde et al. 2017), no information is available on their impact on the microbial communities associated with hyphae.

In the present study, we used root organ cultures of carrots associated with the model AM fungus *Rhizophagus irregularis* to investigate the impact of root-hyphal separation on the diversity and composition of hyphosphere active bacteria. Specifically, we hypothesized that (1) hyphae connection to the root significantly influences the diversity of the bacterial community and the relative abundances of species in the hyphosphere; and (2) separation of hyphae from the root leads to a temporal change in the β-nearest taxon index of hyphosphere bacterial communities.

Materials and methods

Biological material and experimental design

The AM fungus *R. irregularis* MUCL 43194 was provided by the Glomeromycota in vitro collection (GINCO, Belgium). It was maintained in vitro in bi-compartmented Petri plates (90 × 15 mm) on Ri T-DNA transformed roots of carrot (*Daucus carota* L.) clone DC2 on Modified Strullu-Romand (MSR) medium (Declerck et al. 1998), as described in Cranenbrouck et al. (2005). The experimental design consisted of bi-compartmented Petri plates (90 × 15 mm), with one compartment (i.e., the root compartment– RC) containing the carrot root clone DC2 associated with the AM fungus, and the other compartment (i.e., the hyphal compartment– HC), containing only extraradical hyphae (ERH) of the AM fungus (Fig. S1A) (+AM treatment). Twenty-five mL of sterilized (121 °C for 15 min) MSR medium solidified with 3 g L^{-1} Phytagel (Gelzan™ CM, G3251, CP Kelco, USA) was added in the RC. In the HC, 20 mL of the same medium without sucrose, inorganic P, EDTA, calcium and vitamin sources but supplemented with 280 µM organic P in the form of Na-phytate (Phytic acid sodium salt hydrate, 68388, Sigma-Aldrich, MO, US) was added. In Experiment 1, a control treatment was included that consisted of bi-compartmented Petri plates with a 0.5 cm gap in the RC (achieved by removing a strip of MSR medium with a scalpel) to completely avoid the presence of hyphae in the HC (-AM treatment). Every 7 days, the hyphae that extended into the gap was cut with a scalpel to ensure the absence of growth in the HC (Fig. S1A). In Experiment 2 two control treatments were included that consisted of bi-compartmented Petri plates with hyphae cut (with a 0.5 cm gap as above) 3 days before inoculation with bacteria in the HC (C3D treatment) or at the time of bacterial inoculation in the HC (C0D treatment). In both experiments, the composition of the bacterial community was assessed 3-, 6- and 14-days after inoculation in the HC.

Soil type and extraction of bacterial suspension

A fuvo-aquic soil was sampled from a long-term field experiment in Beijing, China (40°08′N, 116°10′E). Five sub-samples weighing approximately 300 g were taken from a depth of 0 to 20 cm, mixed, sieved to 2 mm and homogenized. The

soil was then stored at 4 °C until extraction of bacteria. The physico-chemical properties of the soil were as follows: pH 8.2, 14.74 g kg^{-1} organic matter, 94.10 mg kg^{-1} mineral N (NH_4OAC extractable), 17.79 mg kg^{-1} P ($NaHCO_3$ extractable). The bacterial suspension was extracted from the soil using Tween 80/Tetra Sodium Pyrophosphate (TTSP) and Nycodenz (Burmølle et al. 2003). Specifically, a stock solution of Nycodenz (Nycodenz AG, 1002424, Alere Technologies AS, Oslo, Norway) was prepared by dissolving 16 g of Nycodenz in 20 mL water and sterilizing it by filtration. A solution of Tween80/Tetra Sodium Pyrophosphate (TTSP) was also prepared to a final concentration of 50 mM tetrasodium pyrophosphate and 0.05% Tween 80 in 500 mL water and sterilized by filtration. Four mL of TTSP was added to 2 g of soil in 10 mL centrifuge tubes and vortexed for 1 min. One mL of the liquid phase was transferred to 2 mL centrifuge tubes containing 500 µL of Nycodenz. The tubes were centrifuged at 10,000 g for 10 min. After centrifugation, 1 mL of the upper and middle phase of the solution was transferred to 10 mL centrifuge tubes containing 6 mL of liquid sterilized (121 °C for 15 min) Modified-MSR medium (without sucrose, inorganic P, EDTA, calcium and vitamins). After shaking, the tubes were centrifuged at 5000 g for 10 min at 4 °C. The supernatants were discarded and the bacterial cells re-suspended in 1 mL of sterilized Modified-MSR medium in 10 mL centrifuge tubes (Jin et al. 2024).

Experiment 1: short-time dynamics of bacterial community in the hyphosphere

Bi-compartmented Petri plates were prepared as described above. After 7 weeks of association in the RC, the ERH started to cross the partition wall separating the RC from the HC and developed profusely in the HC. After another 4 weeks, 200 µL of the bacterial suspension described above was inoculated on the surface of the HC containing ERH (+AM treatment) or devoid of hyphae (i.e., the -AM treatment). To ascertain there was no hyphal contamination in the HC of -AM treatment, before inoculating the bacteria, we examined the HC under a stereomicroscope. After 3-, 6-, and 14-days, ERH (for +AM treatment) and the surface of the MSR medium (for -AM treatment) were collected. Three replicates were set up per treatment and harvest time.

Experiment 2: impact of hyphae cutting on the bacterial community in the hyphosphere

Bi-compartmented Petri plates were prepared as described above. In each Petri plate, the hyphae crossed the plastic barrier and developed in the HC. An air gap of 0.5 cm was made in the RC 3 days before bacterial inoculation (C3D treatment) or on the day of bacterial inoculation (C0D treatment). Bacteria were then harvested 3-, 6- and 14-days after inoculation and community composition was evaluated. Three replicates were set up per treatment and harvest time.

Experiment 1 and Experiment 2 used the same growth conditions, with the composition and volume of the medium strictly controlled, as well as the cultivation temperature and time. These standardized conditions helped to standardize the experiments and to minimize any differences between them.

Hyphae and bacteria collection, DNA extraction and sequencing

In the treatments with hyphae connected to roots (+AM treatment) or separated from roots (C3D and C0D treatments) in the HC, the MSR medium in the HC was cut into 4 pieces and distributed equally in two sterile centrifuge tubes (50 mL) containing 35 mL sterilized sodium citrate buffer and vortexed for 4 min for dissolution of the Phytagel. The hyphae from the two tubes were combined, forming a pellet, and rinsed gently twice in a Petri plate containing fresh sodium citrate buffer to eliminate any remaining medium. Sterile blotting papers were used to soak up excess buffer from the hyphal pellet. In the treatment that contained only the bacterial suspension in the HC (-AM treatment) the bacteria were scraped from the surface of the MSR medium. The bacteria were collected in 2 mL centrifuge tubes stored at -80°C for DNA extraction.

Total genomic DNA extraction of ERH and MSR medium was done using the FastDNA SPIN Kit for Soil (MP Biochemicals, Solon, OH, USA), following the manufacturer's instructions. PCR amplification of the bacterial 16S rRNA gene was performed. The PCR amplification of the bacterial 16S rRNA gene V3–V4 region was performed using the forward primer 338 F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Data analysis

All statistical analyses were conducted with R 4.1.1 and IBM SPSS Statistics 21. Microbiome bioinformatics were performed with QIIME2 with slight modifications according to the official tutorials (Bolyen et al. 2019). Briefly, raw sequence data were demultiplexed using the demux plugin followed by primers cutting with the cutadapt plugin (Martin 2011). Sequences were then quality-filtered, denoised, merged and chimeras removed using the DADA2 plugin (Callahan et al. 2016). Non-singleton ASVs were aligned with mafft (Katoh et al. 2002). Taxonomy was assigned to ASVs using the classify-sklearn Naive Bayes taxonomy classifier in the feature-classifier plugin (Bokulich et al. 2018) against the SILVA Release 138 Database (Bolyen et al. 2019). Bray-Curtis metrics (Bray and Curtis 1957) was visualized via principal coordinate analysis (PCoA). Statistical analyses of differentially abundant ASVs were performed using the R packages DESeq2. To clarify, the enriched ASVs were sourced from the harvest times at 3DAI, 6DAI, and 14DAI. The relative abundances of ASVs from the treatments C3D, C0D, and +AM were compared to those from the -AM treatment. All significantly enriched ASVs ($\log_2$-fold change > 1, $P < 0.05$) were collected and presented for comparison, showing the enrichment levels of ASVs under three different hyphae and root connections at 3DAI, 6DAI, and 14DAI, as indicated by the magnitude of the $\log_2$-fold change. Alpha diversity metrics, including observed species and Chao1, were calculated using the 'vegan' package in R (Chao 1984; Gotelli and Colwell 2001).

For Fig. 5A, the data from Experiment 1 of arbitrary pairs of replicates from the -AM or the +AM treatments comprised a sample unit. For Fig. 5B, the C3D, C0D, +AM treatment, and the corresponding -AM treatment samples harvested at the same time were arbitrarily paired as samples. βNTI (Beta Nearest Taxon Index) was calculated for each pair of samples by finding the nearest taxonomic unit in the contrasted sample for each ASV (Amplicon Sequence Variant). The average phylogenetic distance between these nearest taxonomic units is defined as βMNTD (Beta Mean Nearest Taxon Distance). To obtain the null distribution of βMNTD, we randomized the taxonomic units and their relative abundances 999 times in the phylogenetic tree and recalculated βMNTD. This forms the βMNTD null distribution. βNTI is the standardized effect size between the observed βMNTD and the mean of the null distribution. βNTI was constructed using the R packages NST, picante, and ape. The stochastic contribution rate = the number of $|\beta NTI| < 2$ / total number, and the deterministic contribution rate = the number of $|\beta NTI| > 2$ / total number (Stegen et al. 2012).

Results

The in vitro culture system used in this study was primarily designed for cultivable hyphosphere bacteria. Their contribution rates of the deterministic processes determination across different treatments of the two experiments combined was examined. In all, 13 bacterial phyla were detected. They mainly belonged to four phyla: Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes. Together, the relative abundance of these four phyla accounted for 99.9% of the total abundance (Fig. 1). The Actinobacteria had the highest relative abundance, making up 55.9% of the total. In addition, Fig. 1 illustrates the taxonomic classification and relative abundance of bacteria at the class, order, family, and genus levels within the 50 most abundant genera specifically in the hyphosphere (+AM treatment). The proportions of *Streptomyces*, *Pseudomonas*, *Rhodococcus* and *Cellulomonas* are the highest, with relative abundances of 18.9%, 21.9%, 14.3% and 5.4%, respectively (Fig. 1). Similarly, in the hyphae separated from roots treatments, the relative abundances of *Streptomyces*, *Pseudomonas*, *Rhodococcus*, and *Cellulomonas* in the hyphosphere were 23.8%, 12.6%, 11.1%, and 6.1%, respectively (Fig. S2).

Comparing bacterial communities in the hyphosphere of root-connected hyphae (+AM treatment, Experiment 1) and on MSR medium (-AM treatment, Experiment 1), we observed that, among the 10 most abundant genera, the relative abundances of *Streptomyces*, *Pseudomonas*, and *Cellulomonas* associated with the ERH of *R. irregularis* MUCL 43194 were higher than for the controls without hyphae (-AM treatment) at all three time points (Fig. 2A). In contrast, the relative abundance of *Variovorax* was lower in the +AM treatment than in the -AM treatment at all three time points (Fig. 3A). For the genera *Streptomyces* and *Pseudomonas*, the relative abundances in the +AM treatment were significantly higher than -AM treatment at 3 DAI (Fig. 2A). Furthermore, we observed that the relative abundance of bacteria differed between the +AM treatment and the treatment with hyphae separated from the roots. Indeed, the relative abundance of *Pseudomonas* increased at day 14 in the +AM treatment but decreased in the hyphae separated from the roots including C3D and C0D (Fig. 2B). Principal coordinates analysis (PCoA) of Experiment 1 also showed that the bacteria communities in the +AM treatment and -AM treatment were separated along the main axis, with sample attachment type explaining 38% and harvest time explaining 16% of the variance, respectively (Fig. 2C). In Experiment 2, harvest time explained 26% of the variance (Fig. 2D).

Comparison of ASV enrichment in the different treatments showed the impact of hyphae on bacterial ASV relative abundance (Fig. 3A, C, E). A significant enrichment of ASVs from the Actinobacteria class was observed in

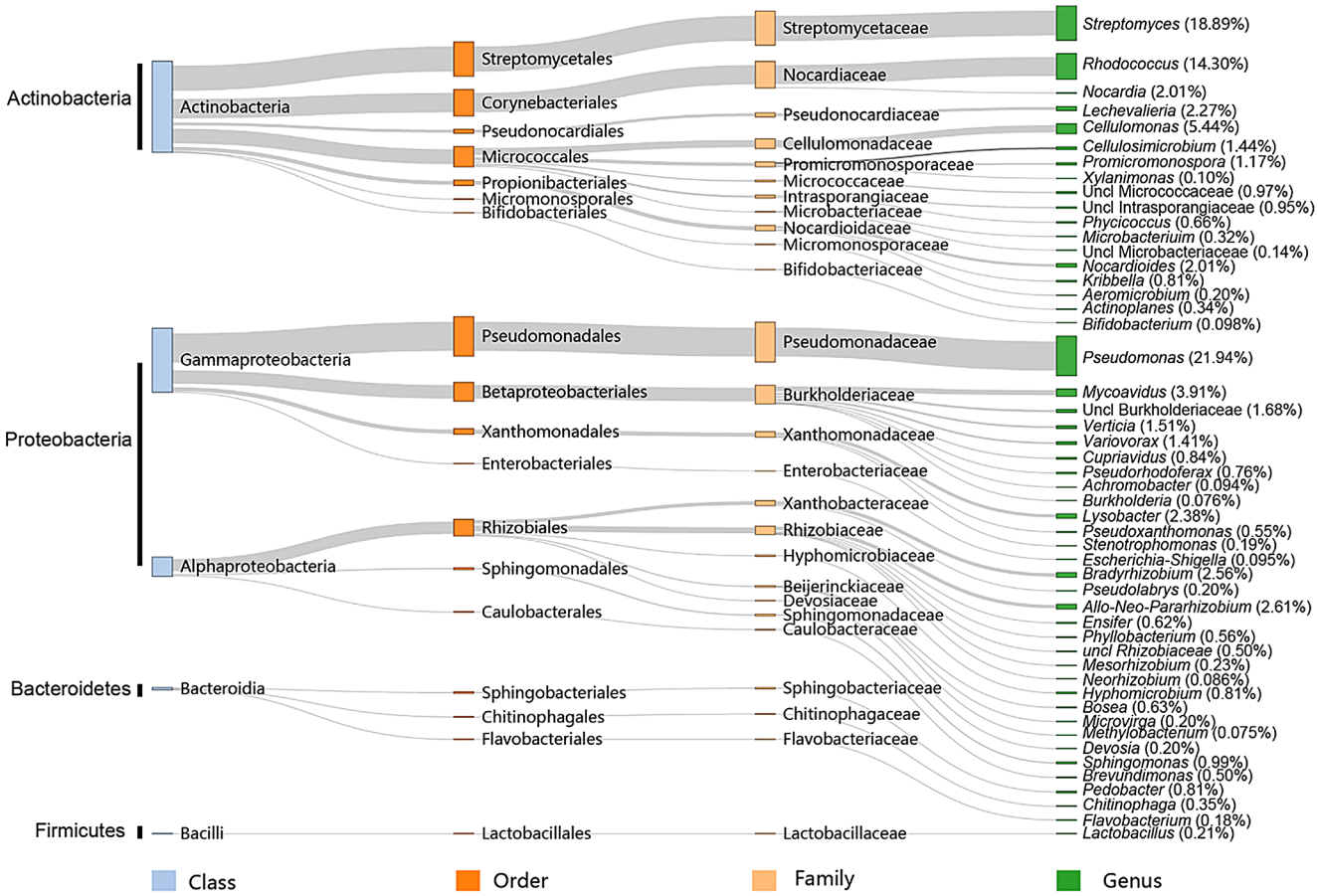
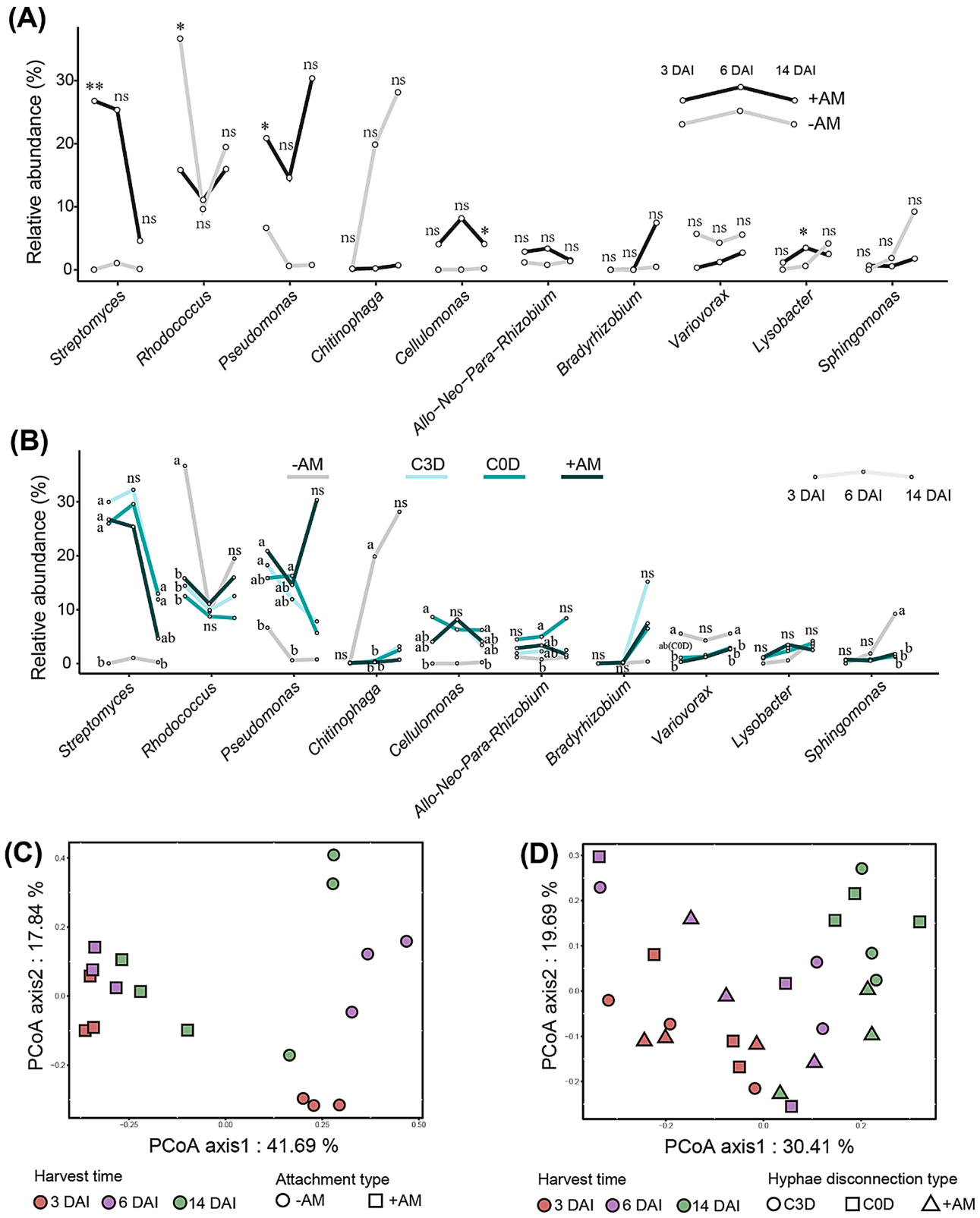


Fig. 1 Species classification of the top 50 most abundant genera in the hyphosphere (+AM treatment, Exp 1) and their proportion at the relevant taxonomic level. The height of the columns represents the relative abundance

the C3D, C0D and +AM treatments. At 3 DAI, the Venn diagram showed that 46 ASVs were commonly enriched in the C3D and C0D treatments, while 29, 29, and 14 unique ASVs were specifically enriched in the C3D, C0D, and +AM treatments, respectively (Fig. 3B). At 6 DAI, the Venn diagram revealed that 38 ASVs were commonly enriched across C3D, C0D and +AM treatments, with 29 unique ASVs in the +AM treatment, and 17 and 15 unique ASVs in the C3D, C0D treatments (Fig. 3D), respectively. At 14 DAI, the Venn diagram further revealed that 27 ASVs were commonly enriched across C3D, C0D and +AM treatments, while each condition also had unique ASV enrichments, such as 29 unique ASVs in the C3D treatment, 37 unique ASVs in the C0D treatment and 18 unique ASVs in the +AM treatment (Fig. 3F).

The Chao1 and Observed species were calculated for the bacterial communities. For both indexes, the bacterial community harvested 14 DAI had a strong and statistically significant linear relationship (i.e., $R^2=0.559$, $P=0.005$ for Chao1 and $R^2=0.680$, $P<0.001$ for Observed species), whereas the bacterial community harvested 3 DAI and 6 DAI exhibited weak and non-significant relationships (Fig. 4A, B). This indicated that the effect of treatments on the bacterial community is most apparent at 14 DAI. At 14 DAI, the α diversity decreased from -AM to C3D, C0D and +AM treatments. It was found that the Observed species of Bacteroidetes were the least in the +AM treatment, and significantly lower than in the -AM treatment (Fig. S3).

To distinguish between deterministic and stochastic processes in the bacterial communities in the different treatments (-AM, +AM, C3D and C0D treatment) 3, 6 and 14 DAI of the bacterial community in the HC, the β-nearest taxon index (βNTI) was calculated. The βNTI values of the bacterial communities in the -AM treatment exhibited a wide range with substantial data dispersion, whereas the βNTI values of the bacterial communities in the treatments with hyphae (+AM treatment) were concentrated within a narrower range. The βNTI values in these treatments were significantly lower than those in the -AM treatment (Fig. 5A). Differences were also observed in the treatments with hyphae in the HC. Specifically, the contribution rates of the deterministic processes for C3D and C0D decreased from 44.4% (3 DAI) to 11.1% (14 DAI) and from 44.4% (6 DAI) to 0% (14 DAI), respectively, while the contribution



rate of the deterministic processes for +AM treatment increased from 0% (3 DAI) to 33% (14 DAI) (Fig. 5B).

Discussion

The functionality of AM fungi to exploit organic nutrients

Fig. 2 (**A**) Relative abundance (%) of the top 10 most abundant bacteria genera at the surfaces of hyphae connected to the carrot root (+AM treatment, Exp 1) or in absence of hyphae (-AM treatment, Exp 1) 3-, 6- and 14-days after inoculation (DAI) of the bacterial community in the hyphal compartment. The significance test was conducted with *t*-tests, comparing the differences between +AM and -AM treatments for each genus at each harvest time. Different asterisks indicate significant differences between the treatments (*t*-test, $P<0.05$ (*), $P<0.01$ (**)). (**B**) Relative abundance (%) of the top 10 most abundant bacterial genera at the surface of root-disconnected hyphae 3 days before bacterial inoculation (C3D treatment, Exp 2) or cut on the day of bacterial inoculation (C0D treatment, Exp 2), root-connected hyphae (+AM treatment, Exp 1) and in the absence of hyphae (-AM treatment, Exp 1), 3- 6- and 14-days after inoculation (DAI) of the bacterial community in the HC. Means followed by the same letter do not differ significantly by LSD ($P<0.05$). (**C**) Comparison of bacterial communities by principal coordinates analysis (PcoA) based on Bray–Curtis distances of harvest time and attachment type. (**D**) Comparison of bacterial communities by principal coordinates analysis (PCoA) based on Bray–Curtis distances of harvest time and hyphae connection type (C3D, C0D and +AM treatments, C3D and C0D treatment from Exp 2, +AM treatment from Exp 1)

is limited, and bacteria in the hyphosphere can enhance the functional activity of the extraradical hyphae of AM fungi (Duan et al. 2024). By breaking down complex organic matter, the bacteria make nutrients available to hyphae, thereby improving the nutrient uptake efficiency of the fungi (Zhang et al. 2014). Moreover, the bacteria's promoting effects enhance the germination of fungal spores and increase the colonization rates of AM fungi (Roesti et al. 2005; Bharadwaj et al. 2008; Bidondo et al. 2016). This relationship between AM fungi and hyphosphere bacteria is vital for the overall health and productivity of the soil ecosystem. The bacteria help in protecting hyphae from potential pathogens and environmental stresses, further contributing to the resilience and stability of the symbiotic network (Sangwan and Prasanna 2022; Jin et al. 2024). Bacteria's roles in nutrient cycling, pathogen defense, and plant growth promotion underscores the intricate and mutually beneficial interactions. Understanding these dynamics is crucial for developing sustainable agricultural practices and ecosystem management strategies.

Hyphosphere visualization is necessary to understand and quantify the community composition of bacteria, but under in situ conditions (e.g., field or greenhouse experiments), clear conclusions are uncertain because of: (1) the continuum between the hypha surface and hyphal-free environment (i.e., there are no sharp borders), (2) the small size of AM fungal hyphae, their obligatory nature and difficulty to distinguish from saprotrophic fungal hyphae, (3) the difficulty of visualizing hyphae without disturbing the system and (4) the impossibility of separating the impact of roots from those of the hyphae. In addition, the impact of AM fungi on hyphosphere bacterial communities and their dynamic changes usually are analyzed using a two-compartment microcosm system under soil conditions, combined with high-throughput sequencing (Zhou et al. 2020; Emmett et al. 2021), but this method has some drawbacks. Soil heterogeneity can lead to significant variations in bacterial communities, potentially masking low-resolution phenomena. Furthermore, while the DNA of all bacteria is sequenced, this does not guarantee that all sequenced bacteria are active, which can affect interpretation of the results. During cultivation, air settling and watering can introduce additional microorganisms into the system, which cannot guarantee the accurate study of bacterial communities in the original soil. To address the challenges mentioned above, a controlled in vitro bi-compartmented culture system has been developed. This system can produce a large number of extraradical hyphae without any unwanted contamination and allows for the visualization of hyphae without disturbing the system (Zhang et al. 2018; Duan et al. 2022). The partition wall in the culture plate creates an ideal space for observing interactions between AM fungi and bacteria by preventing the direct influence of root exudates. It also enables the separation of cultivable bacteria closely associated with hyphae from those on the surface of the medium and distinguishes bacteria associated with root-connected hyphae from those with root-separated hyphae. Finally, strict control of temperature and nutrient conditions ensures precise reproducibility of experimental results, allowing the establishment of a bacterial community under sterile conditions. These improvements make it possible to conduct in-depth, reliable studies of bacterial communities in the hyphosphere.

Here we demonstrated that the hyphosphere of root-connected hyphae exhibits a distinct microbial composition and variation compared to root-separated hyphae and from the medium. The bacterial community in the hyphosphere of root-connected hyphae is less diverse, shows deterministic community assembly, and exhibits different trends of community assembly compared to that on the surface of the medium. Separation of hyphae from the roots also markedly impacts the bacterial community because of the interruption of C flow from plant to hyphae and exudation of carbon compounds at the hyphal surface.

Microbial diversity decreased from the surface of the medium to the hyphae surface

At 14 DAI, the Chao1 and Observed species indices for the bacterial communities showed a strong and statistically significant linear relationship, whereas the bacterial communities at 3 DAI and 6 DAI exhibited weak and non-significant relationships. This indicates that the effect of treatments on the bacterial community is most apparent at 14 DAI. A decrease in the diversity of bacteria in the +AM treatment was observed compared to the diversity in the -AM

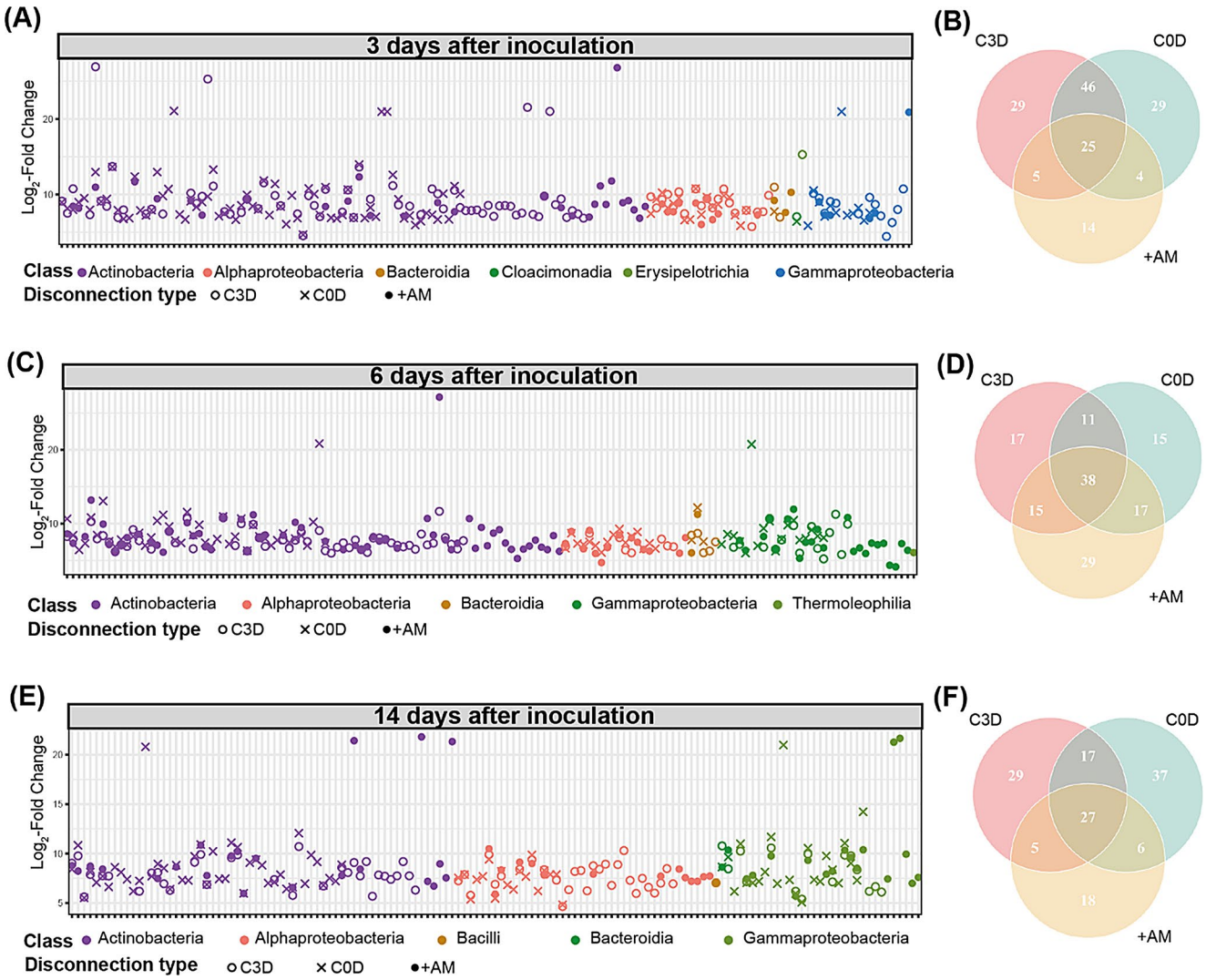


Fig. 3 Log_2-fold change of enriched ASVs on bacterial communities harvested (**A**) 3 DAI, (**C**) 6 DAI and (**E**) 14 DAI in the hyphal compartments of the C3D, C0D and +AM treatments compared to the -AM treatment. The color of the points represents the bacteria classes, while the circles represent C3D treatment, the cross shapes represent C0D treatment, and the solid dots represent+AM treatment. Numbers of enriched ASVs at (**B**) 3 DAI, (**D**) 6 DAI and (**F**) 14 DAI of the C3D, C0D and +AM treatments each compared to the -AM treatment

treatment and hyphae separated from root treatments. This was particularly pronounced 14 DAI with a large number of Bacteroidetes species tending to disappear from the +AM treatment compared with the -AM and hyphae separated from roots treatments. This suggests that hyphae, similar to roots, select bacterial species, resulting in a less diverse bacterial community in the hyphosphere than in the medium. Overall, AM fungal hyphae build their microbial environment. As with the rhizosphere, bacterial diversity decreases from the culture medium (or bulk soil) to the surface of the hyphae (or root) (Edwards et al. 2015). This selective process may be because hyphae and roots recruit and retain microorganisms with specific functions or co-evolutionary relationships, resulting in a lower bacterial diversity in their immediate microscale environment (Xun et al. 2019).

The hyphal exudates of AM fungi affect the hyphosphere bacterial community

In the present study, the bacterial community changed over time (from day 6 to 14) as the exudation of C compounds was quickly accompanied by their utilization by the bacteria. This led to a drastic shift in the most abundant bacterial genera in the hyphosphere, altering the proportions of the most abundant taxa, as earlier reported (Fan et al. 2022). To support this observation, we further compared the bacterial communities of hyphae connected and separated from the roots, because hyphae lose their viability and thus transport capacity of plant-derived C-compounds within ~24 h (Johnson et al. 2002). Surprisingly, we found that the cutting of hyphae did not significantly affect the bacterial community

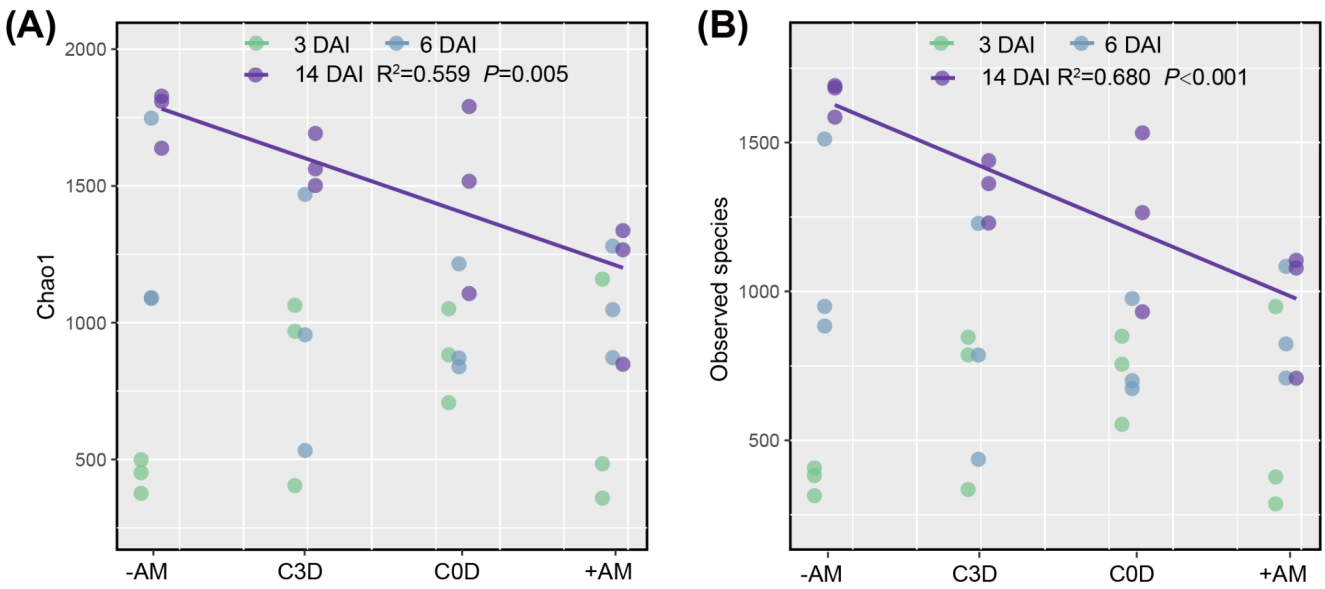


Fig. 4 (**A**) Chao1 and (**B**) Observed species of bacteria in absence of hyphae (-AM treatment, Exp 1), in root-connected hyphae (+AM treatment, Exp 1) and in root-disconnected hyphae 3 days before bacterial inoculation (C3D treatment, Exp 2) or cut on the day of bacterial inoculation (C0D treatment, Exp 2), 3- 6- and 14-days after inoculation (DAI) of the bacterial community in the hyphal compartment

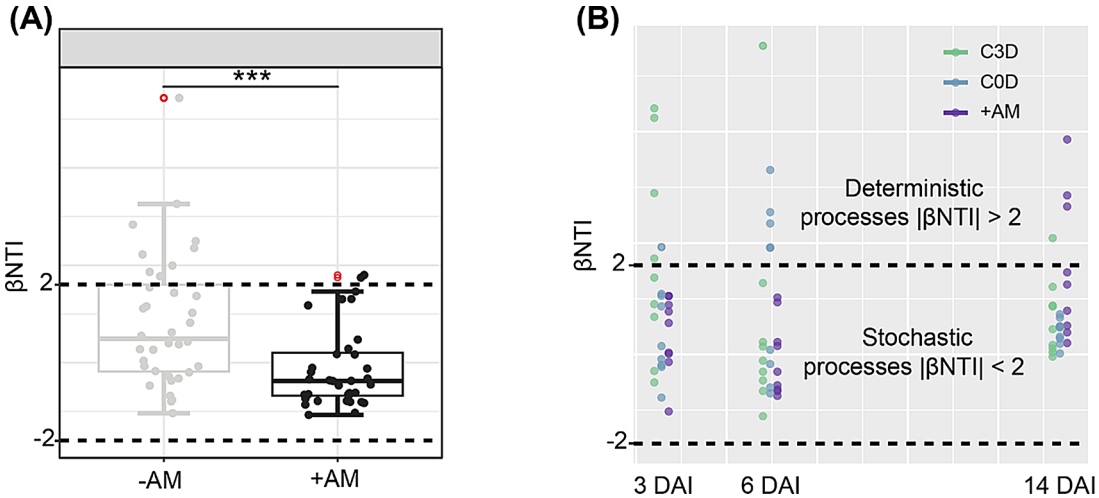


Fig. 5 Relative influence of deterministic and stochastic assembly processes in bacterial community. (**A**) The βNTI of bacteria community in absence of hyphae (-AM treatment, Exp 1) and in root-connected hyphae (+AM treatment, Exp 1) (*** $P<0.001$, *t*-test). The box-and-whisker plots visualize the distribution of βNTI values across different groups. The horizontal line within the box represents the median, the lower line of the box represents the first quartile (Q1, 25th percentile), and the upper line of the box represents the third quartile (Q3, 75th percentile). The whiskers indicate the minimum and maximum values within 1.5 times the interquartile range (IQR), respectively. Points outside the whiskers are considered outliers and are shown as individual red dots. Outliers are not excluded from the analyses. The jitter points are added to display the individual data points for each group. (**B**) Distributions of βNTI in root-connected hyphae (+AM treatment, Exp 1) and in root-disconnected hyphae 3 days before bacterial inoculation (C3D treatment, Exp 2) or cut on the day of bacterial inoculation (C0D treatment, Exp 2) at 3-, 6- and 14-days after inoculation (DAI) of the bacterial community in the hyphal compartment

composition at day 6 but did at day 14. It is not excluded that ruptured hyphae massively release their C contents as they lose their membrane integrity, thus feeding the bacteria at their surface at day 6, a situation that is no longer likely at day 14, because most C sources presumably have been exhausted by then. Based on these results, we suggest that the amounts of hyphal exudates play a significant role in the structure of the bacterial community, contributing to the mechanisms underlying its composition (Emmett et al. 2021; Wang et al. 2023). It is important to note, however, that in our study the hyphae already had been growing for four weeks in the hyphal compartment, and may have secreted a substantial amount of C prior to separation from roots. This probably also influenced the bacterial composition on the

surface of the hyphae at days 6 and 14, whether connected or not. It would therefore be useful to take this aspect into account in future studies in order to obtain more pronounced effects. Indeed, quantifying hyphal exudates is important for understanding the dynamics of hyphosphere bacterial communities and their composition (Zhang et al. 2022; Wang et al. 2022).

It is important not to overlook the soluble cellular contents released from fungal hyphae after they break, which can serve as a rich nutrient source for bacteria in the surrounding environment, potentially promoting the growth and proliferation of certain bacterial species. Furthermore, considering that the fungal cell wall is primarily composed of chitin, we observed an increase in the relative abundance of *Chitinophages* (Fig. 1). Chitin, as a natural polysaccharide, is an important carbon and energy source for many bacteria, and thus, the increase in *Chitinophages* may reflect the bacteria's ability to utilize this resource (Lu et al. 2023).

In addition, because of the lack of a control condition with neither roots, live hyphae, nor hyphal necromass present, we are unable to accurately estimate the independent impact of the MSR medium on the bacterial community. Specifically, we acknowledge that our bacterial inoculum might have been biased by the extraction procedure from the original soil or by exposure to the culture medium. These biases could result in certain bacterial species being over- or under-represented in the experiment, thereby affecting our results and conclusions. To better understand the impact of the medium on the bacterial community, future studies should consider incorporating additional control experiments, including the use of MSR medium alone in the absence of plant roots and fungal cell components, to rule out the influence of other environmental factors. Moreover, refined extraction methods should be employed in subsequent experiments to minimize interference with the original soil bacterial community, and multiple media should be used for parallel cultivation to comprehensively assess bacterial responses under different conditions.

Impact of hyphal connectivity on bacterial community assembly processes

Differences in βNTI values were observed between the medium and the hyphosphere, suggesting that the assembly processes of bacterial communities on the medium and in the hyphosphere are distinct. The contribution rates of the deterministic processes increased in the +AM treatment compare to C3D and C0D treatments at 14 DAI (Fig. 5B), reflecting a pronounced shift towards deterministic community assembly in the presence of hyphal exudates. This divergence could be attributed to the varying C availability and other environmental conditions specific to each compartment, highlighting the complexity of bacterial community assembly in these two niches (Zhalnina et al. 2018). These findings consolidate the hypothesis that the continuous presence of hyphal exudates fosters a stable, deterministic microbial community structure, which transitions towards stochasticity (βNTI<-2 or βNTI>2) as exudates are exhausted.

Because of their extensive development in the medium of the HC, hyphae can quickly absorb nutrients from the medium, including trace amounts of P that may come from Phytagel, water, or other components of the medium. This absorption alters the nutrient composition of the medium, creating a different nutritional environment for bacteria. Our study might have been affected by changes in bacterial communities caused by the absorption of nutrients by hyphae. The absorption activities of hyphae are not limited to nutrients but also include water and other trace elements, further influencing their role in the ecosystem. Under our experimental conditions, the activities of hyphae can have a profound impact on the chemical and physical properties of the medium. For example, absorption by hyphae also affects bacterial growth and metabolism by altering pH and redox potential (Li et al. 2022; Falcão et al. 2023). These changes can lead to adjustments in the composition of bacterial communities, promoting the growth of some bacteria while inhibiting others.

Therefore, when analyzing experimental results, we cannot overlook the influence of hyphal absorption on bacterial communities. These influences are not limited to direct competition for nutrients but also may indirectly affect the dynamic balance of the entire microbial community through interactions and signal transmission between microorganisms (Zhang et al. 2018; Rozmoš et al. 2022). Hyphae's absorption and secretion activities are inseparable aspects that together determine the diversity and stability of the medium environment. Both the secretion and absorption activities of hyphae thus are integral to their ability to shape the environment.

Conclusion

The bi-compartmented in vitro culture system is well suited for studying the diversity of bacterial communities associated with hyphae connected or separated from the host plant, as it avoids the direct influence of root exudates on bacteria. Moreover, that the bacteria were purified directly from agricultural soil without further cultivation, suggests that this is close to their natural activities and therefore to their tendency to interact with the hyphae of AM fungi. The physical disruption of fungal hyphae directly affects the α-diversity of bacterial communities, which may further

affect their ecological functions. The experimental results show that the connection between hyphae and roots significantly influences specific bacterial groups in the hyphosphere, particularly in regulating the relative abundance of certain species and community diversity. The connection between hyphae and roots affects the deterministic and stochastic processes in bacterial community assembly. In treatments where hyphae are connected to roots (+AM treatment), bacterial community assembly processes show high determinism. In contrast, in treatments where hyphae are severed (C3D and C0D treatment), bacterial community assembly processes tend towards stochasticity. This indicates that the physical connection between hyphae and roots has a significant impact on the structure and dynamics of bacterial communities, especially in regulating microbial community assembly.

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Author contributions Z.X.J. and L.Z. planned and designed the research. Z.X.J. performed experiments. Z.X.J., S.L.D. and L.Z. analyzed data. Z.X.J., S.L.D., S.D. and L.Z. wrote the manuscript.

Data availability The authors confirm that the data supporting the findings of this study are available within the online publication document. Sequences data that support the findings of this study (amplicon sequencing) have been deposited in the NCBI SRA database with SRA accession codes SRR30359768–SRR30359800 and BioProject code PRJNA1151855.

Declarations

Ethical approval There are no ethical issues involved in this work.

Competing interests The authors declare no competing interests.

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