

Effects of Rock Phosphate Application on the Composition of Bacterial Communities Associated with Arbuscular Mycorrhizal Fungal Mycelia

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Abstract

Background

Inter-kingdom interactions between arbuscular mycorrhizal fungi (AMF) and bacteria are increasingly recognized for their potential to enhance fertilizer use efficiency in agroecosystems. Here, we investigated the effects of rock phosphate amendment and AMF inoculation on phosphorus (P) nutrition in leek (*Allium porrum* L.), as well as on bacterial communities associated with AMF extraradical mycelium. A bi-compartmental microcosm was used to disentangle root-derived effects from those mediated by AMF mycelium.

Results

Inoculation with *Rhizophagus irregularis* significantly increased total plant biomass ($p < 0.001$), while rock phosphate amendment enhanced arbuscule abundance in roots ($p = 0.03$), leading to higher shoot P content ($p = 0.013$) and photosynthetic activity ($p < 0.0001$). Because rock phosphate was the sole P source, these results indicate that P solubilized in the soil was translocated to the host plant via the mycorrhizal pathway. Rock phosphate amendment also significantly altered the composition of bacterial communities associated with AMF mycelium ($p = 0.01$). Across treatments, bacterial assemblages were dominated by Planctomycetota, Pseudomonadota, Chloroflexota, and Bacillota, with enrichment of *Planctomyces* and *Gemmata* in AMF mycelium, and *Planctomyces*, *Gemmata*, and *Bacillus* in soil. The core bacteriome associated with *R. irregularis* was primarily composed of Planctomycetota and Bacillota, taxa known to form biofilms on AMF extraradical hyphae.

Conclusion

These findings demonstrate the pivotal role of mycorrhizal symbiosis in enhancing P acquisition from rock phosphate and provide new insights into AMF–bacteria interactions that are relevant for developing sustainable fertilization strategies.

Introduction

Agroecosystem productivity relies on the continuous input of phosphate-based processed chemical fertilizers to maintain soil fertility; this is because the depletion of bioavailable phosphorus (P) occurs as the rate of uptake by crop roots often exceeds nutrient dissolution from bulk soils [1]. Advances in plant breeding have led to the selection of crops highly dependent on these chemical fertilizers, particularly commodity crops such as corn, wheat, rice, and potato, without considering the contributions of crop microbiota, which potentially represent an alternative genetic reservoir that has barely been tapped [2]. The root-associated microbiome is a key determinant of soil nutrient availability, plant biomass productivity, and pollutant degradation [3]. Soil microbes have long been known to contribute to plant

health by facilitating nutrient acquisition, inhibiting pathogens, and protecting plant roots against abiotic stresses [2].

Beneficial soil microbes provide important ecological services to natural and agricultural ecosystems [4], and, given their potential to positively influence agricultural productivity, harnessing these microbes is a major goal for environmental and agricultural biotechnologies [5].

Arbuscular mycorrhizal fungi are both root- and soil-inhabiting symbionts and are among the most common soil fungi. They form symbioses with the roots of 71% of all vascular plant species [6], providing invaluable services for the plants, in particular improving the capacity for P uptake in return for carbon from the host plants [7]. Additionally, mycorrhizae can provide plants with increased resistance to root pathogens [8–10], alleviate abiotic stress [11], and improve soil quality by aggregating soil particles and decreasing soil erosion [12, 13]. It is becoming recognized that some of the presumed benefits of mycorrhizal symbiosis, such as P uptake and nutrient cycling, are in fact dependent on the interaction between AMF and their associated microbes [14–18]. AMF hyphae also serve as physical pathways for microbial mobility and dispersal [19], and studies have shown that AMF interact closely with myriad bacteria and fungi that can be exploited to enhance mineral immobilization for plants [20–23].

In AMF–soil bacteria interactions, soil microbes can interact with AMF synergistically, adversely, or commensally. An early study [24] reported evidence of the presence of bacteria on the AMF spore wall, and a later research study showed that bacteria can invade the spore wall layers and survive in the spore cytoplasm [25]. Several subsequent studies have reported that diverse bacterial communities are associated with AMF spores [21, 26–28]. Bacteria belonging to the genera *Burkholderia*, *Pseudomonas*, *Variovorax*, and *Chromobacterium* were more frequently associated with the AMF *Rhizophagus irregularis* [23, 29], while the authors of Scheublin, *et al.* [30] reported that the Oxalobacteraceae family was more specifically associated with the AMF hyphal surface than other bacteria. Moreover, Selvakumar, *et al.* [31] isolated 120 strains of bacteria associated with AMF spores belonging to three species (*Funneliformis caledonium*, *Funneliformis mosseae*, and *Racocetra alborosea*) using a culture-dependent approach, and the authors characterized and tested these bacterial strains for the following spore functional traits: chitinase, protease, cellulase enzymes, and exopolysaccharide production. These authors [31] also found that among the 120 isolated bacterial strains, 113 showed at least one functional trait, while 7 strains showed none.

The cell wall of AMF is composed of chitin, proteins, and polysaccharides [32]. Biopolymer-degrading microbes adhering to the spore and hyphal outer layer might use oligomers of chitin as a carbon source [33], while AMF-associated microbes may also use other cell wall components as a nutrient source. So far, spore-associated bacteria have been isolated from the spore cytoplasm [34], surface sterilized spores and hyphae, and were reported to either stimulate fungal growth or increase plant nutrient uptake [23, 30]. Moreover, the close interaction between AMF and hyphosphere bacteria has been shown to increase plant growth by enhancing phosphate solubility [35, 36] and fixing atmospheric nitrogen [37] and can contribute to AMF spore germination and plant root colonization [34].

It is well documented that AMF identity influences the microbial communities associated with plant roots and the rhizosphere [21]. A recent study by Lahrach, *et al.* [38] demonstrated that the organismal phylogeny of AMF shapes the bacterial communities associated with their mycelia. However, the factors driving shifts in the microbial community associated with AMF mycelia remain unclear. Finely ground soft rock phosphate fertilizers can be used for direct application under specific conditions, such as permanently moist and acid soils [39]. However, in most cases, rock Phosphate (RP) does not show agricultural effectiveness, due to its low rate of dissolution. However, some biological solutions, such as the culture of green manure crops [40] or the use of rhizobacteria can increase the solubilization of RP [41].

In this study, we investigated the impact of RP amendment on AMF–bacteria interactions. We hypothesized that 1) RP amendment influences the bacterial community associated with AMF mycelium, and 2) RP amendment and AMF inoculation enhance the P nutrition of leek plants.

To test these hypotheses, we designed a bi-compartmentalized microcosm to separate root effects from AMF mycelium effects, using leek as the host plant, in a greenhouse trial. We employed metabarcoding targeting the 16S rRNA gene to determine bacterial community changes in the mycelia, bulk soil, and hyphosphere associated with AMF mycelia. This setup allowed us to precisely analyze how RP amendment and AMF inoculation influence bacterial community structure and contribute to the P nutrition of leek plants.

Materials and methods

Microcosm setup and substrate preparation

The investigation utilized microcosm units to partition the soil and ensure the physical separation of AMF mycelia in the root-free compartment (RFC) from the plant roots in the root compartment (RC). Each unit consisted of two pots, measuring 6.5 x 6.5 x 8.5 cm (LxWxH), which were bonded together using silicon glue. One pot served as the plant growth compartment (RC), and the other facilitated hyphal propagation (RFC). The pots had a cut side with a 44 µm membrane (manufactured by SEFAR Incorporation, USA) that enabled interconnection without root passage. Before initiating the experiment, the microcosms were sanitized with 70% ethanol and exposed to UV light under laminar flow for 30 minutes.

A sandy loam soil of low P content was collected from the organic farm of the IRDA research station in St-Bruno in Québec (45°32'59.6" N, 73°21'08.0" W). This soil was characterized by a pH of 6.01 and a Mehlich-3 extractable phosphorus concentration of 0.41 mg/kg of soil [42]. The soil analysis was performed using a commercial service provided by EnvironeX (Longueuil, QC). The chemical properties were described by Renaut, *et al.* [42]. The soil was air-dried, sieved at 2 mm, and then mixed with sand (Bomix company, Laval, QC) at a ratio of 1:1 (v/v) to produce a growth substrate with depleted phosphorus level. To eradicate the indigenous microorganisms, both the substrate and RP were

sterilized using γ -irradiation with a minimum dose of 22.3 kGy and a maximum of 46.2 kGy (Nordion's Gamma Centre of Excellence, Laval, QC). The sterilization was confirmed via the serial dilution method and inoculation of the tryptic soy agar (TSA) plates. Each compartment of the microcosm received around 333 g of the substrate and RP was added where required. An amount of 0.16 g of RP per pot was homogenized with the RFC substrate to provide the equivalent of approximately 100 kg P_2O_5 per hectare. The RP used in this study, as the sole source of P, was the sedimentary RP with a bone phosphate of lime $\geq 54\%$, kindly supplied by OCP Group, Morocco.

Host plant and microbial inoculum

Seeds from leek (*Allium porrum*) (McKenzie Seeds, Canada), a highly mycotrophic plant, were used as a host. Under sterile conditions in a laminar flow, the seeds were surface sterilized with ethanol (70%) for 30 s and then bleached (15%) for 5 min, and were then rinsed abundantly with sterile distilled water. The seeds were kept soaked in the last rinsing water for 2h. Three surface-sterilized seeds were sown into each RC at a depth of 5 mm. The spores of the AMF *Rhizophagus irregularis* DAOM 197198, kindly provided by Premier Tech, Canada, were used to inoculate the seeds at the same time of sowing of each mycorrhizal treatment. To enhance the chance of root colonization by the AMF, a second application was performed one month after the first, directly to the roots after thinning the plants to one per microcosm. The AMF species was chosen based on its *in vitro* production process, its application as a commercial inoculant in agriculture, and its large distribution [43]. In each AMF inoculation, the seeds/seedlings received 200 spores diluted in 300 μ l of sterile distilled water.

To enrich the soil microbial community, a microbial suspension was prepared from natural soil collected from the rhizosphere of organically cultivated crops within the Botanical Garden of Montreal. Soil subsamples were taken separately from potato, leek, tomato, eggplant, and maize during the flowering season and transferred to the lab. The rationale behind using many crop soils was to diversify microbial communities. A composite sample was prepared by pooling equal amounts from each of the five rhizosphere soil samples and homogenizing the mixture. Six kilograms of the composite soil were sieved through a 2 mm mesh to remove roots and rock fragments, and then divided into 3 portions. Firstly, we mixed 1/3 of the soil (2 kg) with 4 L of sterilized distilled water, and the soil was brought into suspension via stirring for 30 min and then kept for at least 2 h at room temperature. The liquid phase of the soil suspension was filtered through autoclaved sieves with mesh widths of 53 μ m and 20 μ m. This filtration excludes AMF spores in the soil suspension. The same operation was used for the second and the third soil portions using the soil suspension obtained from the first and the second filtration, respectively. The final filtrate was collected in a sterile Erlenmeyer flask and considered as the microbial suspension.

The inoculation with the microbial suspension of the RFC substrate was performed two months after the second AMF inoculation using 50 mL of the microbial suspension.

Experimental design

This study represents a subset of an experiment on the recruitment profile of microbes by AMF carried out in a greenhouse using the microcosm units described earlier. The study examined the effect of sedimentary RP on the community structure of bacteria associated with the mycelium of the AMF *R. irregularis* DAOM 197198. Two factors were considered: (1) two levels of RP, with or without RP application, and (2) two AMF levels, inoculated or uninoculated. Consequently, the experiment involved four treatment combinations: M0P0, M0P1, M1P0, and M1P1, where M refers to AMF, P to RP, 0 to absence and 1 indicates presence. The trial was organized using a randomized block design, comprising nine replicates on a mesh table to prevent the risk of cross-contamination through water. Three replicates were established each week over a period of three weeks. Leek plants were grown under conditions of photoperiod (16/8 h day/night) and temperature (21/18°C day/night). An additional set of microcosm units was prepared and inoculated with AMF to monitor plant colonization and the presence of mycelia in the RFCs. A layer of calcinated clay of granules (Turface®), approximately 1 cm thick, was added on top of the microcosm pots to cover the soil surface, and it was renewed weekly to minimize or prevent surface contamination by microalgae. The microcosms were watered to maintain soil moisture at holding capacity. Once a week, the plant compartments received 50 mL of modified half-strength Long Ashton nutrient solution containing 22 ppm (0.71 mM) of P. This concentration of P is known to enhance AMF-induced root colonization [44]. Two months after the second inoculation with the AMF spores (a period allowing the establishment of extraradical AMF mycelia in the RFCs), all RFCs were treated with 50 mL of microbial suspension. Afterwards, the irrigation regime was switched and the diluted nutrient solution without P was supplied once a week to the RFCs. The microcosms were kept watered with tap water as needed throughout the week.

Harvest and plant analysis

Growth parameters and chlorophyll concentration

The harvest was performed five months after setting up the experiment over three weeks to ensure consistent timing across all replicates. Before cutting the shoots, the stem diameter of plants was measured with an electronic caliper at 5 mm above the soil. To verify the effect of the treatments on the plant's photosynthetic activity, an atLEAF chlorophyll meter (FT Green LLC, USA) was used to evaluate the concentration of chlorophyll ($\mu\text{g}/\text{cm}^2$) on the fully expanded third or fourth leaf from the top according to the manufacturer's instructions. Afterward, each microcosm was disassembled, and the RC was carefully discharged. The roots were rinsed with sterile distilled water, and the shoots were cut from the roots, put in a paper bag, and dried in a hot-air oven at 80°C for 72 h. The dry biomass was recorded, and the shoots were ground with a clean dried pestle and mortar for further chemical analysis. The fresh roots were weighed, and to evaluate the mycorrhizal colonization, a small amount of fine roots was placed in a separate 15 mL falcon tube containing 8 mL of 50% ethanol. The remaining roots were weighed a second time and then dried in the oven under the same conditions as the shoots. The second fresh root weight helped to calculate the total amount of dry root weight.

Mycorrhizal colonization and phosphorus content

The mycorrhizal colonization ratio was evaluated using the ink and vinegar staining method [45]. The roots preserved in 50% ethanol were washed with tap water and cut into 1 cm long segments. The root segments were cleared in a 10% KOH solution boiling at 90°C for 3 to 4 min until they became transparent and were then rinsed with distilled water. To remove the KOH residues, the roots were soaked in another bath of 1% acetic acid for 4 min. Cleared roots were placed in a 5% ink–vinegar solution at 90°C for 3 min and rinsed with distilled water and then stored in a lactoglycerol solution for a minimum of 24 h at room temperature to remove the excess of ink solution. The stained root segments were mounted on microscope slides with fine-point forceps, and the degree of mycorrhization was determined via the intersection method [44]. The numbers of arbuscules, vesicles, and intraradical mycelia were counted and the percentages were calculated.

The ground shoots were used to determine the total phosphorus content via the modified dry ash method [46]. Ground shoot aliquots of 0.5 g were incinerated at 500°C for 4 h and digested in 100 mL borosilicate Erlenmeyer containing 6 mL and 1 mL of concentrated sulfuric acid and nitric acid, respectively. The resulting solutions were filtered in an acid-washed flask, filled up to 100 mL with demineralized water, and then homogenized before P concentration analysis via the molybdenum blue colorimetric technique [47]. The shoot P content (μg) was subsequently calculated from the P concentration and shoot biomass.

Extraradical mycelia collection

Prior to harvesting the substrate from the hyphal compartment, Surface layer with the top 1 cm of the substrate was removed along with a 1 cm width strip from the membrane side of the substrate to reduce bacterial communities affected respectively by potential surface contamination and by root exudates near the membrane. Afterwards, about 10 mL of the substrate was collected in a 15 mL falcon tube. To collect the extraradical mycelia, the rest of the substrate was transferred to a 1 L sterile glass jar and covered with cold sterile distilled water to a height of almost 1 cm. The jar was lidded, vigorously shaken, and left to stand for approximately 15 min. During this time, the extraradical mycelia of AMF began to agglomerate due to their adhesive properties. Next, the solution was poured through a sterile 40 μm mesh sieve, and this procedure was repeated four to five times until there were no mycelial aggregates observed. To clean them as much as possible from the attached particles, the extraradical mycelia were rinsed with cold sterile saline solution of 0.9% NaCl, after which they were transferred to a microcentrifuge tube using sterile forceps. Mycelia samples were squeezed to remove any remaining liquids, and the weights were recorded. Regarding the non-mycorrhizal RFCs, no extraradical mycelia were found. All tubes containing substrate and mycelia samples were immediately flash frozen in liquid nitrogen after sampling and kept on dry ice in a cooler box before being stored in the freezer at -80°C for the downstream analysis of nucleic acids.

DNA extraction and PCR amplification

Just before DNA extraction from the substrate, all the samples were transferred into 50 mL falcon tubes with pierced lids and placed in a lyophilizer for 3 days until dried. Then, the samples were sieved with a 1 mm mesh sieve to facilitate substrate particle separation. Hereinafter, the materials resulting from the M1 and M0 treatments were named, respectively, hyphosphere soil and bulk soil.

The bulk and hyphosphere soil DNA was extracted from 250 mg of homogenized soil of each sample using the DNeasy PowerSoil Pro Kit (QIAGEN, Canada) according to the manufacturer's protocol. We performed the DNA extraction from mycelia using the DNeasy Plant Mini Kit (QIAGEN, Canada) following the manufacturer's instructions, starting by grinding about 50 mg of liquid nitrogen-frozen samples in 1.5 mL tubes containing sterile white sand, which facilitates cell lysis. The elution step was performed in 50 μ L of elution buffer for bulk and hyphosphere soils and in 20 μ L for mycelia and stored at -20°C. Therefore, genomic DNA extracts were first visualized via gel electrophoresis on 1% agarose stained with GelRed diluted at a ratio of 1/10000 using the GelDoc System (BioRad, Montreal, QC, Canada). The DNA concentration was determined using the Qubit 2.0 Fluorometer (ThermoFisher, Canada) and the Qubit double-stranded DNA (dsDNA) HS Assay Kit.

To investigate the total bacterial community, the V3-V4 region of 16S rRNA gene was targeted using Platinum™ Direct PCR Universal Master Mix (ThermoFisher, Canada). The amplicon libraries were constructed using the primers pair of 341F (5'-CACCTACGGGNGGCWGCAG-3') and 805R (5'-ACTACHVGGGTATCTAATCC-3') tagged, respectively, with CS1 (5'-ACACTGACGACATGGTTCTA-3') and CS2 (5'-TACGGTAGCAGAGACTTGGTCTG-3'), which permit barcoding [48]. The PCR amplification was performed in a 25 μ L volume reaction mix containing 10 μ L of Platinum Direct PCR Universal Master Mix, 0.25 μ M of each primer, 4 μ L of Platinum GC Enhancer, 7 μ L of water, and 1 μ L of template DNA. Negative controls with only water were included in each PCR run. The thermal cycling conditions were as follows: initial step of activation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 68°C for 20 s, and a final extension at 68°C for 1 min with a hold at 10°C. PCR reactions were amplified in an Eppendorf Mastercycler Pro thermocycler (Eppendorf, Canada). The amplification was confirmed by running the PCR products on a 1% agarose gel. Amplicons were sent for Illumina MiSeq sequencing using 2 × 300 bp paired-end reads which were demultiplexed on the instrument in the NGS platform at the Genome Quebec Innovation Centre (Montreal, QC, Canada).

Bioinformatics and statistical analyses

A bioinformatic analysis was performed using QIIME2 version 2021.4.0 [49]. The pipeline used to process the 16S rRNA gene sequences was DADA2 v1.18.0 [50]. Initially, Cutadapt 3.4 was employed to remove both primer sequences from the 16S rRNA gene amplicons with parameter values “minimum-length = 50”, “times = 2”, “overlap = 6”, and “p-error-rate = 0.1”. Next, we excluded the forward and reverse sequences with less than 220 bp with the command “--p-trunc-len”, as the base quality of the sequences tended to diminish below that threshold in our data. Afterwards, the amplicon sequence variant (ASV)

table was calculated, and chimeras were removed. The taxonomy assignment of the ASVs was performed using the naive Bayesian classifier method on the databases SILVA and RDP, and the identities of the ASVs of interest were verified manually using BLASTn on the NCBI (nr/nt) database.

AMF and plant-related parameters were analyzed employing JMP Pro 17 software (SAS Institute Inc., Cary, NC, USA). Shapiro–Wilk test was used to verify the normal distribution of the data, and Levene's test to determine the homoscedasticity. The data of root dry-weight biomass and root colonization were transformed using Box–Cox method before performing statistical analyses. An analysis of variance (ANOVA) was used to assess the effects of AMF inoculation, RP application, and their interactions, followed by Tukey's HSD test to compare the mean values, which differed at $p < 0.05$. AMF inoculation and RP addition were considered as fixed effects, and the random effect was attributed to the block.

Microbial diversity analysis was performed using R (Version 4.3.2); the rare-curve function from the vegan package v2.6-4 [51] was employed for normalizing the dataset through a random subsampling process that aligned each sample's read data to the minimal read count observed in the dataset. Shannon and Simpson diversity indices were computed to quantify alpha diversity utilizing the vegan package. The influence of RP application on alpha diversity was statistically examined via an ANOVA, followed by post hoc analyses using the agricolae package v1.3-7 [52]. The beta diversity of bacterial ASVs across samples was determined using Bray–Curtis dissimilarity and visualized with principal coordinate analysis (PCoA) plots using the vegan package. The vegan package's ADONIS function was used for the permutational multivariate analysis of variance to evaluate the effects of AMF and RP application on betadiversity, applying a Hellinger-transformation to the data and executing 999 permutations. An indicator species analysis was conducted via the indicpecies package v1.7-14 [53] to identify the taxa associated with the application of RP. In defining the core microbiome, the designation “core bacteriota” was attributed to the bacterial taxa consistently detected in the mycelial or hyphosphere soil samples associated with the AMF *R. irregularis*. The core bacteriome was identified using the microbiome package v1.24-0 [54] with 95% prevalence. The co-occurrence network was built using the R packages [55] and igraph [56] only using ASVs with a total abundance superior to 0.05%, thus retaining only 1567 ASVs. Metabolic pathways associated with the taxonomy of bacteria were calculated using the Picrust2 of Galaxy tool [57]. The taxa associated with treatments were identified using the microbial package in R [58] and its “ldamarker” command, which implements the Linear discriminant analysis effect size (LEFSe) method.

Results

Mycorrhizal colonization and mycelial production

The mycorrhizal colonization was successfully established in the plants inoculated with *R. irregularis*, whereas no colonization was observed in non-mycorrhizal roots. Means were 94.82% and 89.32% under the presence and absence of RP respectively, which explains the quantity of mycelial biomass extracted from the hyphal compartment (Table 1). While no hyphae were found in the absence of *R. irregularis*

inoculation, they multiplied extensively in the hyphal compartment when the plants were colonized (Table 1). RP did not significantly influence the mycelial biomass, the percentages of mycorrhizal colonization and vesicle formation. Surprisingly, the percentage of arbuscules was significantly affected by the RP application ($p = 0.03$).

Table 1

Mycorrhizal colonization and fresh biomass of the hyphae in the RFC. The abbreviations M1P0 and M1P1 indicate samples inoculated with *R. irregularis* without RP and those inoculated with *R. irregularis* and supplemented with RP, respectively.

Treatments	Mycelial biomass (mg/pot)	Mycorrhizal colonization (%)	Arbuscules (%)	Vesicles (%)
M1P0	117.95 ± 27.96 a	89.32 ± 3.66 a	62.86 ± 10.69 a	69.49 ± 6.90 a
M1P1	109.60 ± 19.95 a	94.82 ± 2.37 a	90.12 ± 2.23 b	74.07 ± 4.64 a

Data are presented as the mean value (n = 9) and standard error. The significance of treatments was determined by one-way ANOVA. Lowercase letters indicate significance at the $p < 0.05$ level.

Plant productivity

Plant biomass and stem diameter

Agronomic parameters of *Allium porrum* were affected by the treatments (Fig. 1). The colonization with *R. irregularis* significantly enhanced both root ($p < 0.001$) and shoot dry weight biomass ($p < 0.001$) (Table S1, Fig. 1A, B). However, RP application did not influence shoot ($p = 0.373$) and root ($p = 0.493$) dry-weight biomass (Table S1, Fig. 1A, B). A similar trend was exhibited for stem diameter (Fig. 1C). While the application of AMF spores significantly increased the stem diameter ($p < 0.001$), RP application was statistically insignificant ($p = 0.702$) (Table S1). However, the combined treatment (M1P1) tended to increase the stem diameter ($p = 0.07$) (Table S1). M1P1 treatment improved plant biomass and stem diameter by 125.14% and 51.47%, respectively, compared to the control (Table S1).

Shoot P concentration and photosynthetic capacity

Although RP application tended to increase shoot P concentration ($p = 0.072$), mycorrhization significantly increased the total P acquired by plants ($p = 0.014$) compared to the control (Fig. 2A; Table S2). Moreover, mycorrhizal plants supplied with RP (M1P1) exhibited 135% increase in shoot P content (P concentration × shoot biomass) relative to the control (Fig. 2A; Table S2). Regarding photosynthetic capacity, both RP application ($p = 0.017$) and AMF inoculation ($p < 0.001$) significantly increased chlorophyll concentration compared to the control (Fig. 2B; Table S2). The combined treatment (M1P1) resulted in the highest chlorophyll concentration, representing a 60% increase relative to the control (Fig. 2B; Table S2).

Taxonomic profiles of the bacteria

Raw Illumina MiSeq 16S rRNA gene sequencing generated a total of 2,513,803 bacterial reads. We retrieved 1,004,950 non-chimeric reads from 54 samples, ranging from 1,948 to 91128 reads per sample (two samples were excluded due to insufficient sequencing quality). An extra cleaning of the dataset was performed to remove unidentified ASVs at the phylum level as well as those with fewer than 10 reads in all samples. The final dataset comprised a total of 7514 ASVs, which were subsequently categorized into 31 phyla, 85 classes, 130 orders, 149 families, and 194 genera. The rarefaction curves indicated that all samples had reached saturation (Fig. S1). In each of the habitats, Planctomycetota was the most abundant phylum, followed by Pseudomonadota, with 57% and 10% in the mycelia, 39% and 12% in the hyphosphere soil, and 44% and 11% in the bulk soil, respectively (Fig. 3), while *Parcubacteria candidate phylum* OD1 came in third place with 7% in the mycelia. Bacillota, on the other hand, was in the hyphosphere and bulk soils, with 11% in each of these habitats. Among the mycelia, the prevalent genera were *Planctomyces* (10%) followed by *Gemmata* (9%) and *Pirellula* (4%) (Fig. 3). In contrast, the predominant genera in the hyphosphere soil were *Planctomyces* (9%), *Bacillus* (7%), and *Gemmata* (5%). Similarly, in bulk soil, the dominant genera were *Planctomyces* (11%), *Bacillus* (6%), and *Gemmata* (5%). An important proportion of bacterial genera remained unidentified, with 62% in the mycelia, 55% in the hyphosphere soil, and 55% in the bulk soil.

Furthermore, the Venn diagram analysis (Fig. 4) of bacterial ASVs shared among the three habitats revealed 672 ASVs, accounting for 15%, 22%, and 24% of the bacterial communities in mycelia, the hyphosphere, and bulk soil, respectively. Additionally, 281 ASVs (6% of the mycelial community and 9% of the hyphosphere community) were found exclusively in these two habitats. Similarly, 232 ASVs were common between mycelia and bulk soil, representing 5% and 8% of their respective bacterial communities.

In contrast, the highest number of shared ASVs (1,216) was observed between the hyphosphere and bulk soil, comprising 40% and 44% of their respective bacterial communities. Notably, 3,404 ASVs were unique to mycelia (47%), 845 were exclusive to the hyphosphere (12%), and 616 were found only in bulk soil (9%).

Effect of RP amendment on the bacterial community assemblage in mycelia, hyphosphere, and bulk soil

Neither RP nor sample type affected the α -diversity as measured with Shannon and Simpson indices (Fig. 5 and Table 2). However, a PCoA showed a clear separation of bacterial communities associated with the AMF mycelia from those in the hyphosphere soil and bulk soil samples (Fig. 6A). Similarly, RP application led to a distinct categorization of bacterial communities associated with AMF mycelia (Fig. 6B). Given that the bacterial communities were separated by habitats, an additional PERMANOVA analysis was conducted to determine if RP application affected the bacterial communities within each habitat. The results showed a significant impact of RP application on the bacterial communities

associated with AMF mycelia ($p = 0.01$) and in the bulk soil ($p = 0.015$), accounting for 6.8% and 6.1% of the variation, respectively (Table 3). Another PERMANOVA analysis was conducted on bacterial soil communities to evaluate the effect of AMF spore inoculation. The results revealed a significant effect ($p = 0.004$) on the bacterial assemblage, accounting for 3.5% of the variation (Table 4).

Table 2

α -diversity of bacterial community assessed by Shannon and Simpson indices in different habitats under RP amendment.

Habitats	Index	Source of variation	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(> F)
AMF mycelia	Shannon	RP	0.305	0.305	1	8.000	0.839	0.387
	Simpson	RP	6.92E-05	6.92E-05	1	8.000	0.155	0.704
Hyphosphere soil	Shannon	RP	0.043	0.043	1	16	0.468	0.504
	Simpson	RP	2.04E-06	2.04E-06	1	16	0.106	0.749
Bulk soil	Shannon	RP	4.02E-04	4.02E-04	1	8.000	0.006	0.942
	Simpson	RP	3.98E-06	3.98E-06	1	16	0.094	0.763

Table 3

β -diversity variance partitioning of bacterial community's structure among RP application in permutational multivariate analysis of variance in different habitats.

Variable	Source of variation	Df	SumOfSq	R ²	F	Pr(> F)
AMF mycelia	RP	1	0.444	0.068	1.165	0.01**
	Residual	16	6.092	0.932		
	Total	17	6.536	1		
Hyphosphere Soil	RP	1	0.229	0.044	0.732	0.87
	Residual	16	5.001	0.956		
	Total	17	5.230	1		
Bulk soil	RP	1	0.317	0.061	1.035	0.015*
	Residual	16	4.900	0.939		
	Total	17	5.217	1		

Table 4
 β -diversity variance partitioning of bacterial community's structure among AMF inoculation in permutational multivariate analysis of variance in soil biotopes.

Source of variation	Df	SumOfSq	R2	F	Pr(> F)
AMF	1	0.677	0.035	1.884	0.004**
Residual	52	18.684	0.965		
Total	53	19.361	1		

Patterns of indicator species and core bacteriome

Several bacterial ASVs exhibited consistent shifts in abundance across treatments and sample compartments. Indicator species analysis identified 35 ASVs (Table S3), revealing a strong compartment- and treatment-specific signal. In the absence of RP application, 26 indicator ASVs (74%) were detected, the majority of which were associated with mycelial samples (20 ASVs). These indicators were dominated by Planctomycetota (16 ASVs), with additional representation from the *Parcubacteria candidate phylum* OD1 (three ASVs) and Bacillota (one ASV). Five indicator ASVs were linked to bulk soil, spanning Actinobacteriota, Chloroflexota, Acidobacteriota, Bacillota, and Planctomycetota, while only a single indicator ASV, affiliated with Verrucomicrobiota, was detected in the hyphosphere soil. In contrast, RP application yielded fewer indicator taxa (nine ASVs, 26%), most of which were associated with bulk soil, primarily Planctomycetota (six ASVs) and one *Parcubacteria* (OD1). Only one indicator ASV was detected in each of the mycelial and hyphosphere compartments, affiliated with Planctomycetota and OP3, respectively.

Core bacteriome analysis further highlighted compartment-specific microbial assemblages. Across all samples, 12 core taxa were identified, occurring in at least 95% of samples (Table S4). The mycelial compartment harbored six core taxa, including *Planctomyces* sp., three members of the Pirellulaceae family (genera A17, *Pirellula* sp., and one unclassified taxon), *Bacillus flexus*, and an unclassified Gemmataceae. Similarly, six core taxa were detected in the hyphosphere soil, comprising *Bacillus fumarioli*, AKIW781 (Chloroflexota), *Planctomyces* sp., two Pirellulaceae taxa (A17 and *Pirellula* sp.), and *Gemmata* sp. In bulk soil, five core taxa were identified, including AKIW781 (Chloroflexota), *Bacillus fumarioli*, *Planctomyces* sp., *Pirellula* sp., and *Gemmata* sp. Although no core ASVs were shared between mycelial and hyphosphere compartments, several genera overlapped, whereas two and three shared core ASVs were observed between bulk soil and mycelial samples, and bulk soil and hyphosphere samples, respectively.

Consistent with these patterns, LEfSe analysis identified clear treatment-associated biomarkers. The family Anaerolineaceae and one ASV assigned to the genus *Planctomyces* were significantly enriched under RP amendment ($p < 0.001$), whereas the order JG30-KF-AS9 within the Chloroflexota was significantly associated with the absence of RP addition ($p < 0.001$). These discriminant taxa were predominantly detected in soil samples. At a finer resolution, two ASVs were identified as core taxa

within the mycelial compartment, being present in more than 90% of mycelial samples: ASV2 (*Planctomyces*) and ASV3 (Pirellulaceae). In soil samples, two core ASVs affiliated with the order AKIW781 (Chloroflexi) were consistently detected (Tables S3, S4).

Network analysis and functional profiling

The co-occurrence network of bacterial ASVs was structured into two main groups: one predominantly composed of taxa associated with AMF mycelium and the other composed of soil-associated taxa (bulk soil and hyphosphere). A hub taxon was identified in both soil and mycelial compartments (ASV3100), belonging to the family *Thermogemmatissporaceae* within the phylum *Chloroflexota*, with a betweenness centrality of 2.2×10^5 . This hub taxon did not exhibit significant sensitivity to RP amendment ($p = 0.322$) (Fig. 7).

Discussion

We investigated the influence of RP amendment on the assemblage of soil bacterial communities and their association with the AMF mycelia, and subsequently evaluated how this interaction affects P nutrition and growth of leek plants.

Furthermore, the microcosm design facilitated the suppression of the direct effect of root exudates. Along with the extraction method, this setup effectively extracted large amounts of AMF mycelia. We observed that all plants inoculated with AMF exhibited an increased colonization rate. Additionally, the RP application led to a higher proportion of root arbuscules, which serve as the symbiotic interface between plant cells and AMF intraradical hyphae. These structures play a crucial role in the exchange of materials between plants and AMF, enhancing the plants' ability to absorb higher levels of P [59]. Although mycorrhizal colonization was greater in the treated plants, the RP did not influence mycorrhizal colonization. However, the shoot and root biomass increased with the combined application of AMF and RP; this is consistent with previous results showing that the P concentration does not influence the percentage of mycorrhization but aids in absorbing P from the soil and RP, leading to increased mineralization in *Acacia gummifera* [60]. A study Elhaissofi, *et al.* [61] found that inoculating durum wheat with P-solubilizing rhizobacteria, in combination with RP amendment, enhanced both the root morphology and aboveground plant morphological and physiological traits. Similarly, another study by Mahdi, *et al.* [62] demonstrated that inoculating quinoa with the halotolerant phosphorus-solubilizing bacterium *Bacillus velezensis* improved the plant's resilience and phosphorus uptake under high salt stress induced by NaCl application. Moreover, the mycorrhizal plants with access to RP showed a significantly higher uptake of P and an increase in chlorophyll concentration in their shoots, which could potentially enhance their photosynthetic capacity and plant productivity. This enhanced P nutrition is likely an indirect result of RP solubilization facilitated by AMF and its role in shaping the microbiome associated with its hyphae, given that RP was the only form of P source used in the experiment. This can be explained by the ability of *R. irregularis* to penetrate the pot-separating membranes and explore the RFC soil through its hyphae. These findings are supported by a previous work [63] demonstrating that the presence of AMF in the hyphal compartment was linked to phytate P depletion. This suggests that

the P solubilization in the microcosm was facilitated by the mycelium of AMF. McCormack and Iversen [64] reported that fungi can access more pore spaces than roots, thereby expanding the resource absorption area. A meta-analysis by See, *et al.* [65] estimated that the average AMF hyphal density across different soil systems was 2000 cm/cm³. This extensive network contributes to the distribution of carbon in the soil through the exudation of organic compounds and hyphal turnover, which is a key mechanism driving bacterial abundance in the hyphoplan. This corroborates our findings of enriched bacterial communities in mycelial samples. AMF hyphae secrete various organic compounds, including carbohydrates, organic acids, amino acids, and other substances [66]. Like plants, AMF interact with soil microbes through their extraradical hyphae [67].

Ordóñez, *et al.* [68] found that AMF alone did not significantly enhance P uptake from RP; however, the addition of P-solubilizing bacteria increased P uptake by promoting both intra- and extraradical AMF hyphal growth, underscoring the cooperative relationship between AMF and bacteria. The hyphal network colonizing the RFC supports microbial prokaryotes by providing labile carbon, which facilitates microbial colonization and biofilm formation on the mycelium [36, 69]. The specific organic compounds secreted by AMF hyphae can vary depending on the AMF species and environmental conditions [66]. Additionally, this hyphal network may act as a "highway" for bacterial dispersal, enabling them to access nutrient-rich microhabitats, such as RP particles [17, 68, 70].

Different strategies are used by AMF to acquire P from the soil. Wang *et al.* [71] found that AMF can either produce greater amounts of hyphae or recruit different bacterial communities, which are able to mobilize P from unavailable sources. Our results demonstrated that RP did not influence the biomass of AMF mycelia, but instead, it affected the bacterial communities recruited by its mycelium. Thus, soil P availability is potentially increased through the solubilization of RP in the RFC. The microbes associated with AMF hyphae are not random; rather, they vary according to the host's nutritional requirements and the organic compounds secreted into the soil, which are heavily influenced by the bacterial community associated with the hyphae [65, 69]. Zhang, *et al.* [72] explored the phosphate–carbon (C) exchanges between *Rhizophagus irregularis* and *Rahnella aquatilis* and found that AMF hyphae exuded fructose, which induced phosphatase gene expression in *R. aquatilis*, enhancing phytate mineralization. Furthermore, the AMF phosphate transporter gene was upregulated in the presence of *R. aquatilis*, suggesting that the specific type of AMF hyphal exudates can influence bacterial communities in the mycorrhizosphere. Additionally, Duan, *et al.* [73] demonstrated that interactions within the hyphosphere between *R. irregularis* and *R. aquatilis* facilitate C-P exchange at the peri-arbuscular space in *Medicago truncatula*.

Planctomyces, *Gemmata* and *Bacillus* were identified as predominant genera in our dataset, with more than 50% of bacterial genera remaining unidentified in the mycelia and both hyphosphere and bulk soils. Most bacterial taxa were identified in the soil samples, and only one ASV (*Planctomyces* sp.) was found to be significantly abundant with RP amendment. Candidates from *Planctomycetota* and *Bacillota* were identified as core taxa in the mycelia, and in addition to these phyla, a bacterium from Chloroflexota were identified as core taxa in the hyphosphere soil samples. Planctomycetota are significantly under-

sampled, and while their partial genomes have been found in a variety of environments, most remain uncultivated [74]. Although a small number of slow-growing cultivable bacteria in axenic cultures have been characterized, they remain an enigmatic group due to challenges in obtaining pure cultures, limiting their characterization [75]. Planctomycetota is a phylum of Gram-negative bacteria, within the Planctomycetes-Verrucomicrobia-Chlamydiae (PVC) superphylum, distinguished by their budding reproduction and the absence of peptidoglycan in their cell walls [76–78].

The observed prevalence of Planctomycetota in our experiment can be attributed to their ecological role as symbionts in natural environments, where they typically live in association with other organisms [79–81]. These microbes effectively compete against various microbial communities in challenging habitats by producing specific antimicrobial compounds, such as stieleriactins, which confer a competitive advantage in resource-limited conditions [82]. A study by Liang, *et al.* [83] on P-solubilizing bacteria in ecological restoration noted that certain bacterial groups, including Planctomycetes, were abundant in P solubilization; the authors found that microbes containing the *gcd* gene were highly abundant in 17 genera, with over half of the *gcd*-containing genomes represented in Planctomycetota, Bacteroidota, Acidobacteriota, and Gemmatimonadota. The authors found a strong correlation between the abundance of these bacteria and bioavailable P in the soil, identifying them as the main drivers of P mineralization. In another study on peanuts, Planctomycetota, Chloroflexota, and Acidobacteriota were found to be dominant phyla contributing to nutrient cycling in the presence of RP [84]. A microcosm experiment revealed that phosphorus-solubilizing bacteria were transported along AMF hyphae, facilitating the movement of organic P patches and enhancing P mineralization under soil conditions [85]. This indicates that the mycelial biomass extracted from the hyphal compartment plays a role in acquiring specific bacteria, predominantly Planctomycetes, which form a co-occurrence network to solubilize RP. Additionally, AMF may recruit other bacteria, including those from Planctobacteriota, Chloroflexota or Bacillota, which may contain *gcd* gene bins and have not yet been identified as potential RP solubilizers.

Conclusions

The study demonstrated that inoculation with *R. irregularis* and the addition of RP significantly improved the P content in leeks, supporting our first hypothesis. Additionally, RP amendment altered the community structure of bacteria associated with *R. irregularis* mycelium, which confirms our second hypothesis. Our results underscore the abiotic and biotic factors that drive changes in the bacterial community within the hyphosphere associated with AMF mycelia. It also demonstrates the beneficial effects of AMF, particularly with the addition of RP, in which certain bacteria were shown to be abundant and effective in P solubilization. The P made available through this process was then taken up and transported to the leek root cells by AMF, suggesting that the combined use of AMF and RP could be a promising strategy for RP solubilization, potentially reducing the need for chemical fertilizers.

To validate these findings, field trials should be conducted before these plant-growth-promoting AMF, and bacterial inoculants can be recommended to farmers for supporting sustainable crop production.

This knowledge could be applied to develop bioinoculants for the next generation of fertilizers, enhancing the P efficiency by utilizing RP instead of processed P fertilizers for sustainable agriculture.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author Contribution

ZL designed and conducted the experiments, developed the methodology, performed the data analysis, and drafted the manuscript. JL and BA contributed to the analysis and data interpretation and participated in the manuscript revision. MH conceived the study, supervised the work, and secured the funding. All authors discussed the results and approved the final version of the manuscript.

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Data Availability

The raw reads corresponding to the hyphosphere soil samples and the AMF mycelia samples were submitted to NCBI (<https://www.ncbi.nlm.nih.gov/>), with accession numbers provided under the BioProject PRJNA1147075.

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Figures

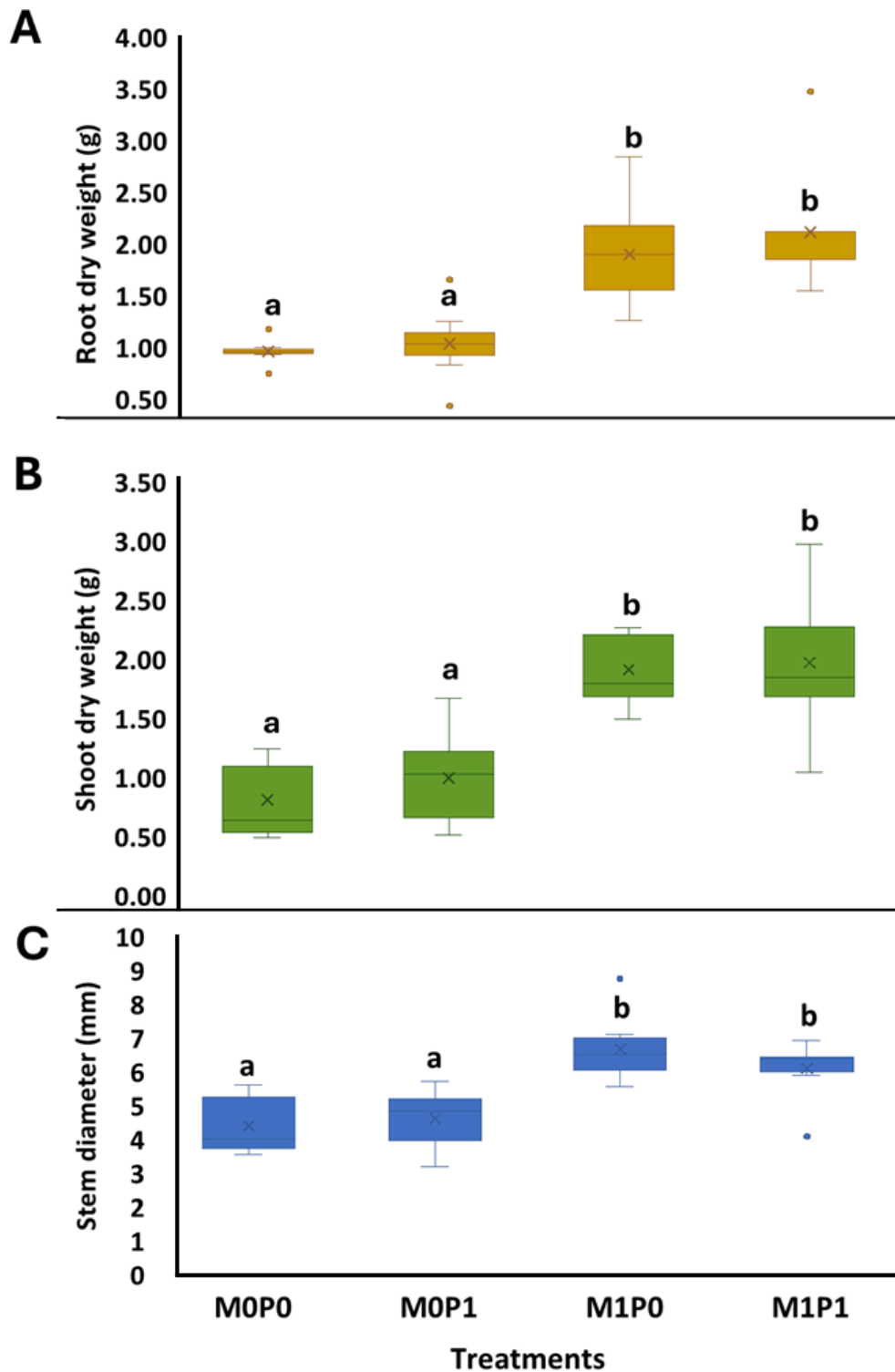


Figure 1

Responses of *Allium porrum* plants to *R. irregularis* inoculation and RP application. (A) Root dry weight, (B) shoot dry weight, and (C) stem diameter (values are means $\pm$ standard errors, $n = 9$). M0P0 (negative control), M0P1 (without AMF and amended with RP), M1P0 (inoculated with *R. irregularis* and without RP), and M1P1 (inoculated with *R. irregularis* and amended with RP). The significance of treatments and interactions were determined via two-way ANOVA.

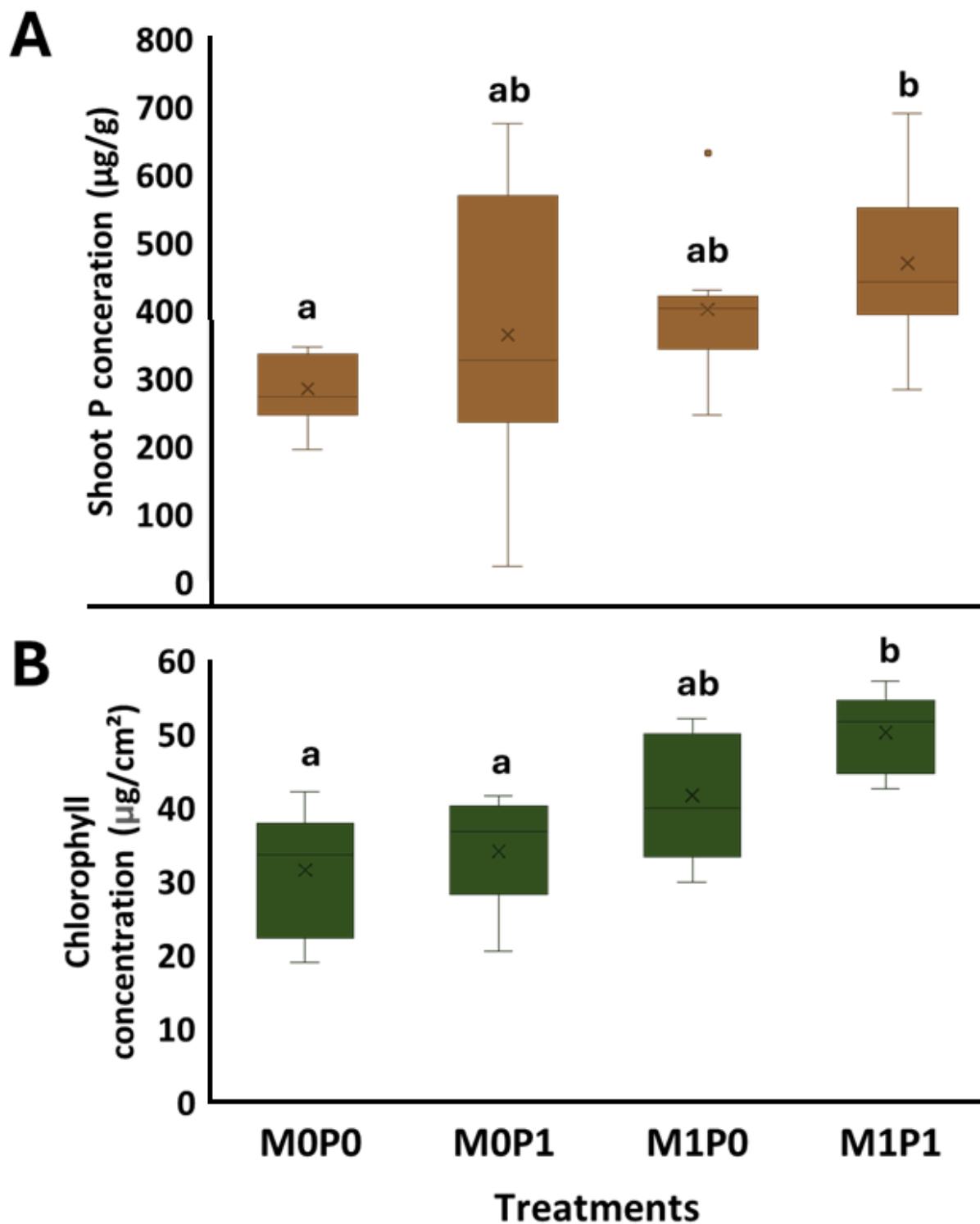


Figure 2

Effects of *R. irregularis* inoculation and RP application on (A) shoot phosphorus concentration and (B) chlorophyll concentration in *Allium porrum* (values are means $\pm$ standard errors, $n=9$). M0P0 (negative control), M0P1 (without AMF and amended with RP), M1P0 (inoculated with *R. irregularis* and without RP), M1P1 (inoculated with *R. irregularis* and amended with RP). The significance of treatments and interactions was determined via two-way ANOVA.

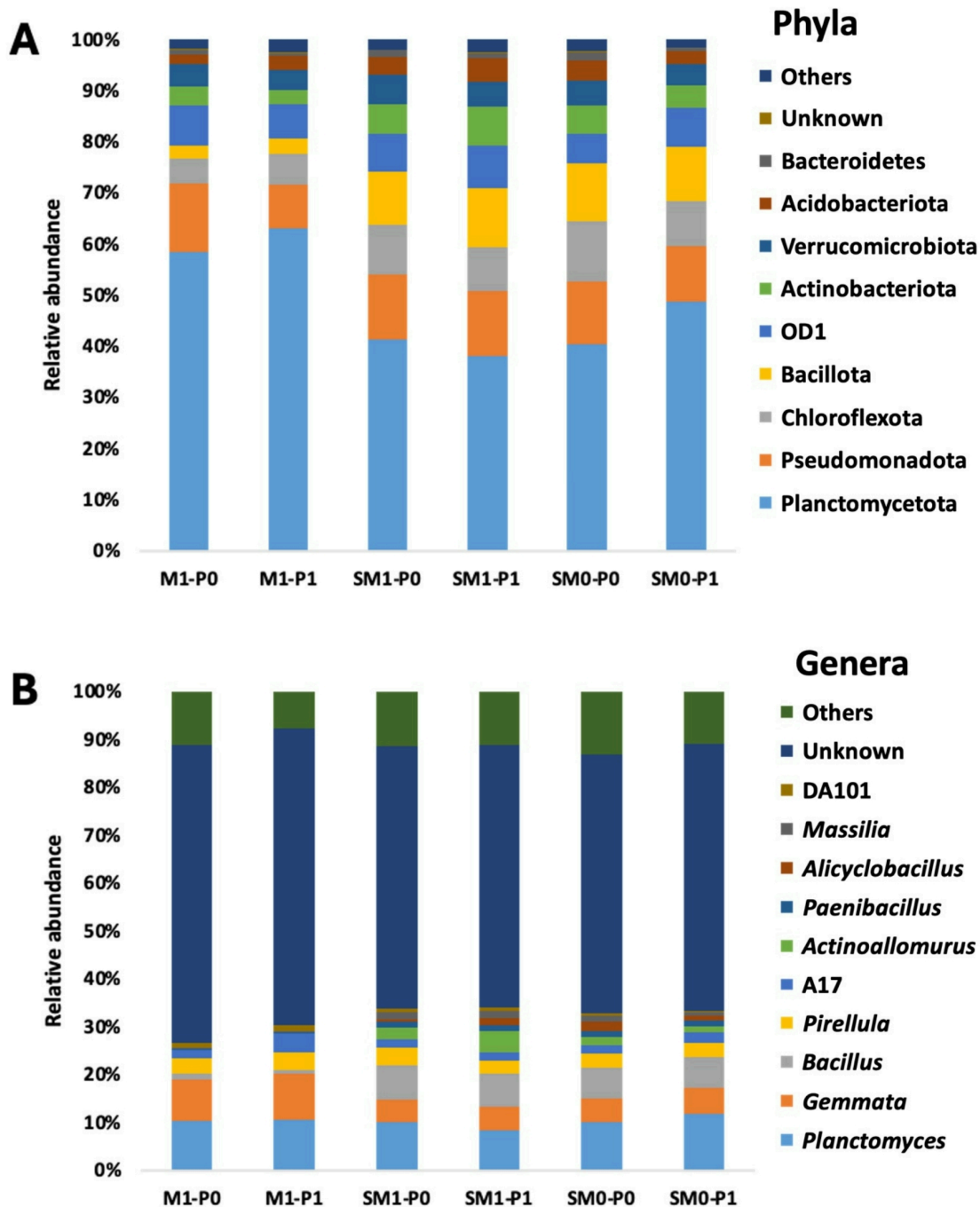


Figure 3

Relative abundances of the 10 most common bacterial taxa in the mycelia (M1-P0, M1-P1), hyphosphere soil (SM1-P0, SM1-P1), and bulk soil (SM0-P0, SM0-P1) under RP amendment at phylum (A) and genus (B) levels, with all remaining taxa grouped under "Others". The abbreviations M1, SM1, and SM0 refer to mycelium, hyphosphere soil, and bulk soil, respectively.

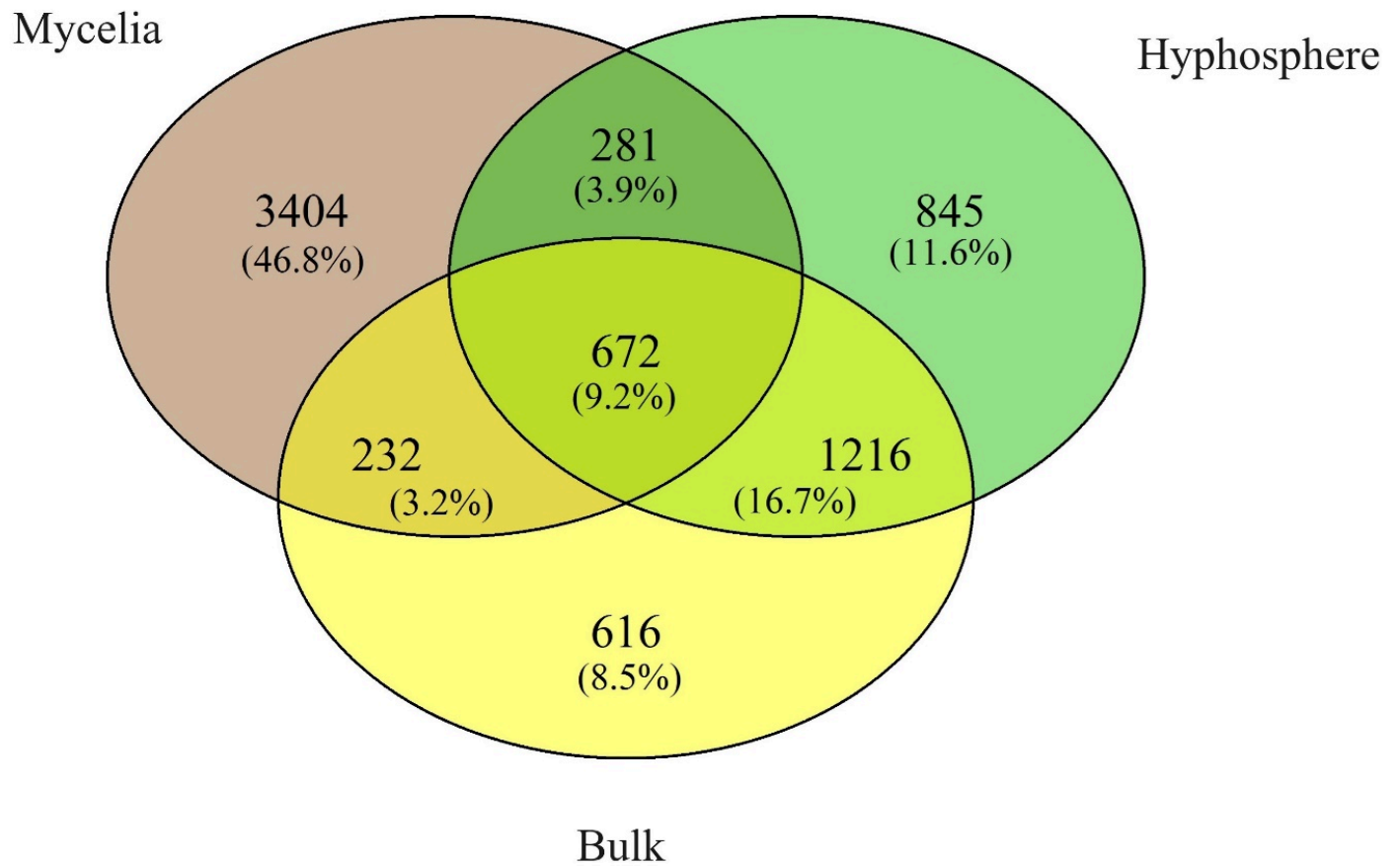


Figure 4

Venn diagram of bacterial ASVs shared between mycelia, hyphosphere, and bulk soil.

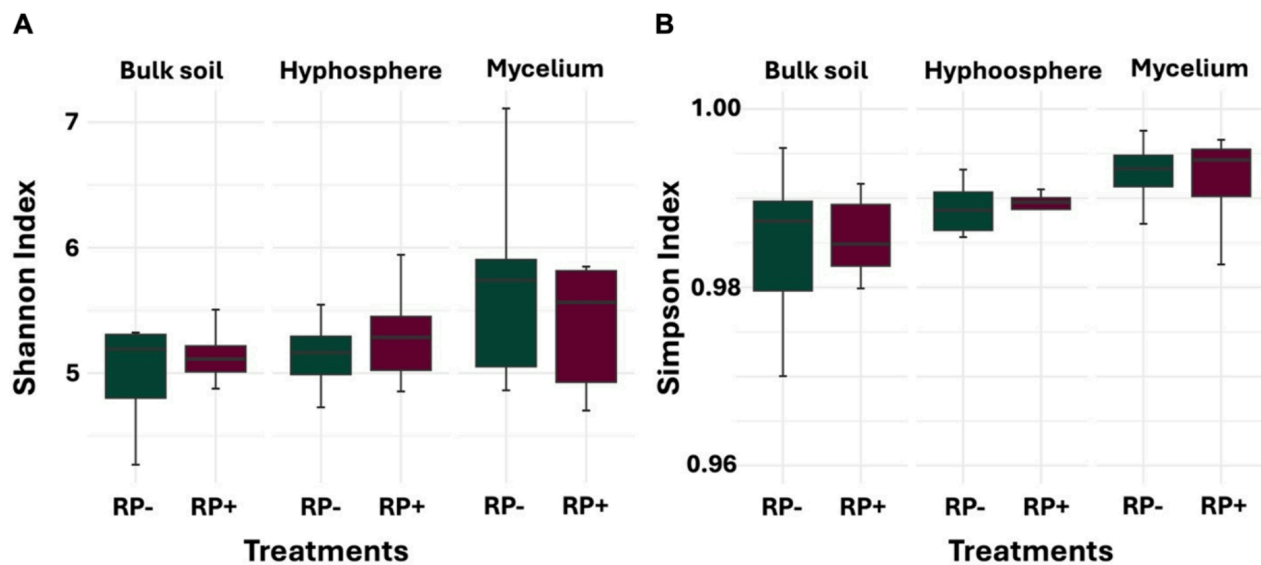


Figure 5

α -diversity of bacterial communities assessed via (A) Shannon and (B) Simpson indices in the AMF mycelium, hyphosphere, and bulk soils under RP amendment.

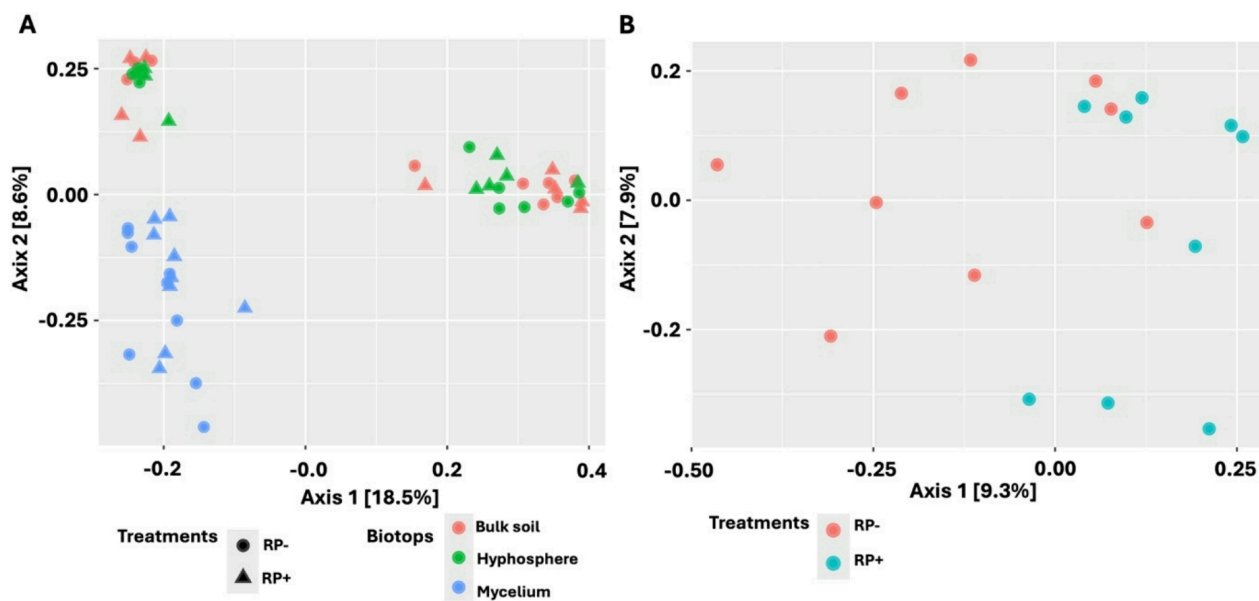


Figure 6

Principal coordinate analysis based on Bray–Curtis distance displays variations in the bacterial communities among (A) sample types and on (B) *R. irregularis* mycelia under RP amendment.

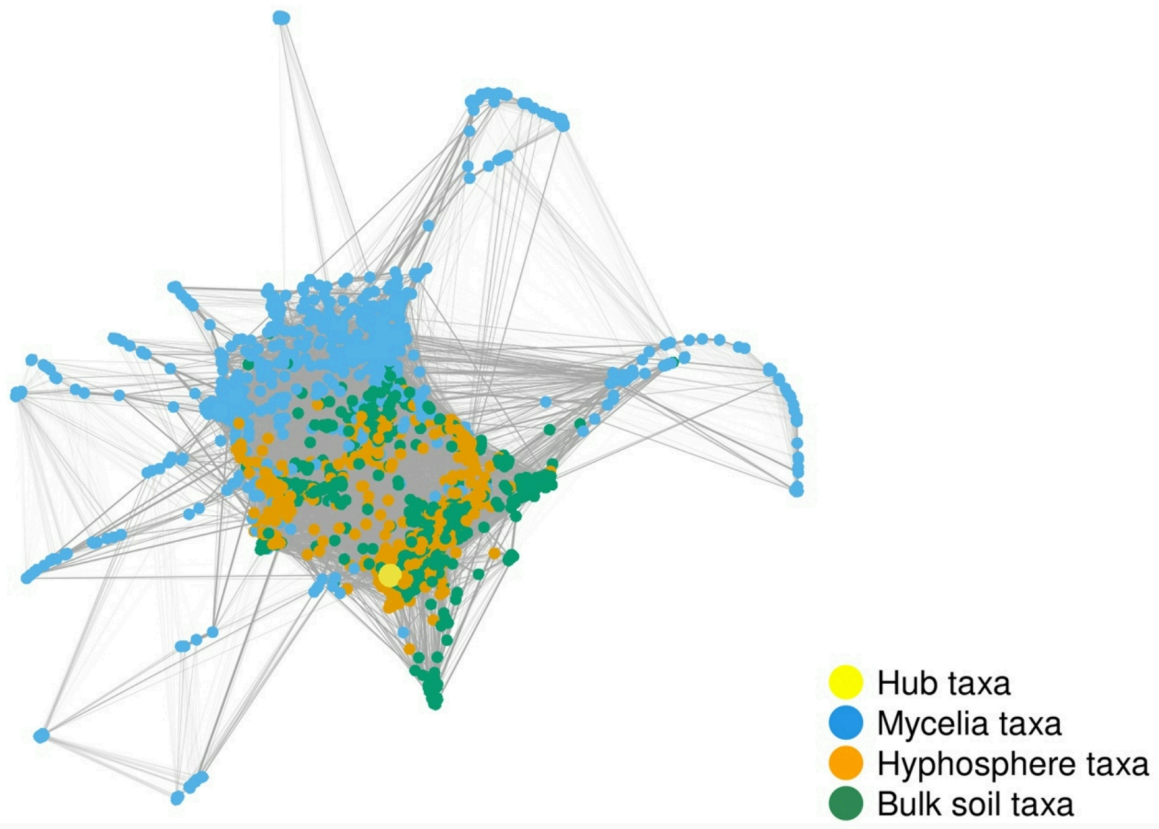


Figure 7

Pairwise co-occurrence network representation of bacterial ASVs in the different compartments. The hub taxon is ASV 3100, belonging to the family Thermogemmatissporaceae, in the Chloroflexota phylum.

Supplementary Files

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