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Evidence of a selective and bi-directional relationship between arbuscular mycorrhizal fungal and bacterial communities co-inhabiting plant roots.

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Summary

Arbuscular mycorrhizal fungi provide plants with vital mineral nutrients and co-exist inside the roots alongside a complex community of bacterial endophytes. These co-existing AMF and bacterial root communities have been studied individually and are known to be influenced in structure by different environmental parameters. However, the extent to which they are affected by environmental parameters and by each other is completely unknown. The current study addressed this knowledge gap by characterizing AMF and bacterial communities inside plant roots from a natural and an agricultural ecosystem. Using multivariate modelling the relative contribution of environmental parameters in structuring the two communities was quantified at different spatial scales. Using this model, it was possible to then remove the contribution of environmental parameters and show that the co-existing AMF and bacterial communities were significantly correlated with each other, explaining up to 36% of each other's variance. Notably, this was not due to presence of know AMF endobacteria, as removal of endobacterial reads maintained the significance of correlation. These findings provide first empirical evidence of a **selective** and **bi-directional relationship** between AMF and bacteria co-inhibiting plant roots and indicate that a significant fraction of this covariation is due to biological and ecological interactions between them.

Significance Statement

This study couples the in-depth characterization of endosphere root microbial communities with sophisticated multivariate analyses to discover a previously uncharacterized relationship between bacteria and AM fungi co-existing inside plant roots.

Introduction

Arbuscular mycorrhizal fungi (AMF, Glomeromycotina) form symbiotic associations with roots of the majority of land plants, including major crop species (Smith and Read 2010). They provision plants with important mineral nutrients (phosphorus and others) in exchange for photosynthesis-derived carbon, and play essential roles in disease protection, soil aggregation and improved water uptake (Delavaux, Smith-Ramesh, and Kuebbing 2017; Marschner and Dell 1994). As a result, AMF are important components of ecosystem functioning (Powell and Rillig 2018) and are of major interest in sustainable agriculture as alternatives to non-renewable mineral fertilizer (Verbruggen et al. 2013; Rillig et al. 2019).

Plants with arbuscular mycorrhizal fungi often show better growth, higher yield and increased nutrient content (Lehmann and Rillig 2015; Pellegrino et al. 2015). Indeed, a global-scale meta-analysis revealed an overall positive effect of AMF on grain yield of major cereal crops (Zhang et al. 2019). Given the urgent need to sustainably increase food production in order to feed the rising world population (Fedoroff 2015), AMF are expected to play pivotal roles in these production goals by improving yields of crops while reducing the need for chemical fertilizer (Rillig et al. 2019). However, in order to make the most of AMF in the agricultural setting, it is important to understand the factors which affect the interaction between AMF, plants and their environment.

The plant-AMF symbiosis is very complex with many factors influencing the effect on plant growth and yield (Hoeksema et al. 2010). Moreover, the plant-AMF interaction occurs in the presence of a diverse assemblage of microbes, including bacteria, which populate the bulk soil, rhizosphere soil and the plant root endosphere (inside the root). Interactions between AMF and these bacteria can play pivotal roles in the plant-AMF symbiosis and ecosystem functioning. For example, provisioning of phosphorus (P) by AMF is facilitated by interaction with P-solubilizing bacteria which can travel along AMF hyphae to reach patches of organic P otherwise inaccessible to the plant (Jiang et al. 2021; Zhang et al. 2014). Moreover, AMF facilitate the translocation of plant-derived carbon to soil microbial communities in the process of acquiring nutrients thus influencing their community structure (Nuccio et al. 2013). Interactions with another group of bacteria, termed mycorrhiza helper bacteria, promote the establishment of the AMF-plant symbiosis with positive effects on plant growth (Frey-Klett, Garbaye, and Tarkka 2007). AMF also harbor intracellular bacteria which live within fungal hyphae and spores (Desiro et al. 2014). Two different AMF endobacteria have been described to date, *Ca.* Moeniiplasma glomeromycotorum (*Ca*Mg, Tenericutes) and *Ca*. Glomeribacter gigasporarum (*Ca*Gg, beta-Proteobacteria)(Naito et al. 2017; Jargeat et al. 2004). *Ca*Gg endobacteria are believed to be mutualists of AMF(Lumini et al. 2007), whereas the role of *Ca*Mg is unknown.

AMF with their associated endobacteria, colonize plant root tissues alongside a community of bacterial endophytes. Bacterial root endophytes have been shown to be important in plant biology providing disease protection, nutrients and stress tolerance (Trivedi et al. 2020; Liu et al. 2017). Their communities are typically clustered phylogenetically, indicating the existence of closely-related microorganisms and a strong selective influence of plant host in structuring their communities(Trivedi et al. 2020). Being the site of nutrient exchange between AMF and the plant, the root endosphere is a hub of important AMF-bacterial interactions. Given the importance of AMF and the breadth of possible interactions with bacteria, it is crucial to understand these communities in nature and determine how they influence each other's structure.

There are well-known drivers of AMF community structure within plant roots including spatial variation, plant genotype and soil parameters (Rasmussen et al. 2018). In the same way, these parameters influence the structure of bacterial root endophyte communities with varying degrees of importance (Trivedi et al. 2020; Liu et al. 2017). However, the extent to which the co-existing AMF and bacterial communities are influenced by environmental parameters and by each other remains virtually unexplored.

To address this knowledge gap in AMF-bacterial interactions we characterized AMF and bacterial communities co-existing within plant roots in two different environments: a dune ecosystem and an agricultural grassland located near each other in Co. Wexford, Ireland. Since typical agricultural ecosystems are often sown as plant monocultures and plant diversity is known to influence the structure of associated microbial communities, we chose the dunes site as the natural plant monoculture counterpart to an agricultural grassland. Using multivariate modelling we asked: (1) “What is the relative contribution of environmental parameters in structuring the AMF and bacterial communities at different spatial scales?” and (2) “Do the co-existing AMF and bacterial communities influence each other’s structure?”. In answering these questions, we aimed at understanding the relative importance of different structural forces (environmental variables, space and the co-existing microbial community) in microbial community assembly inside plant roots.

Materials and Methods

Sampling site description and strategy

The managed agricultural grassland site was located at Teagasc, Johnstown Castle, Co. Wexford, Ireland (52^o 17’ 55’’ N, 6^o 29’ 49’’W) (Sheil et al. 2016). This grassland site was seeded with *Lolium perenne* (perennial ryegrass) and subjected to different levels of long-term phosphorus (P) fertilization starting in 1995. The site was divided into sixteen 10 x 2 m^2 plots, in a randomised block design (Figure S1). Each P treatment had four plots receiving annual fertilisation rates of 0, 15, 30 and 45 kg ha^{-1} yr^{-1} of 16% superphosphate (2 $CaSO_4$ + $Ca(H_2PO_4)_2$) in February of each year. For the purposes of this study they are hereafter referred to as P0, P15, P30 and P45. The aboveground plant material was harvested and removed eight times per year to simulate grazing. After harvesting, all plots received 40 kg N ha^{-1} as calcium ammonium nitrate ($5Ca(NO_3)_2$ NH_4NO_3). Potassium (K) in the form of potash (KCl) was also applied at a rate of 125 kg ha^{-1} yr^{-1} to compensate for K offtake in the herbage and loss from the soil. In 2016, each of the 16 plots was further subdivided into two (giving a total of 32 plots). Half of these were treated with cattle slurry 2 times per year to compensate for C loss ("+ slurry"), the other half were left untreated ("-slurry"). Soil and root samples for this study were collected from the "- slurry" plots in May and November 2018. Three soil cores (7.5 cm diameter and 10 cm deep) were obtained from each plot using a soil corer (AMS 404.45 soil core sampler and slide hammer, American Falls, ID, USA) combined into a single composite sample and transported on ice to University College Dublin where they were stored at -20°C until processing. A total of 32 samples were collected for both time points. Additionally, 5-10 small cores (2.0 cm diameter and 10 cm deep) were collected (AMS 56975 soil probe, American Falls, ID, USA) from each plot and combined in to a single composite sample for soil chemistry analysis at the Teagasc Soil laboratory at Johnstown Castle, Wexford, Ireland.

The dune site was located at Curracloe, Co. Wexford, Ireland (52° 23' 20'' N, 6° 21' 45''W). Samples were collected in May and November 2018. Four transects were laid out starting from the edge of vegetation at the seaward side (corresponding to the end of the beach) and extending 100 m inland (Figure S1). Samples were taken every 20 m by collecting the soil and roots around a single *Ammophila arenaria* (marram grass) plant nearest to each 20 m mark. A total of 40 samples were collected for both timepoints. Due to sand burial and dry soil conditions at the time of sampling it was not possible to obtain samples from the 0 m mark, which is closest to the beach. At each sampling point we recorded the distance to four nearest plants in the 70 cm radius around the area of soil sampling, later averaged to give average nearest neighbour distance (NND). Average NND was used as a measure of plant density. Samples were transported on ice to University College Dublin where they were stored at -20^{o}C until processing.

Soil chemistry analysis

Samples for soil chemical analysis were prepared by oven drying at 40^{o}C for 72 hours. Soil samples were then sieved to 2 mm with stones and plant material removed. Soil pH was analysed for all samples using a ratio 2:1 deionised water to soil and pH determined with a probe (WTW, Germany).

The Mehlich III method (Ziadi and Tran 2007), a modification of the Mehlich II test (Mehlich 1984) was employed to analyse soil nutrient availability for all samples. A 1.5 g sub sample was added to a 50 ml tube. Mehlich III reagent (a dilute acid-fluoride-EDTA solution with a pH of 2.5) was added to the tube in a 1:10 (soil:solution) ratio and placed on a gyratory shaker for 5 minutes at 180 rpm. The solution was filtered through Whatmann no. 42 filter paper. The filtrate was analysed for P, K, Al, Ca, Co, Cu, Fe, Mg, Mn, S, Zn and Cu using inductively coupled plasma-optical emission spectroscopy (ICP-OES).

Decontamination of roots

Roots were separated from soil within each sample making sure to include both fine (<1mm diameter) and thicker roots. These were stored at -80^{o}C until further processing. Thereafter, roots were washed with water to remove soil debris and surface-sterilised using sequential washes with 70% ethanol, 0.5% NaOCl and sterile nanopure water (Lekberg et al. 2018). Surface-sterilised roots were cut with sterile scissors and transferred to 2ml Eppendorf tube to fill approximately 1/3 of the tube, they were subsequently freeze-dried (lyophilized) and ground in a Tissue Lyser II (Qiagen, Germany) using 3 mm steel beads prior to DNA extraction.

DNA extraction, amplicon library preparation and sequencing

DNA was extracted from roots using the DNeasy Plant Mini Kit (Qiagen, Germany) and cleaned with the DNeasy PowerPro Cleanup Kit (Qiagen, Germany). The clean-up step was required because the initially-extracted DNA appeared dark in colour and yielded unsuccessful PCR reactions indicating contamination with PCR inhibitors. Bacterial 16S rRNA gene libraries were prepared for Illumina Miseq sequencing using dual index primers in a one-step PCR method as described in Kozich et al. (Kozich et al. 2013). Briefly, the V4 region of the bacterial 16S ribosomal RNA (rRNA) gene was PCR amplified with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) containing Illumina adapter sequences and index barcodes. Each 20 µl reactions PCR reaction contained 10µl of Phusion Flash High Fidelity PCR master mix (Thermo Scientific, Massachusetts, USA), 8µl dH_2O, 0.5µl of each primer (to give 0.25µM final concentration) and 1µl template DNA. PCR conditions were as follows: initial denaturation at 95◦C for 2 min, followed by 30 cycles of 95◦C for 20 s, 55◦C for 15 s and 72◦C for 5 min with a final elongation of 72◦C for 10 min. All PCR reactions were conducted in duplicate and subsequently pooled. AMF 18S rRNA gene libraries were prepared for Illumina Miseq sequencing using a two-step (nested) PCR method in which PCR1 primes the gene-specific region and adds the Illumina sequencing primer sites (Nextera), the PCR2 adds Illumina indexes and flow cell binding/amplification sites. In PCR1 the 18S rRNA region was amplified using the AMF-specific primers WANDA(Dumbrell et al. 2011) and AML2(Lee, Lee, and Young 2008) flanked by overhang sequences (Table S1). All PCR1 reactions were carried out in 20 µl volumes containing 10 µl of Phusion Flash High Fidelity PCR master mix, 8 µl dH_2O, 0.5 µl of each primer (to give 0.5 µM final concentration) and 1 µl template DNA. Each reaction was performed in duplicate under the following conditions: initial denaturation at 98°C for 10 s followed by 35 cycles at 98°C for 1 s, 68°C for 5s, 72°C for 15s, with a final elongation of 72°C for 1 min. Duplicate reactions were pooled and subsequently diluted ¼ to use as template for PCR2. PCR2 reactions were carried out in 20 µl volumes containing 10 µl of Phusion Flash High Fidelity PCR master mix, 8 µl dH2O, 0.5 µl of each primer (to give 0.05 µM final concentration) and 1 µl diluted PCR1 product. PCR2 cycling conditions were as follows: initial denaturation at 98°C for 10 s followed by 10 cycles at 98°C for 1 s, 68°C for 5s, 72°C for 15s, with a final elongation of 72°C for 1 min.

All PCR products (16S and 18S) were cleaned and normalized using the SequalPrep Normalization Plate Kit (Invitrogen, Massachusetts, USA) according to manufacturer's instructions. The amplicon pool was sent to the Centre for Genomic Research, University of Liverpool for sequencing on an Illumina Miseq 300 PE platform.

Bioinformatic analysis

Sequence processing

The raw Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2 (Martin 2011). The option -O 3 was used, so the 3' end of any reads which match the adapter sequence for 3 bp or more are trimmed. Reads were further trimmed using Sickle version 1.2 (https://github.com/najoshi/sickle/releases/tag/v1.2) with a minimum window quality score of 20. Reads shorter than 15 bp after trimming were removed. If only one of the read pairs passed this filter, both reads were removed from downstream analysis.

Trimmed reads which passed quality control were processed following the mothur Miseq standard operating procedure (Kozich et al. 2013). Briefly, each read was trimmed to a maximum of 330 bp (16S) or 550 bp (AMF). Ambiguous bases were removed and sequences containing homopolymer runs >8 bases were discarded. Reads were aligned to the SSU SILVA reference alignment v132 customized to either the 16S V4 region (for 16S sequences) or to the 18S region targeted by the AML2-WANDA primers (for AMF sequences) followed by identification and removal of chimeric sequences using the VSEARCH algorithm. 16S sequences were classified using the classify.seqs command and the mothur-formatted RDP Classifier reference trainset v.18 files, customized to include known AMF endobacterial sequences. 16S sequences were then clustered into operational taxonomic units (OTUs) at 0.03 cutoff using the ‘cluster’ command with default opticlust method. AMF 18S sequences were classified using the classify.seqs command to the reference AMF files obtained from the Maarj*AM* database (Öpik et al. 2010) and customised into a mothur-compatible format. AMF sequences were then clustered into “phylotypes” according to their taxonomic classification in the Maarj*AM* database at the “Genus” level.

For bacteria, 11 891 reads were obtained from each sample on average. The data was further rarefied to an even depth of 3 261 corresponding to reads in a sample with lowest number of reads.

For AMF, 58 147 reads were obtained from each sample on average and one sample from the agricultural grassland was removed due to low number of reads. AMF data were subsequently rarefied to 25 288 reads corresponding to sample with lowest number of reads.

Multivariate analyses

All the downstream bacterial and AMF community analyses were conducted in R v. 4.0.3 with the phyloseq and vegan package (McMurdie and Holmes 2013; Oksanen et al. 2013). The significance of separation of AMF/bacterial communities by location/sampling time was visualized using Principle Coordinate Analyses, PCoA (depicted in Figures 1 and 2) and the significance of separation was tested based on Bray-Curtis dissimilarity distances using a permutational analysis of variance, PERMANOVA (adonis function). Differences in microbial diversity (Shannon diversity index) by locations/sampling time were conducted using a pairwise Wilcoxon rank sum test through the pairwise.wilcox.test function.

To understand the drivers of microbial communities within plant roots, we modelled the structure of AMF and bacterial endophyte communities as a function of sample position (i.e. spatial autocorrelation), measured soil parameters and co-existing microbes. We performed these analyses separately on AMF and bacterial endophyte communities, using the other co-existing microbial group (bacteria for AMF and *vice versa*) as a predictor of community structure. The model aimed to quantify the relative importance of these three sets of variables (space, soil nutrient levels and the co-existing microbes) in partitioning the total community variance. First, we combined the dunes and agricultural grassland into the same analysis to identify the most general predictors of microbial community structure. We expected the dunes and the agricultural grassland to be characterized by distinct microbial communities associated to the two dominant plant species. Subsequently, we repeated the analysis on the each of the two sites (dunes and agricultural grassland) to identify the best local correlates of microbial community structure and quantify their importance relative to the co-existing microbes associated to the same root samples. We Hellinger transformed the community data matrix (Legendre and Gallagher 2001) to work in an Euclidean space that we could decompose with variance partitioning. We used Canonical Analysis of Principal Coordinates (Anderson and Willis 2003; Legendre and Legendre 2012) to model the effects of the various covariates (spatial, environmental, and co-existing microbes) on microbial communities.

We quantified spatial patterns in the multivariate distribution of microbial communities with Principal coordinate analysis of neighbour matrices (PCNM)(Borcard and Legendre 2002).This analysis is based on an eigenvalue decomposition of the distance neighbour matrix and returns a set of vectors, typically called “spatial vectors” that can be fitted to the data to identify the vectors that mostly account for spatial correlations. To select the vectors that account for majority of the variation we used the R function “ordiR2step” available through the vegan package which maximizes the adjusted R^2 and calculates multiple ordination models with different PCNM combination as “explanatory” variables. The output is a combination of PCNM vectors that explain majority of the variation. Once identified, the resulting vectors were used as correlates to model the community table alongside all other “explanatory” variables or correlates. The spatial vectors could thus be used to quantify community variation that could not be attributed to other measured covariates such as soil parameters or other groups of biota. These spatial vectors were important in our context, because we expected that any unmeasured environmental properties affecting microbial communities would do so through spatial gradients, and could thus also indirectly be accounted by the spatial vectors. In this way, the inherent differences between the dunes and the agricultural sites, including soil type and dominant plant species type would also be captured by the spatial vectors. The vectors might also indirectly account for unmeasured biotic interactions that could cause spatial patterning, as well as stochastic but spatially structured processes such as dispersal of spores.

Given correlation between measured soil parameters, we needed to avoid collinearity and resultant overfitting in the multivariate modelling of the microbial community table. We thus used Principal Component Analysis to reduce the dimensionality of the set of soil variables. The major axes of the PCA (those accounting for >75% of the variation) were interpreted as environmental gradient and used as environmental variables in the multivariate modelling of the microbial community table.

Finally, to identify the effect of co-existing microbes (effect of bacteria on AMF and *vice versa*), we focused on the portion of variance in one microbial dataset (either bacteria or AMF) that was left unexplained by environmental and spatial vectors, in practice the residuals of the model with space and environment. We tried to further account for residual variance of one microbial community using the other community. Specifically, we first reduced the dimensionality of the ”explanatory” data set (e.g. bacterial dataset for AMF) with a PCoA on the Hellinger-transformed taxonomic dataset and we then extracted as many axes as needed to account for at least ¾ of the “explanatory” microbial community data. We then used the ”explanatory” PCoA axes as correlates of the other microbial community residuals to quantify the unique effect of one community on the other community. Data were expressed in terms of fraction of variance accounted for by each of the three sets of correlates (that is space, environment and co-existing microbial group). The overall goal was to, first test whether variation in one microbial community (either AMF or Bacteria) can uniquely (i.e. after removing the effect of spatial and environmental covariates) account for any significant fraction of the variance of the other community, and second quantify the effect of one microbial community relative to the effects of environmental soil factors and spatial patterns. These analyses were repeated at different AMF and bacterial taxonomic levels (Genus/Family/Order/Class/Phylum). This was done by keeping the taxonomic level of the explanatory group constant (e.g. AMF at Genus level) while varying the taxonomic level of the response group (e.g. bacteria at Genus/Family/Order/Class/Phylum level). The analysis was the repeated by varying the taxonomic levels of the explanatory group and maintaining the response group as constant. All effects were tested using the permutational approach for constrained ordination implemented by the function anova.cca, which implements an anova-like permutation test(Legendre and Legendre 2012).

Results

Site characteristics

The dune and agricultural grassland sites differed in soil type, dominant vegetation and soil nutrient content (Table S2). The dune site was characterized by sandy soils, low soil nutrient availability, an alkaline soil pH (8.5) and its vegetation was dominated by *Ammophila arenaria* (marram grass). The clay-rich agricultural soil had a high nutrient content, acidic pH (6.08) and the vegetation was dominated by *Lolium perenne* (perennial ryegrass). The agricultural grassland also had a structured nutrient gradient as it received different levels of Pi fertilization ranging from 0-45 kg ha^{-1} yr^{-1}.

AMF diversity and community structure within plant roots

A total of 99 AMF phylotypes were found within surface-sterilized roots across both sampling sites (dunes and agricultural grassland) and time points (May and November 2018). Rarefaction curves indicated that the sampling effort was sufficient in capturing the total AMF diversity at both sites (Figure S2). Alpha diversity was significantly higher at the agricultural grassland with an average of 31 phylotypes per sample, as compared to the dunes with an average of 20 phylotypes per sample ($P<0.001$) (Figure 1a). AMF communities showed clear separation by site (Figure 1c), with statistically significant difference between groups (PERMANOVA; $P<0.001$, Table S3). The differences were due to the dunes being dominated by AMF belonging to *Scutellospora*, *Gigasporacea*, *Glomus* and *Claroideoglomus*. The agricultural grassland was, instead, dominated by *Glomus*, *Diversisporacea* and *Claroideoglomus* (Figure 1d).

Sampling time did not affect AMF richness and diversity between or within sites (Figure 1b). It also had no effect on the structure of AMF communities across sites (Figure 1c, Table S2) or within the dune site (Figure S2, Table S2). However, at the agricultural grassland site, AMF community structure changed significantly from May to November (PERMANOVA; $P<0.01$) (Figure S3, Table S2). This was marked by an increase in relative abundance of *Claroideoglomus* and *Diversisporaceae* phylotypes, and a decrease in relative abundance of *Glomus* (Figure S4). Interestingly, AMF diversity was not significantly affected by P application *rates* (P0-P45) (Figure S5). The effect of *measured* P on AMF communities was addressed below using multivariate analysis.

Bacterial diversity and community structure within plant roots

Over 18 000 bacterial OTUs were found within surface-sterilized roots across both sampling sites (dunes and agricultural grassland) and time points (May and November 2018). Rarefaction curves showed a high diversity of bacterial endophytes (Figure S2). The observed number of OTUs as well as the overall diversity differed significantly between the dunes and agricultural grassland sites, with an average 903 OTUs per sample in the agricultural grassland, and 754 OTUs per sample in dunes (Pairwise comparisons using Wilcoxon rank sum test, $P<0.0001$) (Figure 2a). Dunes had a higher relative abundance of Planctomycetia and Actinobacteria, whereas Bacilli, Betaproteobacteria and Gammaproteobacteria were higher in relative abundance in the agricultural grassland (Figure 2d). Bacterial endophyte diversity also differed by sampling time being higher in May than in November (Pairwise comparisons using Wilcoxon rank sum test, $P<0.01$) (Figure 2b). Actinobacteria were higher in relative abundance in November at both sites, whereas Acidobacteria were higher in May at both sites (Figure 2d). Interestingly, Betaproteobacteria were only abundant in May at the agricultural grassland site and were almost absent in November.

Bacterial endophyte community structure differed significantly between dunes and agricultural grassland sites (PERMANOVA, $P<0.001$)(Figure 2c, Table S4). Sampling time also had a significant effect on bacterial communities which differed significantly between May and November across both sites (PERMANOVA, $P=0.002$)(Figure 2c, Table S4). This was in contrast to what was observed in AMF communities from same roots which were largely unaffected by sampling time. Interestingly, the family-level composition of endophyte bacteria was very similar between the two sites, the differences mainly being in the relative abundance of the families (Figure 2d).

AMF are known to harbour bacterial endosymbionts, *Ca*Mg and *Ca*Gg, which live inside the fungal hyphae. However, only 251 reads belonging to *Ca*Mg (0.02% total number of reads), and only 20 *Ca*Gg reads (0.002% total number of reads) were recovered in this study.

Patterns of variation in microbial community structure using multivariate analysis

To understand the drivers of AMF and bacterial communities within plant roots, we analyzed the effects of different parameters on microbial community variance at two different levels: (i) combining dunes and agricultural grassland sites into the same analysis to determine *broad scale* patterns, (ii) splitting the analysis by site to identify *local scale* patterns.

AMF: Broad scale correlates

At the broad scale, 35% of AMF community variance was due to the physical distance between the agricultural grassland and dune sites (*i.e.* spatial structure). This was expected, and in fact, a direct result of the sampling design and plant species target for the sampling. The agricultural grassland environment was inherently different from the dunes environment in many aspects including plant type, soil type and soil chemical parameters (Figure S6). Therefore, these inherent differences between sites were expected to drive AMF community separation and were accounted for in our analysis by the physical distance between the sites (*i.e.* spatial structure). After removing the effect of spatial variation, soil chemical parameters alone were not significant in explaining the variance between these sites. It is interesting, however, that after taking into account all of these differences there still remained about 65% of AMF community variance that was not explained by environmental variables (i.e. residuals).

We therefore focused on these residual 65% and asked whether the bacterial community structure within the same plant roots could explain the remaining AMF community variance. We discovered that as much as 21% of the overall AMF community variance across both sites was explained by the bacterial communities co-existing within the plant roots and this fraction of variance was statistically significant at permutation test ($P<0.001$). Importantly, this effect was significant at all bacterial and AMF taxonomic levels, from genus to phylum (Table 1).

We thus hypothesised that the link between AMF and bacterial community structure within the plant roots could be driven by the co-occurrence of specific AMF phylotypes with specific endobacterial OTUs such as those belonging to known AMF bacterial endosymbionts, *Ca*Mg and *Ca*Gg. When we removed sequences belonging to these endobacteria and re-did the analysis, we found that the bacterial community structure was still significant in explaining the same proportion of the AMF community variance (Table 1).

Taken together, these results provide the first evidence of a selective relationship between AMF and bacteria co-inhibiting the same roots.

AMF: local scale correlates

At the agricultural grassland, spatial structure was not a significant predictor of AMF communities. Soil nutrient levels, on the other hand, did significantly correlate with AMF communities. P, K and Mn appeared to be the main nutrients that explained AMF community structure (Figure 3a, Table S5). However, although significant, the combined effect of spatial structure and soil nutrient levels explained only 18% of AMF community variance. Of the remaining variance, 9-22% significantly correlated with bacterial community structure and were confirmed to be significant at all taxonomic levels (Table 1). In addition, removal of known AMF endobacterial sequences resulted in <2% decrease of the variance explained, but the effect remained significant (Table 1).

In contrast to the agricultural grassland, AMF community structure in the dunes was significantly affected by spatial structure (16% of variance explained, $P<0.001$), while soil nutrient levels did not appear to play a role. In the dunes, sampling was conducted along transects laid out perpendicular to the shoreline to ensure that the abiotic gradients that extended inland from the beach were captured. Dunes are a stressful environment characterized by blowing sand and sea salt spray, high soil temperatures, low nutrients and low water holding capacity. The plants located closer to the beach experience higher stress than those further inland, therefore plant density tends to increase as you move inland (Figure S7). When plant density was included as a parameter, it explained 2% of AMF community variance while the proportion of community variance explained exclusively by space dropped to 6%. An additional 10% of variance was explained by the combined effect of space and plant density. It thus appears that both plant density and spatial structure were important independent drivers of AMF communities in the dunes (plant density was also a major driver of AMF community structure in a constrained PCoA ordination plot, Figure 3b, Table S5). However, because plant density was strongly correlated with spatial structure, the effect of space cannot be ignored and plant density and space have to be considered together. Examination of the portion of AMF community variance unexplained by spatial vectors, soil or plant density (±80%, *i.e.* residuals), revealed that the bacterial endophyte community was significant in explaining a portion of the residual variance. At lower AMF taxonomic levels, bacterial endophytes explained 3-5% of the AMF community variance, whereas at higher AMF taxonomic levels, bacteria explained as much as 23% of the variance (Table 1). The significant effect of the bacterial communities on the AMF community variance was still retained when known AMF endobacterial sequences were removed (Table 1).

In conclusion, multivariate modelling demonstrated that bacterial endophyte communities co-existing within the same roots are a sizeable and statistically significant correlate of AMF community structure along with factors such as soil nutrient parameters and plant density.

Bacterial endophytes: broad scale correlates

Like AMF communities, bacterial endophyte community structure within roots was significantly affected by the physical distance between the dune and agricultural grassland sites ($P<0.01$), whereas soil chemical parameters did not play a significant role. After taking into account all of the site-specific differences there remained approximately 70% of bacterial community variance that was not explained by environmental variables (*i.e.* residuals).

Since AMF community variance within roots was significantly affected by the co-existing bacterial communities, we asked whether the reverse was also true. Between 2-7% of the overall bacterial endophyte community variance across both sites was explained by the AMF communities co-existing within the same plant roots and this fraction of variance was statistically significant ($P<0.05$) at all bacterial and AMF taxonomic levels, from phylum to genus (Table 2).

Bacterial endophytes: local scale correlates

At the agricultural grassland site, bacterial communities were not affected by spatial structure or soil nutrients. Instead, as much as 36% of the total variance was explained by the co-occurring AMF communities (Table 2).

In the dunes, bacterial communities were significantly affected by spatial structure (8%, $P<0.01$) and by plant density ($P<0.05$), but not by soil nutrient levels (Figure S8). There was also a 9% variation which was explained by both the spatial structure and soil nutrients and plant density, which was likely due to the structured sampling design that maximized the local abiotic gradients and similar to that observed for the AMF. The AMF community explained a small but significant proportion (2%, $P<0.05$) of the variation in endophyte bacterial communities not explained by environmental parameters (approximately 75%), but only at lower bacterial and AMF taxonomic levels (genus-order). At higher taxonomic levels the proportion explained fell to 1% and was no longer significant (Table 2).

Overall, these results demonstrate the existence of a bi-directional selective relationship between AMF and the bacterial endophytes co-existing within plant roots.

Discussion

In this study we report the first empirical evidence of within-root microbial communities influencing each other's structure. Using our sampling strategy and multivariate analysis it was possible to uncouple the relative contribution of spatial distance and environmental parameters on microbial community structure and show that the co-existing bacterial and AMF communities explained a significant portion of each other's variance. This study provides empirical and statistical support for the hypothesis that a significant fraction of the covariation observed between AMF and bacterial communities in roots is due to biological and ecological interactions between these two groups of microorganisms (Figure 4).

AMF, like other fungi, are known to engage in many interactions with bacteria, from harbouring bacterial symbionts(Pawlowska et al. 2018) to serving as fungal highways for bacterial movement in soil(Jiang et al. 2021). Our data show that the strong covariation between AMF and bacterial communities within roots was not due to the presence of endobacteria within AMF, because the significance of covariation was retained when known endobacterial sequences were removed. We speculate that this covariation could be explained by the presence of a bacterial microbiome associated with AMF. A recent study showed that AMF hyphae in the soil environment establish a specific bacterial microbiome after 2 weeks (Emmett, Lévesque-Tremblay, and Harrison 2021). It is therefore plausible that AMF hyphae populating plant root tissues also establish an analogous bacterial microbiome which is likely assembled from communities of the co-existing bacterial root endophytes.

Another factor that could potentially affect AMF and bacterial community dynamics inside roots is the mode of acquisition of these symbionts by the plant host. AMF are acquired horizontally from the surrounding soil environment whereas bacterial endophytes are acquired both vertically through transmission in seeds as well as horizontally from soil (Truyens et al. 2015). These different modes of transmission could potentially influence the community structure of these microbial groups with the bacterial endophytes being more co-evolved with their plant host than the incoming AMF communities. However, if transmission mode was a major component in the overall bacterial and AMF community dynamics within plant roots it would be expected that AMF and bacterial communities were independent from each other. Our findings that they are in fact strongly correlated implies that transmission mode is not a parsimonious explanation of the observations we collected, further suggesting that there is an interaction (possibly but not exclusively mediated by plants) between these two groups of microorganisms.

The proportion of variance explained by one community of microorganisms on the other differed between AMF and bacteria, and also appeared to be influenced by site and the taxonomic level at which the analysis was carried out. At the broad scale where we combined the agricultural and dunes sites into the analysis, bacterial communities were a larger structural force for the AMF communities, explaining up to 21% of the overall AMF community variance. On the other hand, AMF communities explained only a maximum of 7% of the bacterial community variance. It is unclear why AMF explained a lower proportion of bacterial community variance within roots than *vice versa*, but since plant roots are known to be populated by diverse fungal endophytes other than AMF, it is possible that those fungal endophytes explain an additional proportion of bacterial community variance which was not captured in this study. With our observational data we cannot rule out a contribution by plants, which strongly interact with AMF. Nonetheless, these results emphasize the importance of bacterial-fungal interactions in AMF community structure and highlight bacteria as a previously unknown, but major player in AMF community dynamics, either indirectly (e.g. via plants) or directly.

At the local scale level of each of the two sites considered in the study, correlation between bacterial and AMF communities was higher at the agricultural grassland, with 10-36% of community variance attributed to the co-occurring microbial community. At the dunes site, only 2-5% of community variance was attributed to the co-occurring microbial community, except at higher AMF taxonomic levels where bacteria explained up to 23% of

AMF community variance. Taken together, these data show that the level of covariation between AMF and bacterial within-root communities is influenced by site.

This study also addressed a key challenge of the relative contribution of different parameters on AMF community structure by quantifying the proportion of AMF community variance explained by each factor (Vályi et al. 2016; Horn et al. 2017). The structure of AMF communities is known to be influenced by factors such as spatial distance, plant community composition and environmental parameters (Horn et al. 2017; Davison et al. 2012; Zobel and Öpik 2014). Here, we quantified the relative contribution of different parameters and found that at the broad scale (where both dunes and agricultural grassland sites were included) spatial distance was the most important factor explaining AMF community variance (35%). This was likely due to the inherent differences between the dunes and agricultural grassland sites since spatial separation also encompassed differences in plant community and soil type. Therefore 35% of AMF community variance was attributed to these innate sampling site differences. Interestingly, after accounting for these differences, the co-existing bacterial community explained a further 21% of AMF community variance, whereas soil nutrient levels did not play a significant role. At the local scale, soil nutrient levels and plant density were significant in affecting AMF community structure alongside the co-existing bacterial community, whereas spatial distance was not important. At the agricultural grassland site, our analysis revealed the significant effect of measured soil phosphorus (P), potassium (K) and manganese (Mn) on AMF community variance. Soil nutrient levels, particularly P, have long been known to affect the plant-AMF symbiosis because P is the main nutrient provisioned by AMF to the plant(Antunes et al. 2012; Johnson 2010). For example, under limiting P conditions, AMF can influence plant productivity and community structure but not when P is readily available, presumably because AMF have little influence on plant fitness in P-enriched conditions (Collins and Foster 2009). In this study it was not possible to disentangle which of these soil nutrients (P, K or Mn) was the main driver of AMF community variance because they were correlated with each other (Figure S9). However, these findings highlight the importance of soil P, K and Mn in structuring the AMF communities within plant roots. Interestingly, soil nutrient levels did not play a role in AMF community variance at the dunes site indicating that drivers of AMF community structure are site-specific.

Because AMF are obligately dependent on their plant host, AMF community dynamics are known to be intimately linked with plant community structure, although the nature and direction of this interdependence is influenced by spatial and temporal scales(Zobel and Öpik 2014; Horn et al. 2017). In our study, plant community composition was not expected to play a significant role in AMF community structure because we deliberately sampled in near-monoculture sites. However, our discovery that within-root AMF and bacterial communities can significantly influence each-other’s structure indicates a potential knock-on effect on the host plant community dynamics (Figure 4). It is important to highlight, however, that the evidence provided here is based on strong statistical correlations and does not provide a mechanistic basis for the observed patterns.

The diversity of AMF communities was higher in the agricultural grassland as compared to the natural dune ecosystem (Figure 1a). This was in contrast to what was expected, as AMF diversity is typically lower in conventional agricultural systems as compared to natural systems (Roy et al. 2017). Moreover, the diversity of bacterial root communities was also higher at the agricultural grassland site. These observations cannot be attributed to differences in plant diversity which is usually higher in natural ecosystems compared to agricultural ones, as both sites sampled in our study were primarily plant monocultures. Instead, the differences in microbial diversity are likely explained by differences in soil nutrient content and/or fertilizer application, the latter being known to increase soil microbial diversity (Ge et al. 2008). The dunes site had lower soil nutrient content than the agricultural grassland (Table S2) which was routinely fertilized with P, K and N. Therefore, the higher microbial diversity in the plant roots from the agricultural grassland might be explained by higher soil nutrient content.

The composition of bacterial root communities at both sites was very similar at family-level, with the main difference being in relative abundance of these taxa (Figure 2d). This is indicative of a core set of bacterial taxa that inhabit plant roots and is in agreement with what has been observed in bacterial root endophyte communities of other plants (Trivedi et al. 2020). We also found evidence of temporal shifts in bacterial root endophyte communities at both sampling sites, which changed significantly between May and November 2018. This is also consistent with previous reports of observed changes in bacterial root endophyte communities throughout the developmental lifecycle of the plant (Edwards et al. 2018; Gaiero et al. 2013). AMF communities, on the other hand remained unchanged between the two sampling timepoints in the dunes site, while changing significantly at the agricultural grassland site. This could be explained by differences in grazing, a known driver of AMF community change (Faghihinia et al. 2020). The agricultural grassland was a simulated grazing site that was harvested more frequently (monthly) in summer months (May-August/September) and less frequently in winter (one harvest) (October-March) due to slower grass growth. By contrast, the *A. arenaria* grass dominating the dunes is rarely grazed (Huiskes 1979). These observations provide preliminary evidence of seasonal microbial community shifts at the two sampling sites.

Finally, it is important to note that known AMF endobacteria, *Ca*Mg and *Ca*Gg were represented by very low read numbers in the plant roots collected in this study. This was not due to primer bias against these taxonomic groups, as we were able to amplify high numbers of endobacteria directly from AMF spores (Lastovetsky *et al. In Prep*). Instead, it indicates that AMF endobacteria represent only a small proportion of the bacterial communities within plant roots.

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Author Contribution:

OAL, FPB, DPB and ED designed research; OAL, DPB, FPB and SM performed research; OAL and TC analysed data; OAL, TC and ED wrote the paper.

Data Availability

All sequencing data has been submitted to the Sequence Read Archive (SRA) National Center for Biotechnology Information (NCBI) database under the project number PRJNA823564. Reviewer link:

https://dataview.ncbi.nlm.nih.gov/object/PRJNA823564?reviewer=b59f0frmfpcvdbevvjphb

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Conflict of Interests

The authors declare no competing financial interests.

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Table 1: Significant proportion (%) of the residual AMF community variance explained by the bacterial communities co-existing within the same roots. Results of analyses shown at different bacterial and AMF taxonomic levels. Values in brackets indicate proportion explained after removal of endobacterial sequences.

	Genus	***Family***	***Order***	***Class***	***Phylum***
BACTERIA					
Both locations	8 *(8)*	5 *(4)*	3 *(3)*	3 *(3)*	1 *(1)*
Agricultural grassland	18 *(16)*	16 *(16)*	18 *(18)*	19 *(18)*	9 *(9)*
Dunes	3 *(5)*	5 *(5)*	5 *(5)*	ns *(ns)*	ns *(ns)*
AMF					
Both locations	8 *(8)*	8 *(9)*	11 *(11)*	21 *(21)*	*
Agricultural grassland	18 *(16)*	21 *(22)*	21 *(22)*	22 *(22)*	*
Dunes	3 *(3)*	12 *(7)*	15 *(15)*	23 *(24)*	*

* no variability at this taxonomic level (AMF all belong to Glomeromycotina)
ns, not significant

Table 2: Significant proportion (%) of the residual bacterial community variance explained by the AMF communities co-existing within the same roots at different bacterial and AMF taxonomic levels. Results of analyses shown at different bacterial and AMF taxonomic levels. Values in brackets indicate proportion explained after removal of endobacterial sequences.

	Genus	*Family*	*Order*	*Class*	*Phylum*
BACTERIA					
Both locations	6 *(6)*	7 *(7)*	6 *(6)*	6 *(6)*	5 *(5)*
Agricultural grassland	23 *(23)*	28 *(28)*	33 *(32)*	36 *(36)*	33 *(33)*
Dunes	2 *(<1)*	2 *(<1)*	2 *(<1)*	ns (*<1*)	<1 (<1)
AMF					
Both locations	6 *(6)*	2 *(1)*	2 *(1)*	2 *(<1)*	*
Agricultural grassland	23 *(23)*	12 *(12)*	10 *(12)*	<1 *(<1)*	*
Dunes	2 *(<1)*	<1 *(<1)*	*ns (<1)*	<1 *(<1)*	*

* no variability at this taxonomic level (AMF all belong to Glomeromycotina)
ns, not significant

Figure Legends

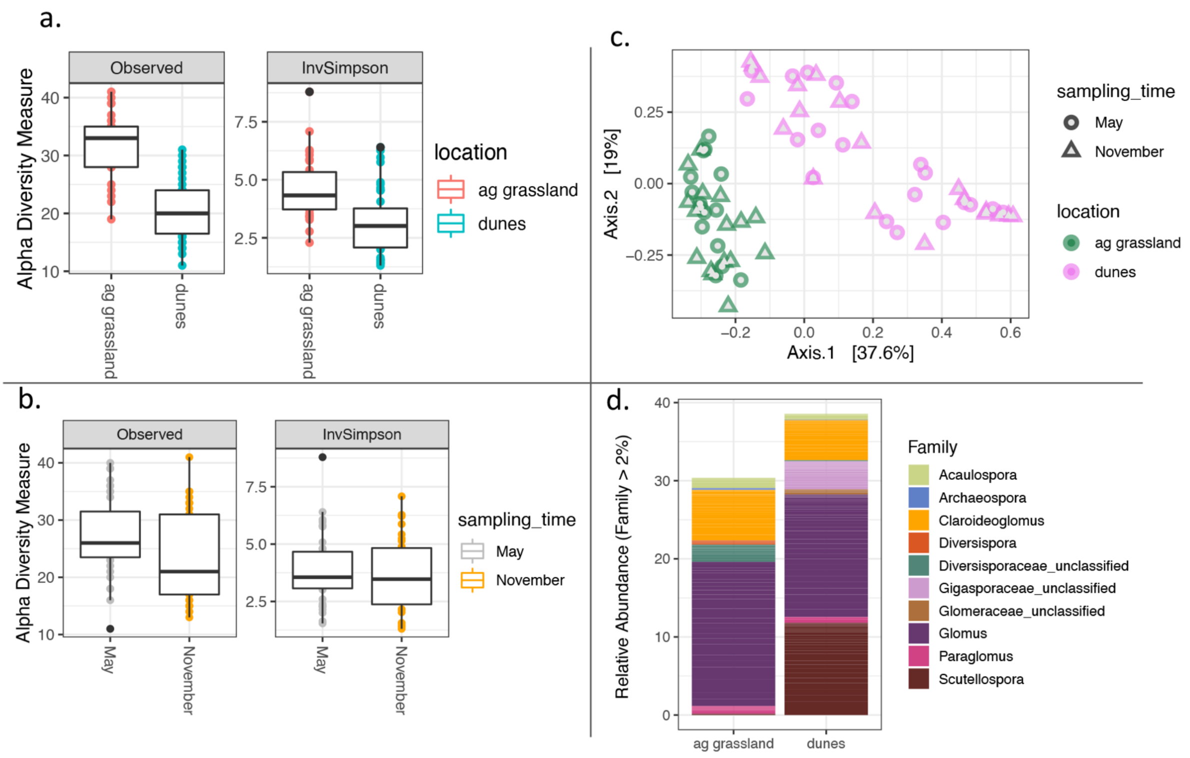
Figure 1. Diversity of AMF inside plant roots in dunes and agricultural grassland locations. (a) Richness (observed) and inverse Simpson (InvSimpson) measures of diversity in the dunes and agricultural grassland locations; (b) Richness and inverse Simpson measures of diversity in May versus November; (c) Principal Coordinate Analysis (PCoA) plot of AMF communities inside plant roots from dune and agricultural grassland locations collected in May and November. PCoA was performed on a Bray-Curtis distance matrix. Each point represents an AMF community found within a single sample; (d) Relative abundance of AMF taxa grouped a Family level in the dunes and agricultural grassland locations.

Figure 2. Diversity of bacteria inside plant roots in dunes and agricultural grassland locations. (a) Richness (observed) and inverse Simpson (InvSimpson) measures of diversity in the dunes and agricultural grassland locations; (b) Richness and inverse Simpson measures of diversity at both sites in May versus November; (c) Principal Coordinate Analysis (PCoA) plot of bacterial communities inside plant roots from dune and agricultural grassland locations collected in May and November. PCoA was performed on a Bray-Curtis distance matrix. Each point represents a bacterial community found within a single sample; (d) Relative abundance of bacterial taxa grouped a Class level in the dunes and agricultural grassland locations split by sampling time.

Figure 3. Constrained principle coordinate analysis (PCoA) plots depicting the relative importance of environmental parameters in structuring the AMF communities inside plant roots. (a) agricultural grassland, model accounted for 25% of variance; (b) dunes, model accounted for 26% variance. Axes values depict proportion of that variance explained by each axis; ANOVA results of relative importance of components are reported in Table S5. The constraining factors were: Longitude (X), PCNM3, PCNM5, PCNM7 for the spatial components (these were derived from a PCNM of the sample distance matrix and then selected through a modelling selection procedure as detailed in the methods); “PC1 (Soil Mn vs Fe)”, “PC2 (Soil P vs K)”, “PC1 (Plant density) , “PC2 (Soil Nutrients)” were the soil nutrient and plant biotic components which appeared as the main drivers of sample separation along PC1 and PC2 in the PCA analysis (See Figure S6). Plant density was measured as average nearest neighbour distance in cm; K, potassium; P, phosphorus; Fe, iron; Mn, manganese.

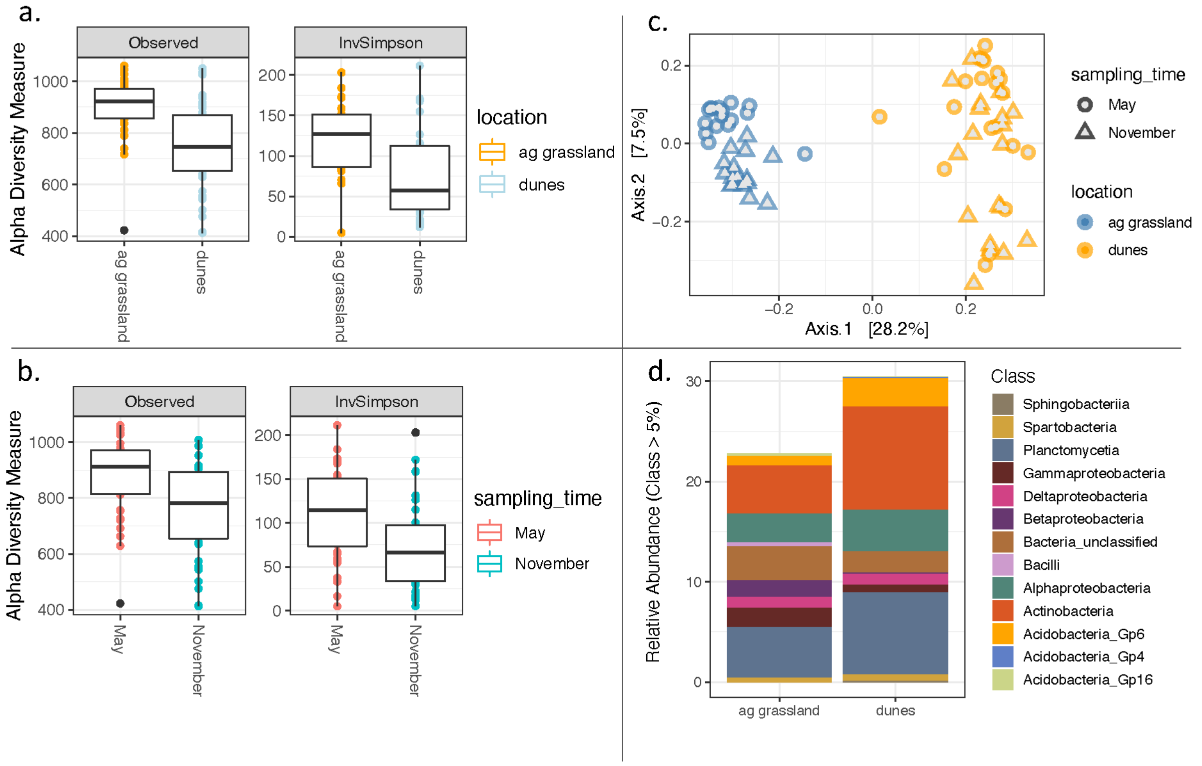
Figure 4. Schematic representation of plant-AMF-bacterial community dynamics. Within-root AMF and bacterial communities have a significant effect on each other’s structure (red arrows, shown in this study) which is turn can influence the biology and the community structure of the plant hosts (grey arrows, potential effect).

Figure 1.



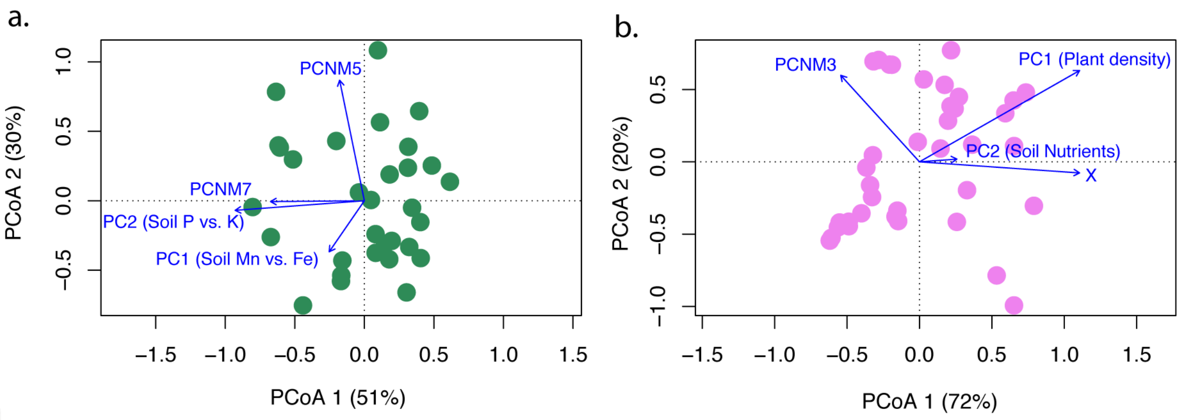
EMI_16227_Figure 1.tif

Figure 2.

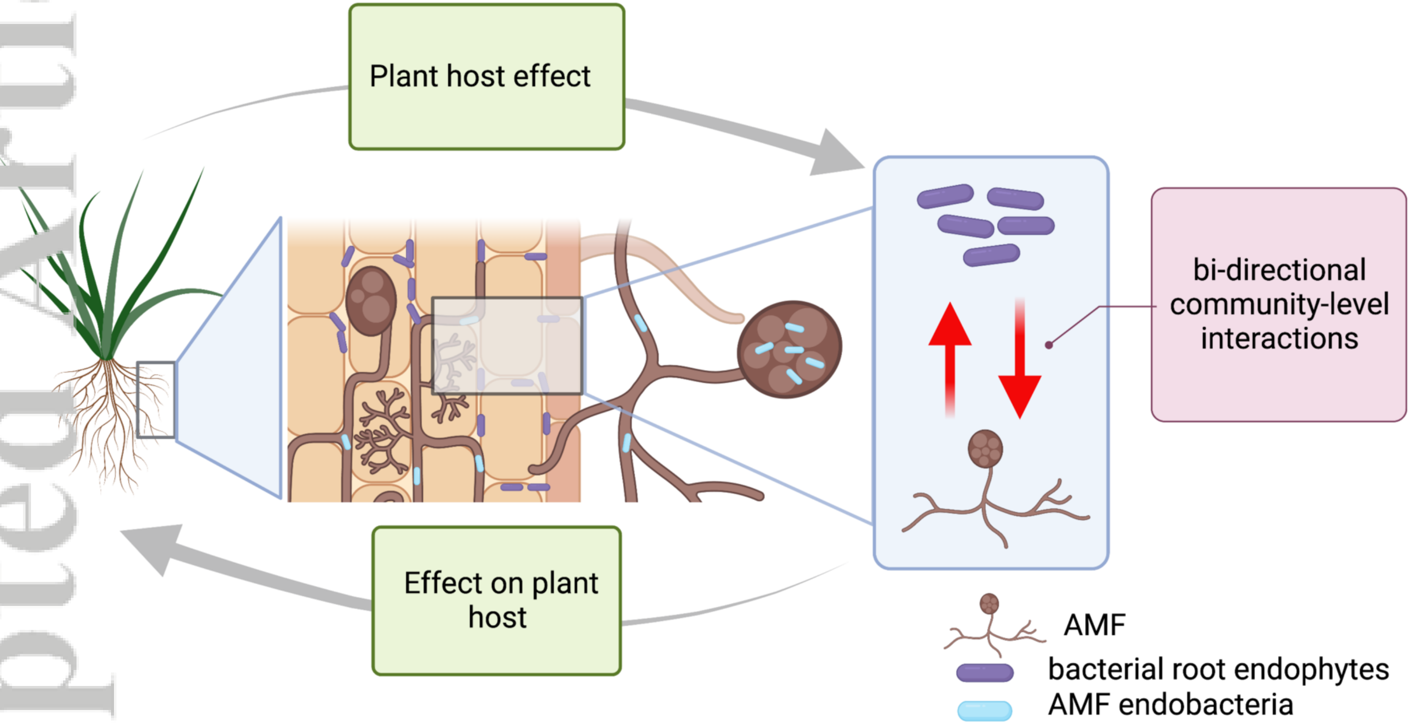


EMI_16227_Figure 2.tif

Figure 3.



EMI_16227_Figure 3_smaller.tif



EMI_16227_Figure 4.png