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Functional Ecology

Plant neighbors shape fungal assemblages associated with plant roots: a new understanding of niche-partitioning in plant communities

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Authors contribution

CM and PV conceived the project and with VG set up the methodology of the study. VG, CM, CR and OJ conducted the experiments. All the authors contributed to data collection. VG performed the sequence analysis with the help of PV. CM performed the statistical analyses and wrote the first draft of the paper. All the authors interpreted the results and to drafting the paper and gave their final approval for publication.

Data availability statement

Ecological data are available on Figshare at <https://doi.org/10.6084/m9.figshare.11828307.v1> (Mony et al., 2021).

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Plant neighbors shape fungal assemblages associated with plant roots: a new understanding of niche-partitioning in plant communities

Abstract

1-Understanding the assembly rules of mycorrhizal fungi is crucial, given their tremendous importance in plant nutrition and health. Differentiation in plant-associated arbuscular mycorrhizal fungi (AMF) is likely driven by a host-preference effect. Coexisting plant species may then affect a focal plant microbiota through fungal dispersal among plants, and plant preferential recruitment of AMF. Both mechanisms are likely shaped by the plant's phylogenetic and functional strategies.

2-We expected that (i) the structure of AMF assemblages associated with a focal plant depends on the identity of the neighboring plant species; (ii) this effect would be predicted by the phylogenetic and functional similarity between the focal and neighboring plant species. These predictions were tested during the first stages of growth, by simulating the early development of plants within a community

3-Using an experimental matrix-focal plant species design testing 15 neighboring plants from five taxonomic families, we demonstrated that the neighboring plants provided different species pools for the focal plant, *Medicago truncatula*, and influenced AMF communities associated to focal plant, especially in terms of richness but not relative evenness. *M.*

truncatula grown with *Brassicaceae* or other *Poaceae* species displayed respectively no or low AMF richness compared to those grown with *Rosaceae* and *Asteraceae* species. These effects were weakly dependent on the phylogenetic distance from the neighboring plant but were predicted by the functional proximity. AMF assemblages were enriched and bore more resemblance to the neighboring plants when the neighboring plants were functionally dissimilar from the focal one. Functional dissimilarity was only a significant predictor when based on traits characterizing the nutrient use and uptake strategy rather than on a more integrated growing strategy of the plant.

4-Microbiota composition was shown to be dependent on the identity of the neighboring plant, particularly on its functional belowground niche. At the colonization stage, when the plant arrives in a community, plant mycobiota might be influenced by the spatial distribution of plants already present in the community. This work suggests a new view of the concept of niche partitioning in space for plants based on microorganism-plant interactions.

Keywords

AMF assembly rules, plant composition, neighboring effect, plant-fungi interactions, host-plant preference effect.

Introduction

The importance of symbioses for plant growth and health has long been acknowledged (e.g. Vannier et al., 2015). Arbuscular mycorrhizal fungi (AMF) are among the most widespread symbionts of plants, colonizing roots of approximately 80% of all terrestrial plants (Smith and Read, 2008). In exchange for carbon, these fungi form obligate symbiotic associations with plants, providing the host plant with phosphorus, nitrogen and trace elements (Smith & Read, 2008). Apart from improving plant nutrition, recent works have demonstrated their key role in plant resistance to both abiotic stresses (Lenoir et al., 2016) e.g. salinity (Porcel et al., 2012), drought (Ruiz-Lozano et al., 2016) and biotic stresses, including pathogens (e.g. Jung et al., 2012; Schoenherr et al., 2019; Vannier et al., 2019). AMF communities that are associated with plant roots have then a tremendous effect on plant productivity, phenology and morphology (van der Heijden et al., 1998; Streitwolf-Engel et al., 2001; Vandenkoornhuysen et al., 2015). AM fungal compositions have been shown to vary among plant species (Bever et al., 1996; Vandenkoornhuysen et al., 2003), and even locally among host plants of a given plant species (Lê Van et al., 2017; Bittebiere et al., 2020).

Different species of AMF often co-occur in nature and colonize the same host plant (Maherali & Klironomos, 2007). However, preferential fungal assemblages have been described for a certain number of plant species (e.g. Eom et al., 2000; Martínez-García & Pugnaire, 2011), even co-occurring in the same habitat (Vandenkoornhuysen et al., 2002, 2003). This preferential plant-host effect is linked with the recruitment process of AMF in plant roots. Plants differ in their rooting systems, which may present physical and chemical barriers that prevent fungi, especially AMF, from colonizing them (Vandenkoornhuysen et al., 2015). For instance, some plant species generate indeed allelochemical compounds that can prevent the germination of spores or the development of the mycorrhizal association (Stinson et al., 2006; Callaway et al., 2008). Kiers et al. (2011) also demonstrated the existence of a rewarding C allocation by the host plant to the best cooperating AMF, i.e. the species that provides the plant with the most phosphorus. This reward process is expected to sort the AMF colonizers depending on their cooperative behaviour. All these effects contribute to a differentiation in fungal assemblages among plant species.

Plant individuals recruit AMF that are present in the surrounding soil. These AMF propagules can infect plant roots located close by through spore germination or hyphae growth (Camargo-Ricalde, 2002). The composition of this soil reservoir is determined by

environmental drivers (Bahram et al., 2015), but can also be influenced by plant species distribution (passenger hypothesis, Hart et al., 2001) due to the combined actions of plant-root spatial patterns and host preference effect. Indeed, fungi associated with a focal host plant are likely to be influenced by the identity of the plants that develop close by. Empirical and experimental evidence tends to support this influence. For instance, in the context of invasive plants, a set of studies demonstrated that AMF associated with native plant species were impacted by the presence and density of invasive plant species even at a short distance (Mummey & Rillig, 2006; Shi et al., 2014). Similar results were also obtained with indigenous plants species: recent works in grassland communities indeed demonstrated that the identity of plants growing within a distance of a few centimeters affected the richness and composition of the endophytic root mycobiota associated with a graminean plant species (Bittebiere et al., 2020; Mony et al., 2020). However, in the field, it is difficult to disentangle the direct effect of neighboring plants on the microbiota assemblages from their indirect effect through modifications of the abiotic environmental conditions (i.e. Habitat theory in Zobel & Opik, 2014) or through plant-plant interactions (synergetic or antagonist effects) that drive the microbiota compartment. Experimentations is thus a better way to focus on the effect of plant neighbor identity on microbiota though such studies remain scarce (but see Johnson et al., 2004).

The effect of close neighboring plants on AMF community composition in the soil and roots has been poorly predicted to date. During evolution, AMF have accompanied plant diversification (Selosse & Le Tacon, 1998). Phyla are known to differ in their ability to associate with a large number of AMF taxa (Soudzilovskaia et al., 2020). Despite no known AMF host-specificity, it is likely that preferential assemblages associated with lineages followed the evolutionary radiations of plants. Indirect evidence for the effect of species identity and phylogeny on soil fungal composition has been demonstrated in tropical trees (Barberán et al., 2015) and could be extended to herbaceous species. Hence, it can reasonably be assumed that phylogenetically related plants would display similar AMF assemblages. AMF colonizing a focal plant species would then likely be more similar to AMF inhabiting roots of neighboring plants alike or of close phylogenetic proximity; while phylogenetically distant neighboring plant species would be more dissimilar in their AMF microbiota.

AMF community composition also depends on plant phenology, growth and physiological status, probably because of their interplay in many plant ecological functions. AMF recruitment would then be expected to vary depending on the functional strategy of the

plant species concerned. One of the very few studies on the topic by Burrows & Fleger (2002) empirically demonstrated in a spore-based analysis that AMF composition differed between legumes and C4 grasses. To date, no study has dealt with a larger set of functional strategies, whereas, considering their key role in plant nutrition, the relationship between AMF composition and the way plants exploit the soil resources would be expected to be even stronger. Root type, morphology and architecture is indeed an important driver for AMF colonization and composition in woody plants (Eissenstat et al., 2015). Some authors also suggested that plant soil exploitation relied on a trade-off between investing in root development or recruiting and promoting the development of AMF (Liu et al., 2015). AMF colonizing a focal plant species would then more likely resemble AMF inhabiting roots of functionally close neighboring plants, while functionally distant neighboring plant species would be more dissimilar in their microbiota.

The aim of the present study was to analyze the effect of neighboring plants on a focal plant species using a plant-matrix design, and to determine the significance of phylogenetic or functional predictors of this effect. The experiment was conducted during the early growth of *Medicago truncatula*. We specifically tested the two following hypotheses:

- 1.) The identity of the neighboring plant at least partially controls the AMF that colonize *Medicago truncatula*,
- 2.) The effect of neighboring plant on *M. truncatula* microbiota might be predictable through the phylogenetically and/or functional proximity between the focal plant and its neighbors. When the neighboring plant is phylogenetically or functionally close to the focal plant, *M. truncatula* microbiota should be more similar to the neighboring plant microbiota.

Methods

We used *Medicago truncatula* as the focal plant species, as it is a well-known mycotrophic plant model species (Cook, 1999). The experiment included a set of 16 treatments corresponding to *M. truncatula* grown in 15 different monocultures of matrix plant species (i.e. neighbor plants), and alone (i.e. without neighbor plant). The matrix species included 15 treatments corresponding to 15 different plant species selected in five taxonomic families (*Fabaceae*, *Asteraceae*, *Rosaceae*, *Brassicaceae*, *Poaceae*) chosen to represent a gradient of phylogenetic distances from *M. truncatula* (Figure S1).

Plant cultures

To inoculate matrix individuals with natural-occurring AMF assemblages, their seeds were germinated and grown in separate pots on a mixed substrate comprising soil (90%) and sterilized vermiculite (10%). The soil was taken from a controlled mesocosm established in 2009 by Benot et al. (2013) containing a species-rich mixture of grassland species with known and controlled composition comprising 12 species: *Brachypodium pinnatum*, *Lolium perenne*, *Elytrigia repens*, *Agrostis stolonifera*, *Agrostis capillaris*, *Holcus lanatus*, *Holcus mollis*, *Festuca rubra*, *Dactylis glomerata*, *Ranunculus repens*, *Anthemis vulgaris*, *Centaurea nigra*. Sampled soil was sieved through a 5mm mesh and carefully homogenized before use. Matrix species seedlings were watered every second day with sterile water and every three weeks with a sterile 1 x Hoagland solution (Arnon & Hoagland, 1940). After 45 days, the seedlings were harvested, carefully washed with sterile water to remove soil particles and rhizosphere microorganisms, and then transplanted in a 0.8 L pot (12cm diameter, 12cm height) filled with sterile vermiculite substrate. Six washed seedlings of the same species were planted in each pot following an hexagonal plantation design to ensure balanced competitive interactions (Birch et al., 2007).

Seven days later, when the seedlings had recovered from the stress of transplantation, one seedling of *M. truncatula*, grown in sterile conditions, was added to the center of the pot. Each treatment was replicated 10 times. The treatment with individual *M. truncatula* without neighbor plant was treated as a negative control, i.e. to ensure that there was no aerial contamination of AMFs in the experimental growth chamber. The 160 pots (15 treatments with matrix species + 1 control with no matrix species) were randomly placed in a strictly controlled growth chamber ($22.5 \pm 0.5^{\circ}\text{C}$; 12L:12D; 5200 lux). Pots were watered with a standard quantity of water (100ml, 150ml and 200ml/week depending on the progress of the experiment) and a 50%-phosphorus deprived Hoagland solution (50ml, 75ml, 100ml/week depending on the advancement of the experiment) to promote root mycorrhization. All the plants were harvested 10 weeks after the beginning of the experiment. In each pot, 100 mg of roots of *M. truncatula* were sampled for molecular analyses. The rest of root system and aboveground part of the plant were dried at 65°C for 72 h to measure dry biomass.

Calculation of phylogenetic and functional distances between the matrix and focal species

For each matrix plant species, we calculated the pairwise phylogenetic and functional distances to the focal plant species *M. truncatula*.

Phylogenetic distances were calculated using a reference phylogenetic tree (Zanne et al., 2014). This phylogenetic tree contained 32,223 plant species. For each pair of *M. truncatula* – neighbor species, we calculated the patristic distance, i.e. the distance between the two species as the path-length distance along the branches of the tree from one species to another. The distribution of phylogenetic distances across the 15 species included in our dataset reflects partly the strong dichotomy in phylogenetic distance between monocots and dicots, while phylogenetic distances among the four dicots families are more closely related.

Functional distances were calculated using traits measured on single individuals (one plant per pot and 10 replicates) grown in the same experimental conditions as the previous experiment and also lasting 10 weeks. These traits were measured in the 16 species (*M. truncatula* + 15 matrix species) that were grown in mycorrhizal conditions. We selected eight traits related to growth and resource uptake to characterize the plant's functional niche. These traits characterised the whole individual plant (Total biomass, Root:Shoot), aboveground organs (Height, Specific Leaf Area, Leaf dry matter content) and belowground organs (Length of the primary root axis, Root dry mass, Root dry matter content) (Table S1). Traits were measured using the standard protocols of Cornellissen et al. (2003). Species differed widely in their traits (Table S2). We calculated two functional distances, one based on the whole set of eight traits that reflected the general plant resource use strategy; one only focused on traits linked with the root system (Root:Shoot, length of the primary root axis, root dry mass and root dry matter content, Table S1). These functional distances were measured by calculating the Euclidean distance between *M. truncatula* and the other species in the multidimensional space formed by the traits (one axis per trait). Traits were standardized to give similar weight to each trait in the multi-dimensional space (Villéger et al., 2008). Phylogenetic and functional distances are shown in Figure S1. Functional distances based on belowground traits were partly coinciding with taxonomic families, especially for the *Rosaceae* species.

Molecular analysis of AMF assemblages

Root-DNA extraction

DNA was extracted from roots sampled from both *M. truncatula* and neighbor species (16 treatments including 15 mixtures and the control, *M. truncatula* alone, 10 replicates). DNA

was extracted from roots from 306 samples instead of 310 because four samples had insufficient roots (the 4 samples with insufficient root mass were not related to a given treatment as they included one *M. truncatula* sample grown respectively with *B. perennis*, *P. pratense*, *S. minor* and *T. repens*). Roots for each sample were collected on the focal plant (*M. truncatula* sample), and by mixing 3 neighboring individuals picked at random among the six in the pot (Neighbor plant sample). Roots were separated from the above-ground part and washed. A subsample of 100mg (fresh weight) was then selected to be representative of the whole rooting system and cut into small pieces. Root surface was cleaned in a 2% Triton X100 solution for 5 min then twice with tap water and finally rinsed with sterile distilled water. After the roots were ground into powder, total root DNA was extracted using a standard protocol with magnetic bead (Sbeadex mini plant kit, LGC Genomics) and using an automated protocol (oKtopure robot platform, LGC Genomics) at Gentyane platform (<http://gentyane.clermont.inra.fr/>). DNA concentrations were normalized to 12.5 ng/ μ L after a fluorimetric Hoechst DNA assay.

Preparation of amplicons for mass sequencing

A first PCR amplification of a 540 bp fragment of the 18S rRNA gene was performed using a specific primer set for targeting Glomeromycetes, AML2 (5'-GAACCCAAACACTTTGGTTTCC) and NS31 (5'-TTGGAGGGCAAGTCTGGTGCC) (Lee et al., 2008). On 5' the 2 primers contained the TAKARA adaptors needed to perform multiplexing. PCRs were performed with Illustra™ PuReTaq Ready-to-go (GE Healthcare®) within a final volume of 25 μ L using 0.2 μ M of primers and 25 ng of extracted DNA. Amplicons were purified using AMPure XP (Beckman Coulter) using an automated protocol on a BRAVO Automated Liquid Handling Platform (Agilent®). Purified amplicons were normalized to the same concentration after quantification (Quant-iT™ PicoGreen™ dsDNA Assay Kit) (Thermo Fisher Scientific). On each amplicon, a second PCR was performed from the adaptors to add multiplex identifiers (Takara) using a SmartChip instrument (Takara). After an initial denaturation step at 97°C for 3 min, the cycling regime was 8 denaturation cycles (97°C for 30 sec), hybridization (55°C for 30 sec) and elongation (72.4°C for 15 sec). The last elongation step at 72.4°C for 5 min was followed by a final step at 15°C for 1 min. After a final AMPure purification and final preparation of the amplicon library, pair-end sequencing 2x250 (Illumina®, Miseq) was performed. The sequences have been uploaded at European Nucleotides Archive ENA (<http://www.ebi.ac.uk/ena>) under accession number ERP119552.

Sequence clustering and construction of a contingency matrix

Sequence data were analyzed using FROGS (Escudié et al., 2017) under a Galaxy instance. In FROGS, the sequence-cluster delineation was performed using SWARM (Mahé et al., 2014), which has the advantage of not needing a cut-off threshold. It is thus not a standard OTU delineation but rather sequence-clusters, meaning the method is closer to amplicon-sequence variants (Callahan et al., 2017). To limit the bias of existing artificial sequence-clusters, a sequence was considered as true if 100% identical sequences were found in at least five different samples and at least two sequences per sample, i.e. a minimum of 10 identical sequences. The most central sequence in the delineated cluster was then compared to the Silva132 18SrRNA gene database (Quast et al., 2012) to associate the sequence with taxonomic information. The contingency matrix was prepared by counting the reads of each sequence-cluster in each sample. After checking the abundance curves for each sample, a normalization to 1000 sequences per sample was performed as we rapidly observed sampling saturation (Figure S2), leading to the contingency matrix.

AMF diversity within the host plant and its neighbors

Based on this matrix, we calculated the mycorrhization status for each sample, whether it was mycorrhized (having AMF sequence-clusters) or non-mycorrhized (having no AMF sequence-cluster). We also calculated AMF sequence-cluster richness at two different scales, the total number of sequence-clusters present considering a given treatment (i.e. grouping of the 10 replicates of *M. truncatula*; γ -diversity), and at the replicate scale (i.e. a given individual *M. truncatula*; α -diversity). To assess the relative abundance of sequence-clusters within the assemblage, we measured evenness based on the Smith and Wilson index (Smith & Wilson, 1996). This index is independent of species richness and can handle a wide range of variations in species abundance values, such as those typical of microorganism data (Heip et al., 1998). The similarity between *M. truncatula* and the neighboring plant AMF (β -diversity) was measured as the number of sequence-clusters shared both by the focal plant individual and the neighboring individual plant and the proportion of shared sequence-clusters (Koleff et al., 2003).

Statistical analyses

We described the differences among AMF assemblage composition among the different treatments for the *M. truncatula* samples and for the neighboring plant samples using db-

RDA analysis and permanova permutation tests. These analyses were used to compare the beta diversity of the AMF assemblages among samples based on the Bray–Curtis distance matrix after an Hellinger transformation of the data. We analyzed the effect of the taxonomic family on AMF assemblages using a mixed model with family as a fixed effect and species as a random factor. The model used was a linear mixed model for the evenness index. We used a generalized mixed model for three variables: the presence/absence of detected fungal DNA in sample (Binomial family type, binary data), sequence-cluster richness and sequence-cluster number shared between the focal and matrix plant species (Poisson family type, count data) (Tables 1, 2 and 3). In all the models, we included *M. truncatula* root mass as a covariable to account for a possible effect of the amount of roots available for colonization by AMF (AMF response variable ~ family*root mass; species as a random factor). Root mass indicated the degree of depletion of mass production due to competitive interactions with the matrix plant species. We tested for significant differences among families using least square means.

To test for a predictive effect of phylogenetic or functional distances on AMF assemblages, we used both linear and generalized models; and used *M. truncatula* root mass as a covariable (AMF response variable ~ Phylogenetic or Functional distance*root mass). To test for variable collinearity, we checked beforehand that these distances were not strongly correlated using Pearson correlation tests. Phylogenetic distance was independent of functional distance based on the whole set of traits ($r=0.02$, $p\text{-value}=0.87$) and weakly correlated to functional distance based on belowground traits ($r=0.21$, $p\text{-value}=0.04$). Both functional distances were independent ($r=0.04$, $p\text{-value}=0.72$). For all models, we checked the normality of residuals visually. We tested model significance with ANOVA type II. Finally, we used canonical correspondence analyses (CCA) (ter Braak, 1987) to investigate whether the fungal sequence-cluster composition depended on phylogenetic or functional distances. We coupled the matrix of cluster-sequences composition in presence/absence under the constraint of each of the three distances. The significance of the effect of phylogenetic or functional distances in the CCA structure was tested using ANOVA-like permutation tests. Statistical analyses were performed using 3.4.4. version of R software with the packages *car* (Fox & Weisberg, 2011), *lme4* (Bates *et al.*, 2015), *lmer* (Kuznetsova, 2017), *lsmeans* (Lenth, 2016), *vegan* 2.5-6 (Oksanen, 2019) and *microbiome* (Lathi *et al.*, 2017).

Results

Description of fungal assemblages associated with M. truncatula

We detected 148 sequence-clusters of Glomeromycetes in total, with 105 sequence-clusters in *M. truncatula* roots and 124 in neighboring plant roots. It included Archaeosporales, Diversisporales and Glomerales (Figure 1). Eighty-one sequence clusters were common to the fungal pool of matrix plant species and *M. truncatula*, while 24 sequence-clusters were only detected in *M. truncatula* plants. 43 sequence clusters were only detected in the matrix plant species with respectively 3 only in *T. pratensis*, 2 in *M. lupulina*, 0 in *L. corniculatus*, 1 in *B. perennis*, 4 in *A. millefolia*, 3 in *L. hispidus*, 3 in *S. minor*, 2 in *P. erecta*, 2 in *F. vesca*, 2 in *D. glomerata*, 2 in *H. lanatus* and 0 in *P. pratensis*. Six out of the 105 sequence clusters detected in *M. truncatula* (~6% of sequence clusters) were present in all treatments in at least one replicate, and also in the neighboring plant roots. We did not detect any fungus on *M. truncatula* grown alone (control treatment) or grown with *Brassicaceae* species (*Brassica nigra*, *Barbarea vulgaris*, *Capsella bursa-pastoris*). The absence of AMF DNA sequence cluster detected in the negative control treatment confirmed the absence of contamination from aerial spores. Neighbor plant species corresponded to AMF pools described in Figure S3. Fungal composition among samples differed among neighboring plants, but also among *M. truncatula* individuals grown in the different treatments (Figure 2). The pool of sequence clusters detected in *M. truncatula* comprised a minimum of 16 sequence clusters for the treatment with *Phleum pratensis* to a maximum of 48 sequence clusters for the treatment with *Fragaria vesca* (Figure 3). *M. truncatula* produced the maximum biomass when grown alone (no competition stress) and the minimum biomass when grown with *Holcus lanatus* (Figure 3).

Effect of the neighboring plant taxonomic family on AMF assemblages in Medicago truncatula roots

The number of mycorrhized samples and the richness of the AMF sequence-clusters depended on the taxonomic family of the neighboring plant at both the γ and α scales (Table 1). The number of mycorrhized *M. truncatula* ranged from no when growing with *Brassicaceae* to more than 90% of the replicates in treatments with *Fabaceae*, *Asteaceae* and *Rosaceae* (Figure 3). The number of mycorrhized *M. truncatula* seemed not to be related to the richness of sequence-cluster. For instance, when only considering mycorrhized plants, we observed that the total richness of sequence-cluster (γ diversity) was lower in *M. truncatula*

growing with *Fabaceae* and *Poaceae* than with *Rosaceae* (Figure 4). Results for mean AMF sequence-cluster richness (α -diversity) was quite similar, though there was a significant interaction effect with root mass (Table 1). Across the results, we detected no effect of the plant taxonomic family on the evenness of AMF assemblages of *M. truncatula*. Thus, only AMF community composition and not the relative abundance among species was influenced by the neighboring plants. The number of AMF common to the individual focal plants and the neighboring plant species (β -diversity) differed between the taxonomic family of the matrix species, with *Fabaceae* species having the lowest value, and *Asteraceae* the highest, while the proportion of shared sequence-clusters was independent from these factors (Table 1; Figure 4).

Effect of phylogenetic distance between focal and matrix species on AMF assemblages of M. truncatula

The number of mycorrhized *M. truncatula* individuals decreased with increasing phylogenetic distance to the neighboring plant species (Table 2). AMF community richness depended on root biomass and on its interaction with phylogenetic distance (Table 2). AMF community richness decreased with phylogenetic distance in *M. truncatula* individuals with the smallest root dry mass while it increased in individuals with higher root dry mass. Evenness and the number of sequence clusters shared by both focal and matrix plant species did not depend on their phylogenetic distance, while in proportion it varied in interaction with root mass. The composition of AMF at the treatment and replicate scales (γ and α -scale) was independent of the phylogenetic distance (Table 4)

Effect of functional distance on AMF assemblages in M. truncatula roots

The number of mycorrhized *M. truncatula* individuals increased with increasing functional distance to the neighboring plant species whatever the distance used (above and belowground traits; or belowground traits only; Table 3). All the variables that characterized the structure of *M. truncatula* microbiota were independent of the functional distance measured for the whole set of traits, or on its interaction with root mass. However, we detected an increase in mean sequence-cluster richness in plants with more root mass. Conversely, the functional distance calculated based on the belowground traits influenced the total and mean individual

AMF richness (γ and α -diversity). The total AMF community richness increased with increasing functional distance, while the mean individual sequence-cluster richness increased only in individuals with intermediate and high root mass. The number of sequence-clusters shared between the focal and the matrix plant species but not their proportion, increased with the functional distance. The AMF community composition in *M. truncatula* roots was dependent on functional distances at the replicate scale, but with a very low explained variance (Table 4).

Discussion

Variability in AMF assemblages in M. truncatula

M. truncatula AMF assemblages differed widely between individuals. We have demonstrated that the neighboring plants modified the pool of AMF recruited by *M. truncatula*, especially in terms of richness while evenness remained similar among treatments. This enrichment of the AMF pool to be recruited through neighboring plants overrides *M. truncatula* host preference in shaping the fungal assemblages. The pool of AMF detected in *M. truncatula* individuals was not totally nested in the pool of AMF detected in the matrix plant species. This cannot be due to initial colonization of AMF in *M. truncatula* seeds used for setting up the experiment. The absence of cluster sequences detected in the negative control treatment indeed suggests that the presence of sequence clusters in seeds previous to germination is unlikely as are other possible sources of contamination. This apparent non-fully nested AMF community may be due to the limitation of the sequencing process to detect sequence-clusters that could be at a very low abundance in the plant. It can also be due to slight differences in successional patterns between AMF communities inhabiting both plant individuals. The results we observed should then be interpreted in the timeline of the experiment, which corresponds to young developmental stages. In *M. truncatula*, microbiota composition changes during plant development, and especially between vegetative and reproductive stages, the transition stage being when symbiotic associations are at their highest level (Mougel et al., 2006).

Effect of neighboring matrix plants on AMF assemblages in M. truncatula

AMF assemblages in *M. truncatula* differed depending on the taxonomic identity of the neighboring plants. The matrix plant species inoculated the focal plant species either by direct contact between their roots while growing, or by enriching the substrate in AMF that were subsequently recruited by *M. truncatula* roots. Neighboring plants belonging to *Brassicaceae* species or to a lesser extent to some *Poaceae* species such as *H. lanatus* or *P. pratense* did not supply AMF to *M. truncatula*. This effect was indeed only detected in part of the samples for the *Poaceae* treatments. *Brassicaceae* species are known to be very low mycotrophic plants. Evidence has been produced that *Brassicaceae* can produce secondary compounds that negatively affect mycorrhizal infectivity potential, hyphal growth and spore germination (Lankau et al., 2011) leading to mycorrhizal degradation (Pakpour & Klironomos, 2015), a way for this plant family to outcompete and colonize habitats. Similarly, in a mesocosm experiment testing grassland mixtures, Bittebiere et al. (2020) demonstrated that the occurrence of *H. lanatus* in the close neighborhood had a negative effect on AMF richness in *B. pinnatum*. The mechanisms underlying this negative effect is not yet known but might provide an advantage to *H. lanatus* to maintain itself and compete with its neighbors.

The treatments with the *Rosaceae* species were related to the highest values of AMF richness of the focal *M. truncatula* both at the treatment scale and at the individual pool scales while *Poaceae* species were related to lower values. By providing a diverse AMF pool from which *M. truncatula* recruited, *Rosaceae* species might improve the probability of having beneficial AMF and provide wider functional diversity for plant growth and resistance to different kinds of stress (Vannier et al., 2015). Higher similarity, due essentially to the enhancement of AMF community diversity in *M. truncatula* when *Rosaceae* or *Asteraceae* species are neighbor also suggests that these plant species may be moderately 'selective' in the AMF pool they recruit, in contrast to *Fabaceae* species that would more strongly select fungal partners.

Effect of phylogenetic and functional distances between plants on AMF assemblages in M. truncatula

The *M. truncatula* AMF assemblage was dependent on the phylogenetic distance only for the richness of sequence-cluster and the proportion of shared sequence cluster (in interaction with plant mass). Contrary to our hypothesis, the occurrence of phylogenetically distant plants in the neighborhood increased the AMF sequence-cluster richness in *M. truncatula* roots for

individuals with intermediate or high root mass. If plants reward and enrol particular AMF based on their level of cooperation (Kiers et al., 2011) or because of their particular functions, this selective rewarding would be expected to lead to AMF filtration and exclusion (Duhamel & Vandenkoornhuysse, 2013). This demonstrated plant rewarding process to AMF (Kiers et al., 2011) could thus be independent of the phylogenetic proximity among plants but ruled by AMF functions and efficiency. These results need however to be confirmed over larger datasets including more variable phylogenetic distances.

The composition, richness and similarity of *M. truncatula* AMF assemblage with its neighbor plant were explained by *M. truncatula* functional proximity to the neighboring plants. The very low explanation of the functional distances compared with sequence-cluster composition suggests that this neighboring plant effect does not cause a species shift in the assemblages associated with the focal plant species but rather affects the richness of the pool recruited, acting mostly on rare species. This enrichment was demonstrated but in the opposite way to our working hypothesis, since AMF assemblages in *M. truncatula* were more similar when this neighboring plant was functionally dissimilar from the focal plant. Except for sequence-cluster composition, the effect of functional proximity on the other response variables was only detected when the functional nutrient-use and uptake strategy were taken into account, rather than based on a more integrated plant growth growing strategy. This may be due to the important and primary involvement of AMFs in plant nutrition, especially in the particular case of young colonization stages. Because *M. truncatula* displayed a low root:shoot ratio, with a long primary root and low root water content, plant neighbors with a higher investment in roots and with shorter primary axis, and hence likely to have several dense lateral roots, improved *M. truncatula* microbiota richness and similarity with the neighboring plants. The observed results could be interpreted as a change in the probability of root contacts between *M. truncatula* and the other plants. It can be also an increase in the number of niches to colonize by AMF when rooting systems differ and correspond to different types of roots morphologies. The enrichment of AMF assemblages in treatments with *Rosaceae* species that display similar functional aboveground traits might be related to such effect. Further work is required to deepen this understanding, for instance by integrating in the description of the belowground functional niche of plants using traits that characterize the architecture and morphology of roots more precisely (Eissenstat et al., 2015).

Understanding and predicting the effects of plant-plant interactions on AMF assemblages

Analyzing and predicting microbiota variability for a given host plant is a key issue in microbial ecology because of the consequences of these microbiota for plant nutrition and health. Some authors argue for a host-plant preference effect leading to particular AMF composition among species (e.g. Vandenkoornhuyse et al., 2002; 2003; Gollotte et al., 2005), while others demonstrated that most variability in microbiota composition is linked with other factors such as environmental conditions, genotypic variability, or even randomness (Bahram et al., 2015; Powell & Bennett, 2016). Using the mycotrophic *M. truncatula*, we experimentally demonstrated for the first time that the composition of root mycobiota of a focal plant is under the influence of its neighboring plants. In this particular situation where focal plants was under the only influence of the neighboring plant species, we have shown that a AMF community fraction was dependent on the identity of the neighboring plant, and especially on its functional proximity to the focal plants in terms of belowground resource use and uptake strategy. Because of the experimental design in which germinated *M. truncatula* seeds were added after neighboring plants had grown, these results help understand microbiota assembly at the colonization stage, when the plant arrives in a community through seed dispersal and during the first steps of development. In these early developmental stages, the “priority effect” due to the order of arrival of AMF species and niche-root occupancy on subsequent AMF assemblages, should be important (Werner & Kiers, 2015). Complementary studies are needed of more advanced plant development stages to confirm the neighboring effect on later stages of the AMF successions. In addition, to focus on the analysis of the neighboring plant effect, the present experiment was done in simplified culture conditions, where substrate was initially sterile, and focal plant grown with one species of neighboring plants. To be closer to field situations, further steps should be conducted using real soil and multispecies mixtures.

At the broader scale of a plant community, the present work suggests a new understanding of the importance of plant spatial patterns in plant coexistence. The demonstrated dependency of the AMF community composition on the identity of the neighboring plants can also explain the apparent heterogeneity of a focal plant microbiota in a diverse floristic neighborhood (Bittebiere et al., 2020). Heterogeneous distribution of plants that are functionally different has been put forward as an important driver for maintaining species diversity in a community because it affects the probability of encountering species with different competitive ability (i.e. spatial niche complementarity, Chesson, 2000). Our

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results suggest extending this niche partitioning effect in space by providing insights into how functionally divergent pairs of plants may enrich AMF assemblages in plants, likely impacting their performance. Beyond the theoretical aspect of a better understanding of the rules governing AMF assemblages in plants, the existence of a niche-partitioning mediated by microorganisms, if validated in real field conditions, might help in determining possible plant species mixtures to increase the microbiota pool in the soil to support the soil fertility ecological service (Guo et al., 2020), with a wide range of future applications in agriculture.

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Table 1: Summary of the models used to test the effect of the taxonomic family (4 modalities) over M. truncatula variables. Dependent variables tested were the number of mycorrhized samples per treatment (i.e. over 10 replicates), fungal sequence-cluster richness at the scale of the treatment (10 replicates); or at the replicate scale, evenness, number and proportion of sequence-cluster shared by both M. truncatula and the neighboring plants calculated over the M. truncatula AMF assemblages. Model parameters provided are the model type (GLMM: generalized linear mixed model, GLM: generalized linear model, LMM: linear mixed model), the type of distribution (Binomial, Poisson or Gaussian), the estimated coefficient (Est.) and the significance (Xhi-2 or F-test and related p-values) for the independent variables. Independent variables include root mass and its interaction with the taxonomic family in all models except those that test effects at the treatment scale. Significant models are in bold.

	Model type	Distribution type	Family		Root mass		Family*Root mass	
			Xhi-2/ F	p-value	Xhi-2/ F	p-value	Xhi-2/ F	p-value
<i>Treatment scale</i>								
N° of samples with AMF sequences	GLMM	Binomial	13.47	0.009				
Sequence-cluster total richness	GLM	Poisson	21.47	8x10⁻⁵				
<i>Replicate scale</i>								
Sequence-cluster richness	GLMM	Poisson	2.08	0.0001	1.47	0.23	11.18	0.01
Evenness	LMM	Gaussian	2.24	0.52	0.30	0.59	0.51	0.58
N° of shared sequence-clusters	GLMM	Poisson	14.33	0.002	0.84	0.36	1.12	0.77
Proportion of shared sequence-clusters	LMM	Gaussian	2.51	0.47	0.18	0.68	6.99	0.07

Table 2: Summary of the models used to test the effect of the phylogenetic distance between *M. truncatula* and the matrix species over *M. truncatula* variables characterizing its AMF assemblages. Dependent variables tested were the number of samples with detected sequences of AMFs over the treatment (i.e. over 10 replicates), sequence-cluster richness at the scale of the treatment (10 replicates); or at the individual scale, evenness, number and proportion of sequence-cluster shared by both *M. truncatula* and the matrix plants calculated over the *M. truncatula* AMF assemblages. Model parameters provided are the type of model (GLM: generalized linear model, LM: linear model), the type of distribution (binomial, Poisson or Gaussian), the estimated coefficient (Est.) and significance (Chi-2 or F-test and related p-values) for the independent variables. Independent variables include root mass and its interaction with the phylogenetic distance in all models except those testing effects at the treatment scale. Significant models are in bold.

	Model type	Distribution type	Int.	Phylogenetic distance			Root mass			Phyl. Dist.*Root mass		
				Est.	Chi-2/ F	P-value	Est.	Chi-2/ F	P-value	Est.	Chi-2/ F	P-value
<i>Treatment scale</i>												
N° of samples with AMF sequences	GLM	Binomial	2.16	-0.006	11.53	0.0007						
Total sequence-cluster richness	GLM	Poisson	3.46	-0.0003	0.44	0.51						
<i>Replicate scale</i>												
Sequence-cluster richness	GLM	Poisson	2.30	-0.002	3.65	0.06	-12.2	7.44	0.015	0.08	13.19	0.0003
Evenness	LM	Gaussian	0.21	-0.0002	1.27	0.26	-0.19	0.25	0.62	-0.002	0.02	0.89

N° of shared sequence-clusters	GLM	Poisson	1.61	-2.69	1.44	0.23	-3.99	0.02	0.87	0.021	0.52	0.47
Proportion of shared sequence-clusters	LM	Gaussian	0.50	0.0007	1.97	0.16	7.60	0.06	0.80	-0.04	6.91	0.01

*Table 3: Summary of the models used to test the effect of the functional distance between *M. truncatula* and the matrix species over *M. truncatula* variables characterizing AMF assemblages. Two functional distances (Funct. Distance) were calculated for both the set of above and belowground functional traits and for the set of belowground functional traits only. Dependent variables tested were the number of samples with detected sequences of AMFs over the treatment (i.e. over 10 replicates), sequence-cluster richness at the scale of the treatment (10 replicates); or at the individual scale, evenness, number and percentage of sequence-cluster shared by both *M. truncatula* and the matrix plants calculated over the *M. truncatula*'s AMF assemblages. Model parameters provided are the type of model (GLM: generalized linear model, LM: linear model), the distribution type (binomial, Poisson or Gaussian), the estimated coefficient (Est.) and significance (Xhi-2 or F-test and related p-values) for the independent variables. Independent variables include root mass and its interaction with the phylogenetic distance in all models except those that test effects at the treatment scale. Significant models are in bold.*

	Model type	Distribution type	Int.	Functional distance			Root mass			Funct. Dist.*Root mass		
				Est.	Xhi-2/ F	P-value	Est.	Xhi-2/ F	P-value	Est.	Xhi-2/ F	P-value
Funct. Distance (above and belowground)												
<i>Treatment scale</i>												
N° of samples with detected AMF sequences	GLM	Binomial	-0.09	1.9*10⁻⁶	7.70	0.006						
Sequence cluster total richness	GLM	Poisson	3.46	-0.0003	0.44	0.51						

Replicate scale

Sequence cluster richness	GLM	Poisson	2.13	-2.5×10^{-7}	0.51	0.47	1.32	6.23	0.01	5.4×10^{-6}	0.47	0.49
Evenness	LM	Gaussian	0.19	-4.6×10^{-9}	0.99	0.32	-1.93	0.12	0.73	2.8×10^{-6}	0.01	0.54
N° of shared sequence clusters	GLM	Poisson	1.55	5.4×10^{-8}	2.92	0.09	7.35	0.02	0.89	-1.3×10^{-5}	1.39	0.24
Proportion of shared sequence clusters	LM	Gaussian	0.65	-7.3×10^{-9}	0.02	0.90	-0.64	0.03	0.86	7.1×10^{-7}	0.02	0.90

Funct. Distance (Belowground)*Treatment scale*

N° samples with detected AMF sequences	GLM	Binomial	-0.09	0.16	8.39	0.004						
Sequence cluster total richness	GLM	Poisson	3.08	0.047	12.79	0.0003						

Replicate scale

Sequence cluster	GLM	Poisson	2.13	-0.01	9.30	0.002	-5.51	1.76	0.18	1.02	5.02	0.02
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richness

Evenness	LM	Gaussian	0.19	-0.002	0.47	0.49	0.24	0.01	0.92	-0.049	0.03	0.86
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N° of shared	GLM	Poisson	1.32	0.05	6.70	0.01	2.64	0.56	0.45	-0.59	0.90	0.34
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sequence clusters

Proportion of	LM	Gaussian	0.53	0.02	0.09	0.76	3.79	0.006	0.94	-0.53	2.62	0.11
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shared sequence

clusters

Table 4: The effect of the plant phylogenetic and functional distances (whole: based on the whole set of functional traits, belowground: based on the root functional traits) on the fungal microbiota inhabiting M. truncatula. Percentages of explained deviance (Expl. Dev.), F-ratio and significances were calculated using ANOVA-like permutation tests with canonical correspondence analysis (CCA). CCA were performed on the sequence-cluster matrix (Presence/Absence) and in sequence relative abundance among sequence-clusters. Values are provided for each CCA.

	Presence/Absence			Sequence relative abundance		
	Expl. Dev.	F	p-value	Expl. Dev.	F	p-value
<i>Treatment scale</i>						
Phylogenetic distance	0.11	1.21	0.08	0.12	1.32	0.25
Funct. Dist. (whole)	0.09	0.96	0.58	0.07	0.76	0.63
Funct. Dist. (belowground)	0.11	1.20	0.11	0.06	0.90	0.84
<i>Replicate scale</i>						
Phylogenetic distance	0.013	1.23	0.06	0.016	1.53	0.06
Funct. Dist. (whole)	0.015	1.46	0.02	0.009	0.90	0.57
Funct. Dist. (belowground)	0.014	1.37	0.01	0.007	0.72	0.82

Fig. 1: Community description at the order, family and genus (for Glomeraceae) scales (Percentage of sequences and Number of sequence clusters) in *M. truncatula* individuals.

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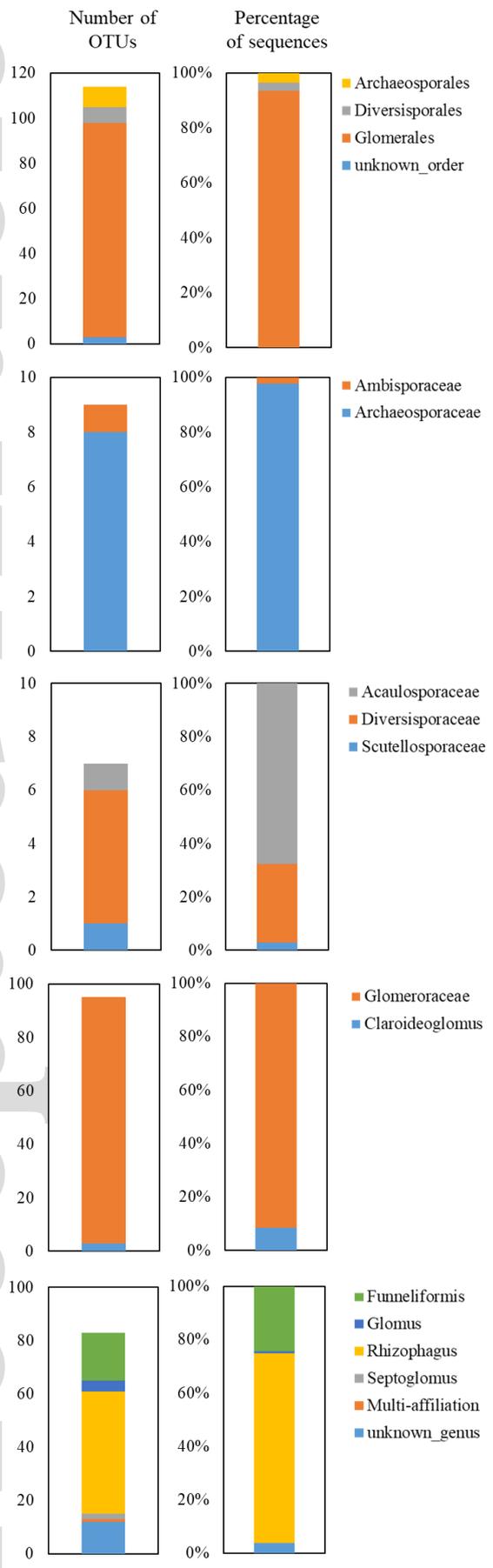


Fig. 2: Distance-based redundancy analysis (db-RDA) plot. Analysis was done on the Bray-Curtis distance of the AMF assemblages after Hellinger transformation. A.) Analysis on focal plants depending on the treatment of neighbor plant *M. trunculata* was grown with; b.) Analysis on neighbor plants. Results from the permutation test testing the effect of the treatment are described on the plot (F-value, *: $p < 0.05$; ***: $p < 0.001$; p-values into brackets) TPRA: *Trifolium pratense*; MLUP: *Medicago lupulina*; LCOR: *Lotus corniculatus*; BPER: *Bellis perennis*; AMIL: *Achillea millefolium*; LHIS: *Lotus hispidus*; SMIN: *Sanguisorba minor*; PERE: *Potentilla erecta*; FVES: *Fragaria vesca*; BNIG: *Brassica nigra*; CBUR: *Capsella bursa-pastoris*; BVUL: *Barbarea vulgaris*; DGLO: *Dactylis glomerata*; HLAN: *Holcus lanatus*; PPRA; *Phleum pratense*.

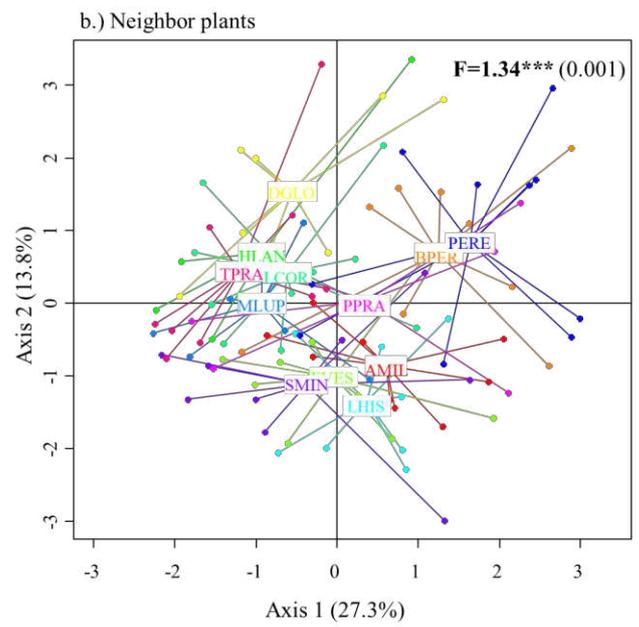
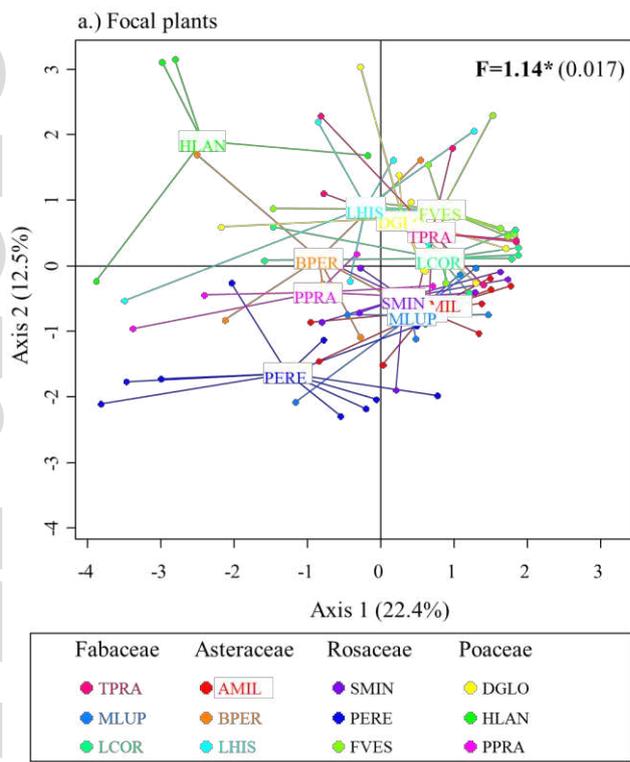


Fig. 3: Total dry mass, total number of mycorrhized samples and total number of sequence-clusters of *M. truncatula* individuals in each treatment (15 matrix species). We added the treatment *without neighboring plant* (“without”) in the upper graph. No AMF sequences were detected for the plants grown alone and for the treatments with Brassicaceae species. TPRA: *Trifolium pratense*; MLUP: *Medicago lupulina*; LCOR: *Lotus corniculatus*; BPER: *Bellis perennis*; AMIL: *Achillea millefolium*; LHIS: *Lotus hispidus*; SMIN: *Sanguisorba minor*; PERE: *Potentilla erecta*; FVES: *Fragaria vesca*; BNIG: *Brassica nigra*; CBUR: *Capsella bursa-pastoris*; BVUL: *Barbarea vulgaris*; DGLO: *Dactylis glomerata*; HLAN: *Holcus lanatus*; PPRA; *Phleum pratense*.

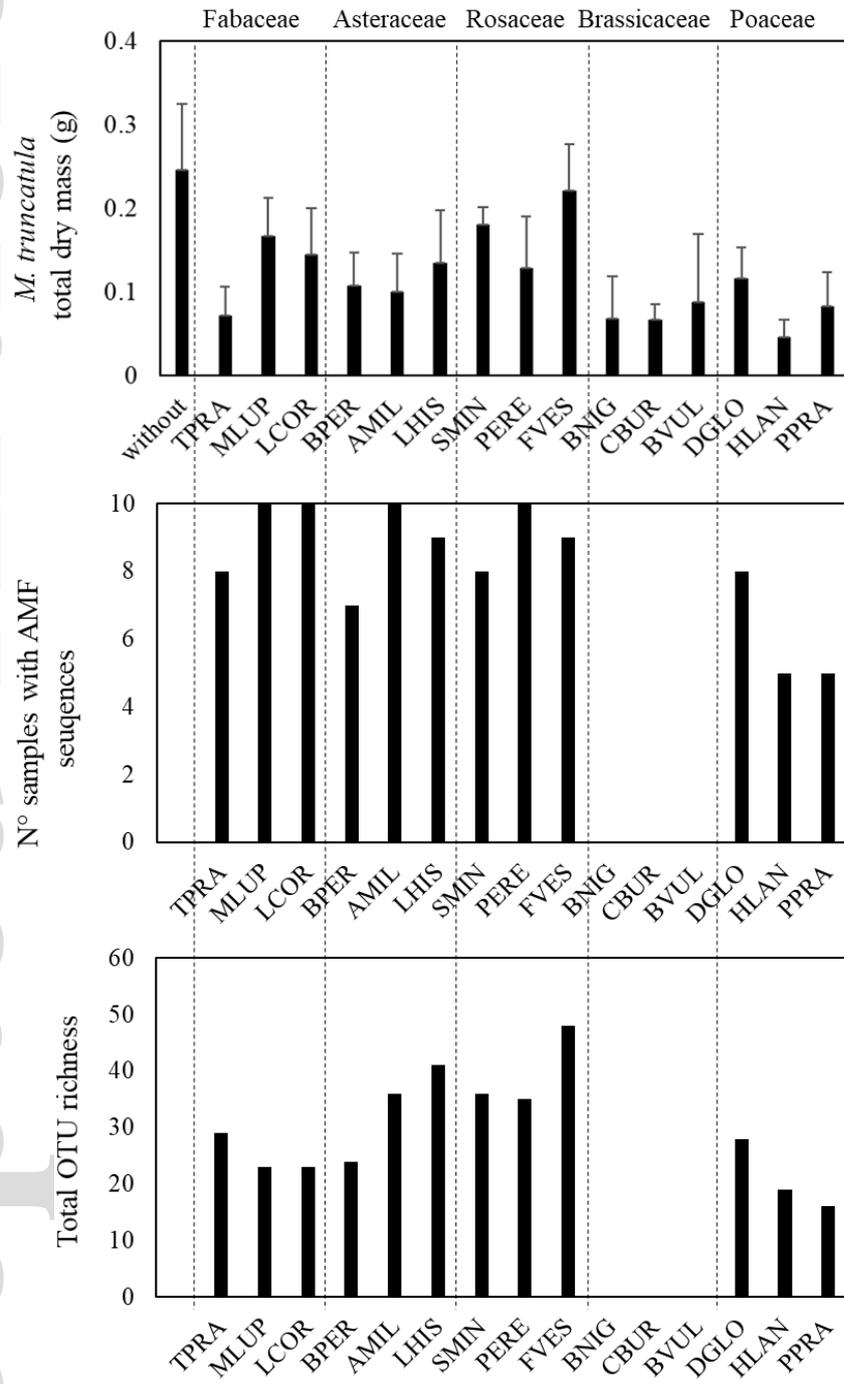


Fig. 4: Effect of the taxonomic family of the neighboring plants on AMF assemblages on *M. truncatula* individuals. Small letters indicate significant differences (Tukey post-hoc test based on lsmeans, see Statistical Analysis methods for details on the models used). Tukey post-hoc test for sequence-cluster richness was done fixing root mass at the mean value.

