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Arbuscular mycorrhizal inoculation and plant response strongly shape bacterial and eukaryotic soil community trajectories

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi and terrestrial plants form one of the most important and ubiquitous symbioses on the planet. Although the central role of AM fungi in rhizosphere processes is well established, the extent of their influence on the development of the whole soil microbial community is less well characterized. We assessed the temporal dynamics of the bacterial and eukaryotic soil microbial communities in mesocosms where AM fungi were inoculated on a grass (*Holcus lanatus* L.) and a clover (*Trifolium pratense* L.). This allowed us to evaluate whether 1) inoculation with AM fungi changes the overall structure of soil communities and 2) if these changes are partially mediated by the altered plant phenotype, as indicated by increases in growth and photosynthetic activity. We observed changes in the community composition of both microbial groups, largely associated with relative decreases in Proteobacteria, Nematoda and some protistan groups (Ciliophora and Lobosa) and relative increases in Gastrotricha and Firmicutes. Plant productivity doubled with mycorrhizal inoculation, while other plant performance measures increased by 10–50%. We conclude that the interaction between AM fungi and plants shapes the surrounding soil microbial communities, probably because enhanced growth of host plants changes the amount, timing, and form of carbon inputs into soil.

1. Introduction

The symbiosis with arbuscular mycorrhizal (AM) fungi is one of the most ancient and prevalent associations of land plants; it is estimated that around 250,000 plant species worldwide are capable of forming this symbiosis (Smith and Read, 2010). For AM fungi the symbiosis is obligate, as they require carbohydrates and lipids from their host plant, for which they provide essential nutrients in return (Keymer et al., 2017; Lekberg et al., 2018). They also influence their hosts via hormone pathways that improve plant health by providing protection against pathogens (Barea, 1996; Torelli et al., 2000). It is estimated that plants colonized with AM fungi invest around 2–20% of their annual photosynthate production into their fungal partners (Read et al., 2002). This influx of carbon and energy into the soil then becomes available to other soil organisms that either directly associate with AM fungi and/or host plants, or indirectly through carbon and nutrient cycling in the "microbial loop" (Koller et al., 2013). In addition to this, plants secrete carbon in the form of exudates that are generally easily degradable and thus a valuable energy source for soil communities. These plant-based carbon inputs into soil drive biological processes (Högberg and Read, 2006) and can have large impacts on soil bacterial (Baudoin et al., 2003; Eilers et al., 2010) and fungal communities (De Graaff et al., 2010). These basal trophic groups are then consumed by other soil organisms, expanding the influence of soil carbon inputs throughout the soil food web (Albers et al., 2006).

Arbuscular mycorrhizal fungi can influence the wider soil community by altering bacterial community composition (Marschner and Baumann, 2003; Rodríguez-Caballero et al., 2017), as well as the composition of other groups such as protists (Henkes et al., 2018), fungi (Trabelsi and Mhamdi, 2013) and nematodes (Veresoglou and Rillig, 2012). However, the influence of AM fungi extends far beyond single pairwise interactions between them and other microbes, as their activity

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also alters many physico-chemical characteristics of the soil environment. For example, AM fungal presence and type are important predictors of soil organic carbon and nitrogen storage in soils (Tatsumi et al., 2020), and are generally found to stimulate nutrient cycling compared to other mycorrhizal types (Craig et al., 2018). The mechanism by which AM fungi influence other soil organisms may be either direct or indirect. Direct influences include competition for space on plant roots (De La Peña et al., 2006), secretion of exudates (Singh, 2012; Toljander et al., 2007) and acting as a suitable habitat (Scheublin et al., 2010), or food source (Caravaca and Ruess, 2014) for other microbes. Indirect effects include changing the surrounding soil structure (Siddiky et al., 2012), and changing the nutritional status of the host plant (Karagiannidis et al., 2002). Despite these well-known influences of AM fungi on several aspects of a soil ecosystem, most studies of community-level effects have focused on single groups of (micro)-organisms or guilds, at a limited number of time-points. Studies that do assess the whole community tend to use non-specific measures such as microbial biomass, enzyme activity or respiration (Wang and Wang, 2018; Xu et al., 2019). Given the relatively fast changes that are commonly reported (e.g. Gao et al. 2019, 2020), along with potential time-delayed effects such as indirect plant-mediated processes and slow growing microbial groups, frequent sampling is necessary in order to fully capture the influence of AM fungi on other soil microbes. It is currently not known how the effects of AM fungi propagate through the entire soil community through time.

To causally tie AM fungi to changes in the surrounding soil community, their presence needs to be controlled in an otherwise identical setting. In this study we present data from a mesocosm experiment that tracks the development of the whole soil microbial community (eukaryotes and bacteria) in the presence (one or three species of AM fungi) or absence of mycorrhizal inoculum under standardized conditions over a 10 week plant growing season whilst simultaneously measuring several soil and plant productivity parameters. This allows us to evaluate the importance of plant- and soil-mediated influence of mycorrhizal fungi, while taking into account the dynamic nature of the plant-soil system.

2. Methodology

2.1. Experimental design

The experiment was conducted during the summer of 2016 in Wilrijk, Belgium (51°09′N, 04°24′E) in a grass field located on the campus of the University of Antwerp. Average annual precipitation at the site is 832 mm, evenly distributed throughout the year, and average summer air temperature is 18 °C (Royal Meteorological Institute Belgium). A sandy soil (pH = 8.4, <0.3% organic C, 5.33 mg N l^{-1}, 206 mg P l^{-1}, 108.5 mg K l^{-1}, 373 mg Mg l^{-1}, 22.25 g Ca l^{-1}, 109 mg Na l^{-1}, bulk density 1.556 kg l^{-1}), mixed thoroughly to limit heterogeneity and pasteurized at 80 °C with a pro-grow sterilizer model SS-60 (Pro-Grow Supply Corp., Brookfield, Wisconsin, USA) was evenly distributed over 30 PVC mesocosms (Ø 40 cm, 40 cm deep, white to prevent heating, with perforated bottom lids to allow water drainage, elevated 15 cm relative to the underlying soil to prevent root and AM fungal contamination). The soil had a volumetric water content of 0.248 m^3 m^{-3} at field capacity (pF 2), 0.062 m^3 m^{-3} at the point of reduced water availability (pF 3.3), and 0.049 m^3 m^{-3} at wilting point (pF 4.2). All mesocosms were placed in a random arrangement under a rainout shelter (6 m × 9 m x 3 m) that was covered with 0.18 mm thick polyethylene foil attached to a metal frame raised 1 m above the ground surface to allow air circulation but prevent precipitation from entering pots, and excessive heating.

Our mesocosms were planted with two common Belgian AM grassland plant species, *Holcus lanatus* L. and *Trifolium pratense* L. These species were selected because 1) they are common species in Belgian grasslands, 2) they are associated with intermediate soil moisture (Ellenberg et al., 1991) and 3) their confirmed AM status (Wang and Qiu, 2006). Plant seeds (Cruydt Hoeck; Nijeberkoop, Netherlands), were surface-sterilized by agitation in a 4% sodium hypochlorite, 1% Tween 20 solution for 10 min, after which the seeds were thoroughly rinsed with sterile distilled water. Seeds were planted on May 14, day of the year (DOY) 134 in an orthogonal pattern, alternating 1:1 between species and leaving ± 4 cm between seeds, resulting in a total of 68 seeds per mesocosm. Overall initial seedling survival exceeded 90%, and individuals that did not germinate or died within a week after germination were replaced.

Inocula containing either none (nonmycorrhizal mix nr K/32), or one of three mycorrhizal species [*Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot, previously named *Glomus intraradices*) strain nr 22, (Schlaeppi et al., 2016); *Funneliformis mosseae* (T.H.Nicolson & Gerd.) strain nr 11/BEG161 (Mozafar et al., 2002), and *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) strain nr 12/JJ132 (Mozafar et al., 2002),] were obtained via AGROSCOPE (see Supplementary Information for more details). Inoculation treatments of zero, one and three mycorrhizal species were selected to increase the odds of having at least one successful mycorrhizal inoculation on plants and microbes. As a secondary effect, the increase in mycorrhizal species should increase soil hyphal occupation, which could result in differences in plant and microbe responses (Boyer et al., 2015; van der Heijden et al., 1998). Ten mesocosm replicates were used per inoculation treatment.

Inoculum containing no AM fungal species and inoculum containing *R. irregularis* were used as is for the experiment and inoculum containing all 3 AM fungal species was created by mixing each single species AM inoculum in a 1:1:1 ratio. Around 4 cm^3 of inoculum containing either zero, one (*R. irregularis*) or three (*R. irregularis, F. mosseae and C. claroideum*) mycorrhizal species was applied to each mesocosm directly in the planting holes around the seeds to enhance mycorrhizal colonization potential while minimizing the quantity of inoculum needed. It is important to note that the nonmycorrhizal inoculum used was not sterile, as it was grown in exactly the same way as the mycorrhizal inoculum and thus contained a developed microbial community (see Supplementary Information for more details). As an additional way to limit variation in (non-AM fungal) microbial communities that may exist between inocula, a microbial wash was prepared. It consisted of an equal mixture of mesocosm soil prior to pasteurization and each of the mycorrhizal inocula together, filtered through a 10 μm synthetic mesh (Sefar AG, Heiden, Switzerland). This mesh size excludes AM fungal spores (Daniels et al., 1981) but allows passage of most prokaryotes and spores of other fungi. This microbial wash was added to each mesocosm (both nonmycorrhizal and mycorrhizal) shortly after germination of the seeds. All mesocosms were kept at approximately 20% volumetric soil water content (SWC) at 10 cm depth by watering 2–3 times per week with tap water.

2.2. Measurements

Plant available nitrate-N, ammonium-N and phosphate-P were estimated by ion exchange strips inserted into the soil of each mesocosm according to Jasrotia and McSwiney (2009). Stomatal conductance and leaf area were measured per species starting on the 16th of August, [days after planting (DAP 95)] until 27th of September (DAP 137) and mortality (defined as the fraction of dead leaf area) was measured weekly per species starting on the 16th of August, (DAP 95) until the end of the experiment on October 25th (DAP 165), at which point total aboveground biomass per species was also measured and photographs of the mesocosms were taken (Supplementary Fig. S1). Soil water content (SWC) and total green cover were measured weekly per mesocosm starting on the 16th of August, (DAP 95) until the end of the experiment on October 25th (DAP 165). Samples were taken from pasteurized and unpasteurized soil, as well as from each mycorrhizal inoculum and microbial wash and frozen for subsequent microbial analysis. These samples represent the starting point for the soil microbial communities (DAP

0). Weekly soil samples, starting on the 16th of August, (DAP 95) until the end of the experiment on October 25th (DAP 165), were taken for pH measurements and the remainder was frozen for microbial analysis. Each sample was taken in the center of the 4 cm by 4 cm squares between the plants, avoiding the outer rows to limit edge effects. A root sample was taken at the end of the experiment (October 25th DAP 165) for staining and checking for mycorrhizal colonization following Vierheilig et al. (2005). A more detailed description of the methods used is given in the Supplementary Information.

2.3. Library preparation and sequencing

DNA was extracted from approx. 0.25 g of experimental soil or inoculum, or the equivalent in the case of the microbial wash, using the MoBio Powersoil kit according to the manufacturer's protocol (MoBio, Carlsbad, CA, USA). DNA was amplified targeting the hypervariable V4 region of the 18 S rRNA gene and the V3–V4 region of the 16 S rRNA gene for eukaryotes and bacteria, respectively. The general protocol followed De Gruyter et al. (2020), using the same primers as described by Stoeck et al. (2010): TAReuk454FWD1 and TAReukREV3 and Klindworth et al. (2013): S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21. The same plant blocker was also used to limit plant DNA amplification (PNA 5′-GCTCAAAGCAAGC-3′). The pooled library was quantified by qPCR and sequenced on an Illumina Miseq using 2 x 301 cycles paired-end sequencing. To further confirm the presence of our AM fungal species, a subtest was done with ITS specific primers ITS1f (Gardes and Bruns, 1993) and ITS2 (White et al., 1990) as the general eukaryotic primers used lack the specificity to detect different AMF species. For this subtest, two mesocosms were randomly selected from each inoculation treatment (See Supplemental Information).

2.4. Bioinformatics

Forward sequences were trimmed to a length of 200 bp to account for diminishing read quality towards the end of reads and reverse reads were discarded for the same reason. Primer sequences were removed, and reads were quality-filtered allowing for a maximum expected error of 1, leaving 14 M filtered reads. De novo OTUs were generated from forward reads at 97% similarity using the UPARSE algorithm (Edgar, 2013) in USEARCH10 (Edgar, 2010) after de-replication and singleton removal. After chimera removal (leaving 1836 and 14,371 non-chimeric OTUs for eukaryotes and bacteria, respectively) all original reads were mapped to the non-chimeric OTUs using USEARCH10 to create an OTU table.

All eukaryotic OTUs were first aligned to the Protist Ribosomal Reference database version 4.6 (PR2) (Guillou et al., 2013) using the default UCLUST function in QIIME (Caporaso et al., 2010; Edgar, 2010) with an 80% sequence similarity threshold to compensate for potential higher divergence from known sequences. Non-protist OTUs were subsequently aligned to the SILVA rRNA database version 128 (Quast et al., 2013) to increase non-protist coverage, as the PR2 database contains fewer non-protist sequences (Guillou et al., 2013), after which the results from both databases were merged. Eukaryotic OTUs were classified as protists, Fungi or Metazoa based on their best hit against the databases. Bacterial sequences were matched to the SILVA rRNA database using the UCLUST function in QIIME with a 97% similarity threshold. Plant sequences were removed from the dataset.

2.5. Statistical analysis

To avoid library size-related artefacts, OTU tables were rarefied by random subsampling of the original OTU tables, with a rarefaction threshold of 2000 (see Supplementary Figs. S2–S3). A two-lines test (Simonsohn, 2017) was performed for the bacterial richness over time due to an observed unimodal pattern in the data (see Supplementary Fig. S4). Because the significant outcome of this test, a second order term was added in this model. All biotic (leaf area, stomatal conductivity, mortality, biomass and total green cover) and abiotic parameters (soil water content at 10 cm depth, pH, nitrate-N, ammonium-N and phosphate-P) were tested for inoculation treatment effect using a mixed-effects model, with mesocosm identity as random effect. A two-lines test was also performed for *T. pratense* stomatal conductance because of observed unimodality in the data (see Supplementary Fig. S5) and also here a second order term was added.

Nonmetric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities was performed on log transformed OTU counts (Molik et al., 2018; Weiss et al., 2017) to visualize overall patterns in microbial community composition in both samples, inoculum and microbial wash (see Supplementary Figs. S6–S7). Principle coordinate analysis (PCoA) was performed on Bray-Curtis dissimilarity of log transformed OTU counts, after which the first two axes were selected for both eukaryotes and bacteria (explaining 16.4% and 25.2% of the variation of the data respectively). Axis scores were analysed with a mixed effects model, with mesocosm identity as random effect, containing the simultaneously measured plant (stomatal conductivity, mortality and total green cover) and soil (soil water content at 10 cm depth and pH) parameters, as well as time and inoculation treatment. Explicitly modelling both the inoculation treatment together with plant and soil parameters, allows us to estimate the relative contribution of plant-mediated effects of inoculation treatment. This method using ordination axis scores with mixed models was selected as PERMANOVA tests are inappropriate for repeated measure designs. Total green cover was selected as a measure for biomass as this corresponded best with the biomass measured at the end of the experiment. Significance values were calculated using the package LMERTEST version 3.1–1 (Kuznetsova A, Brockhoff PB, 2017) and model simplification was performed retaining only significant parameters (p-value < 0.05). Finally the R-squared of this final model was calculated using the r.squaredGLMM function from the package MuMIn version 1.40.0 (Barton, 2019). To check if the observed divergence in microbial communities was not caused by inherent differences in inoculum, an analysis was performed to identify indicator OTUs of inoculation treatments and crosscheck these OTUs with the inoculum used. This method is described in detail in the Supplementary Information.

OTU reads were aggregated at the phylum level and plotted against inoculation treatment to visualize any influence of mycorrhizal inoculation on relative abundance of bacterial and eukaryotic phyla. In order to test which OTUs responded significantly to mycorrhizal inoculation individually, nonrarefied OTU tables were subjected to a permuted multivariate abundance analysis using the MVABUND package version 4.0.1 (Wang et al., 2012). To account for repeated measurements, we restricted permutations so that observations were contained within individual mesocosms but allowed shuffling of mesocosms between inoculation treatments. The order of measurements was preserved in the randomization so that time effects could also be tested. OTUs with a significant response to inoculation treatment were crosschecked with all OTUs present in inocula and the microbial wash to ensure that observed significance was not due to pure inoculation effects (i.e. for a significant OTU to be retained it had to be present in all inocula or in the microbial wash, or absent from both).

3. Results

3.1. Mycorrhizal influence on microbial community structure and richness

Both bacterial and eukaryotic communities changed through time and were affected by inoculation with AM fungi (Figs. 1 and 2). For eukaryotes, time was by far the most important factor in both the first ($F_{1,88.2} = 66$, $p < 0.0001$) and the second PCoA axis ($F_{1,84.4} = 17$, $p < 0.0001$). In the presence of other independent variables that correlate with inoculation treatment (see "Mycorrhizal influence on plant and soil parameters" below) a pure mycorrhizal inoculation effect was not found

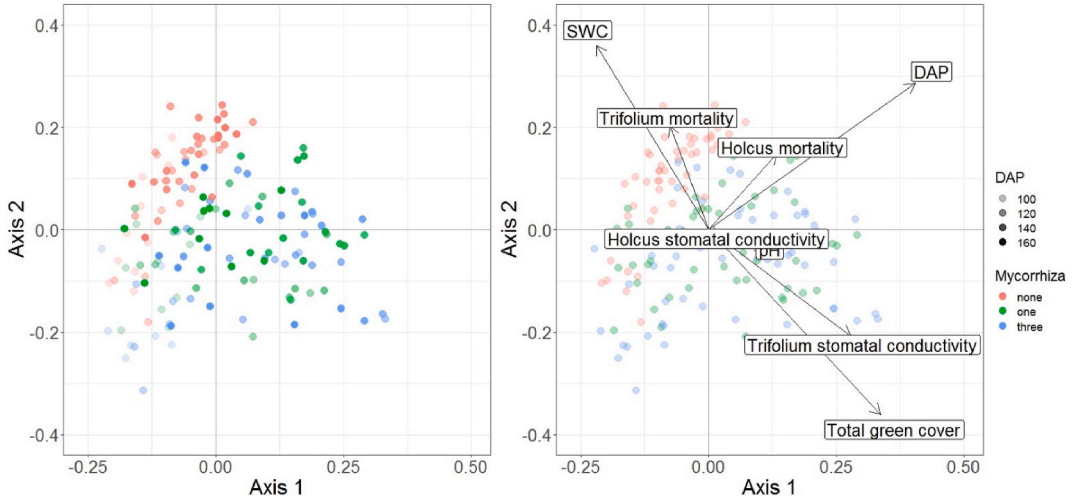

Fig. 1. Biplots of the first two axes of the PCoA ordination of the soil eukaryotic community. Color represents inoculation treatment. Left: biplot of the first two axis of the PCoA of the soil eukaryotic community with shading representing time, expressed as number of days after planting (DAP). Right: the same biplot with vectors representing correlation with the explanatory variable vectors overlaid. All points were shaded equally for clarity of the explanatory variable vectors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

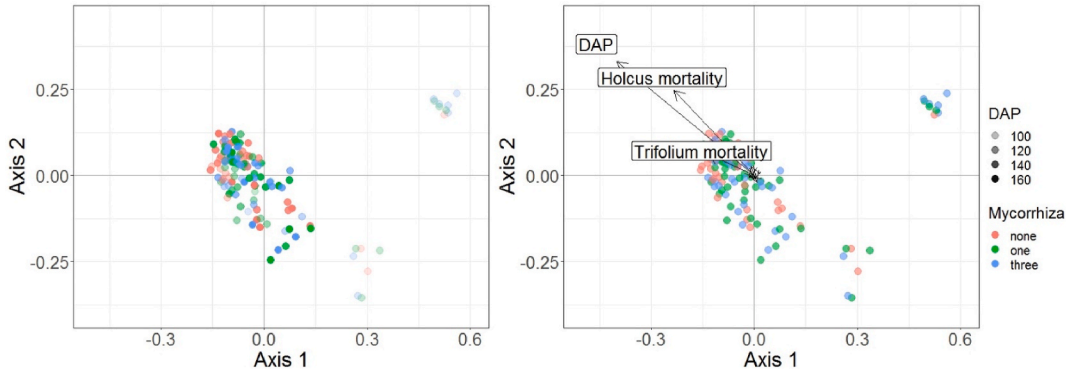

Fig. 2. Biplots of the first two axis of the PCoA ordination of the soil bacterial community. Color represents inoculation treatment. Left: biplot of the first two axis of the PCoA of the soil bacterial community with shading representing time, expressed as number of days after planting (DAP). Right: biplot of the same data of the soil bacterial community with explanatory variable vectors overlaid. All points were equally shaded for clarity of the explanatory variable vectors. Note that only the vectors for DAP, Holcus mortality and Trifolium mortality are displayed due to the very small size of the remaining vectors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in the first axis of eukaryotes. However, in the second axis inoculation treatment was significant as part of an interaction with total green cover ($F_{2,89.7} = 3.71$, $p = 0.028$). The main contrasts appear between the nonmycorrhizal inoculation treatment and the inoculations with either one or three mycorrhizal species (Fig. 1).

For bacteria, time was also the most important factor in both the first ($F_{1,77} = 31.8$, $p < 0.0001$) and the second PCoA axis ($F_{1,80} = 7.97$, $p = 0.006$). However, in contrast to eukaryotes, inoculation treatment also contributed significantly to the variation contained within the first axis, as part of an interaction with *T. pratense* mortality ($F_{2,77} = 4.6$, $p = 0.013$). Similar to the result found for eukaryotes, the main apparent contrasts are between the nonmycorrhizal inoculation treatment and the inoculation treatments with one and three mycorrhizal species (Fig. 2). Inoculation treatment was not a significant predictor for the second axis.

Similarly, the multivariate abundance analysis indicated that inoculation with AM fungi had a significant effect on both the eukaryotic (*likelihood-ratio test (LRT)* = 6,802, $p = 0.001$) and bacterial soil community (*LRT* = 39,321, $p = 0.001$). Nine eukaryotic OTUs out of 320 responded significantly to the inoculation treatment. These OTUs belonged to Cercozoa (three OTUs), Ciliophora (two OTUs), Cryptophyta, Fungi (two OTUs) and Metazoa. At the phylum level, the biggest changes were in Ciliophora, Gastrotricha, Lobosa and Nematoda. Ciliophora decreased by 8.5% and 9.2% when inoculated with one and three AM species compared to the nonmycorrhizal inoculation treatment. Lobosa decreased by 1.8% and 2.1% and Nematoda decreased by 5.3% and 8.6%. In contrast, Gastrotricha strongly increased when AM fungal inoculum was present, increasing by 15.1% and 21.5% (Supplementary Fig. S8). Eight bacterial OTUs out of 1647 responded significantly individually to the inoculation with AM fungi. These OTUs belonged to Chloroflexi (two OTUs), Cyanobacteria, Bacteroidetes and Proteobacteria (two OTUs). Two OTUs remained unassigned. Notable is that one of the OTUs (OTU 7155) which increased from Proteobacteria was identified as *Rhizobium* sp. (Fig. 3). At the phylum level, the main differences appeared within Firmicutes, which moderately increased in mycorrhizal inoculation treatments with 5.4% and 5% when comparing the treatments with one and three AM species with the nonmycorrhizal inoculation treatment. Proteobacteria showed the opposite trend, decreasing by 4% and 4.6% in the mycorrhizal inoculation treatments (Supplementary Fig. S9).

Both inoculation treatment and time significantly affected richness for total eukaryotes ($F_{2,27} = 3.54$, $p = 0.043$ and $F_{1,119} = 44.48$, $p < 0.001$, respectively), with lower richness in the mycorrhizal inoculation

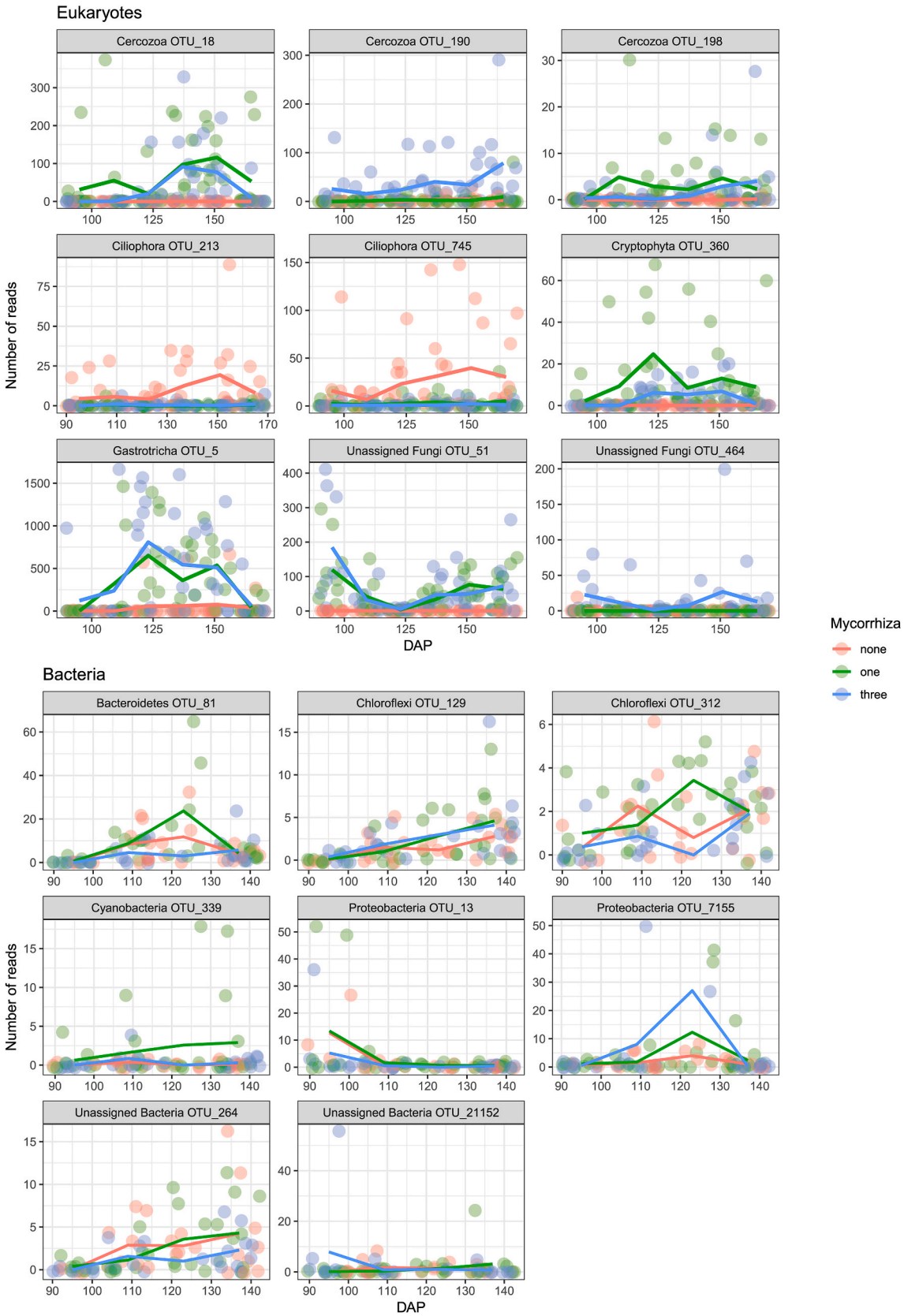

Fig. 3. Effect of mycorrhizal inoculation on the nine eukaryotic and eight bacterial OTUs that differed significantly between inoculation treatments. For each OTU, a phylum level identification is given.

treatments and richness increasing over time (Fig. 4 A). Protist richness was mainly responsible for this mycorrhizal inoculation effect, although this might be due to the higher number of protist taxa observed in the mesocosms compared to fungal and metazoan taxa (Supplementary Fig. S10).

Bacterial richness was not affected by inoculation treatment but significantly correlated with time ($F_{1,108} = 11.15$, $p < 0.0001$), first increasing until September 27 (DAP 137) and then decreasing until the end of the experiment on October 25 (DAP 165) (Fig. 4 B).

3.2. Influence on plant and soil parameters

At the mesocosm level, time and inoculation treatment had an interactive effect on soil water content (Table 1), increasing over time in the nonmycorrhizal inoculation treatment while decreasing in the mycorrhizal inoculation treatments of one and three species despite adjusted watering based on SWC values (Fig. 5 A). pH was slightly but significantly affected by inoculation treatment (Fig. 5 B), but not by time (Table 1). Plant-available nitrate and phosphate were not altered by mycorrhizal inoculation, but ammonium increased significantly (Table 1 and Fig. 5 C–E). Likewise, total green cover significantly increased in the mycorrhizal inoculation treatments, which showed an interaction with time (Table 1); total green cover increased over time when mesocosms were inoculated with either one or three mycorrhizal species, but decreased over time when inoculated with no AM fungi (Fig. 5 F).

Both inoculation treatment and time had a significant influence on *T. pratense* stomatal conductivity (Table 2) which increased with the addition of mycorrhizal inoculum and over time (Fig. 6 D). *T. pratense* leaf area increased over time but at different rates in the different mycorrhizal inoculation treatments as indicated by the significant interaction between time and inoculation treatment (Table 2). Indeed, leaf area in the nonmycorrhizal inoculation treatment barely changed over time, causing a significant difference from the inoculation treatments of one and three mycorrhizal species, which saw a large increase in leaf area (Fig. 6 C). The opposite was true for *T. pratense* mortality, where mortality increased over time in the nonmycorrhizal inoculation treatment but decreased when either one or three AM fungi were inoculated (Fig. 6 B), as shown by the significant interaction (Table 2). Total aboveground biomass of *T. pratense* was significantly higher in the mycorrhizal inoculation treatments (Table 2, Fig. 6 A).

Neither *H. lanatus* stomatal conductivity, leaf area and biomass were significantly affected by mycorrhizal inoculation (Fig. 6 E, G and H), but its leaf area did increase significantly over time (Table 2). Mortality of *H. lanatus* also increased over time, but did so differently depending on mycorrhizal inoculation status. Mortality increased more over time in our nonmycorrhizal inoculation treatment compared to the treatments with one or three AM fungal species (Fig. 6 F).

3.3. Influence of biotic and abiotic parameters on microbial community structure

The PCoA analysis shows that, besides time and inoculation treatment, several of the measured biotic and abiotic parameters also had a significant influence on the bacterial and eukaryotic soil community variation. For the first axis of eukaryotes the final model also included soil water content ($F_{1,70.2} = 4.6$, $p = 0.035$), pH ($F_{1,70.2} = 4.6$, $p = 0.018$), total green cover ($F_{1,97.5} = 5.9$, $p = 0.017$), *T. pratense* stomatal conductivity ($F_{1,96.1} = 9.39$, $p = 0.0028$) and *T. pratense* mortality ($F_{1,96.4} = 5.81$, $p = 0.018$). This final model explained 57.7% of the variation for the first PCoA axis. Although inoculation treatment itself was not significant, its effects are still included in the soil water content, pH, total green cover, *T. pratense* stomatal conductivity and mortality as these each responded significantly with mycorrhizal inoculation (Tables 1 and 2). For the second axis, as mentioned before the final model contained time and the interaction between inoculation treatment and total green cover. Besides these, soil water content ($F_{1,99} = 5.5$, $p = 0.021$) and pH ($F_{1,83.7} = 4.8$, $p = 0.032$) were also significant factors. This final model of the second eukaryotic axis explained 50.5% of the variation contained within the axis.

In bacteria, the final model for the first axis included total green cover ($F_{1,77} = 12.8$, $p = 0.0006$) besides time and the interactions between inoculation treatment and *T. pratense* mortality, and explained 54.8% of the variation contained within this axis. The final model for the second axis only included soil water content as a significant contributor ($F_{1,80} = 3.98$, $p = 0.049$) beyond time and only explained 14.5% of the

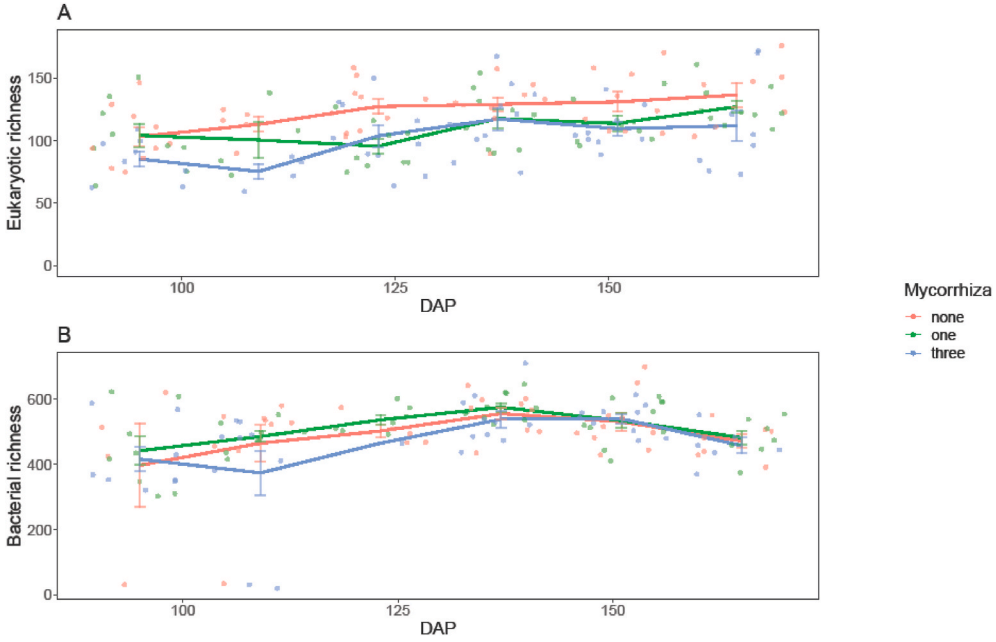

Fig. 4. Effect of inoculation treatment on average (±SE) richness, defined as the number of OTUs per sample, of A: eukaryotes and B: bacteria throughout the experiment. Only eukaryotes responded significantly to mycorrhizal inoculation. Lines represent average values with standard error bars. Points represent individual mesocosm measurements.

Table 1
Effect of inoculation treatments and time on total mesocosm biotic and abiotic parameters. Significant p-values are highlighted in bold. Nitrate-N, Ammonium-N and Phosphate were only measured at two timepoints. See also Fig. 5 for graphical representation of these variables according to inoculation treatments.

	Time			Inoculation treatment			Interaction		
	DF	*F* statistic	*p*-value	DF	*F* statistic	*p*-value	DF	*F* statistic	*p*-value
Soil water content	1147	5.15	**0.025**	2,27	29.87	**< 0.0001**	2147	71.46	**< 0.0001**
pH	1147	3.05	0.083	2,27	5.15	**0.013**	2147	0.73	0.49
Nitrate-N	*NA*	*NA*	*NA*	2	0.67	0.52	*NA*	*NA*	*NA*
Ammonium-N	*NA*	*NA*	*NA*	2	4.2	**0.02**	*NA*	*NA*	*NA*
Phosphate-P	*NA*	*NA*	*NA*	2	0.95	0.39	*NA*	*NA*	*NA*
Total green cover	1147	13.66	**< 0.0001**	2,27	99.33	**0.0003**	2147	47.92	**< 0.0001**

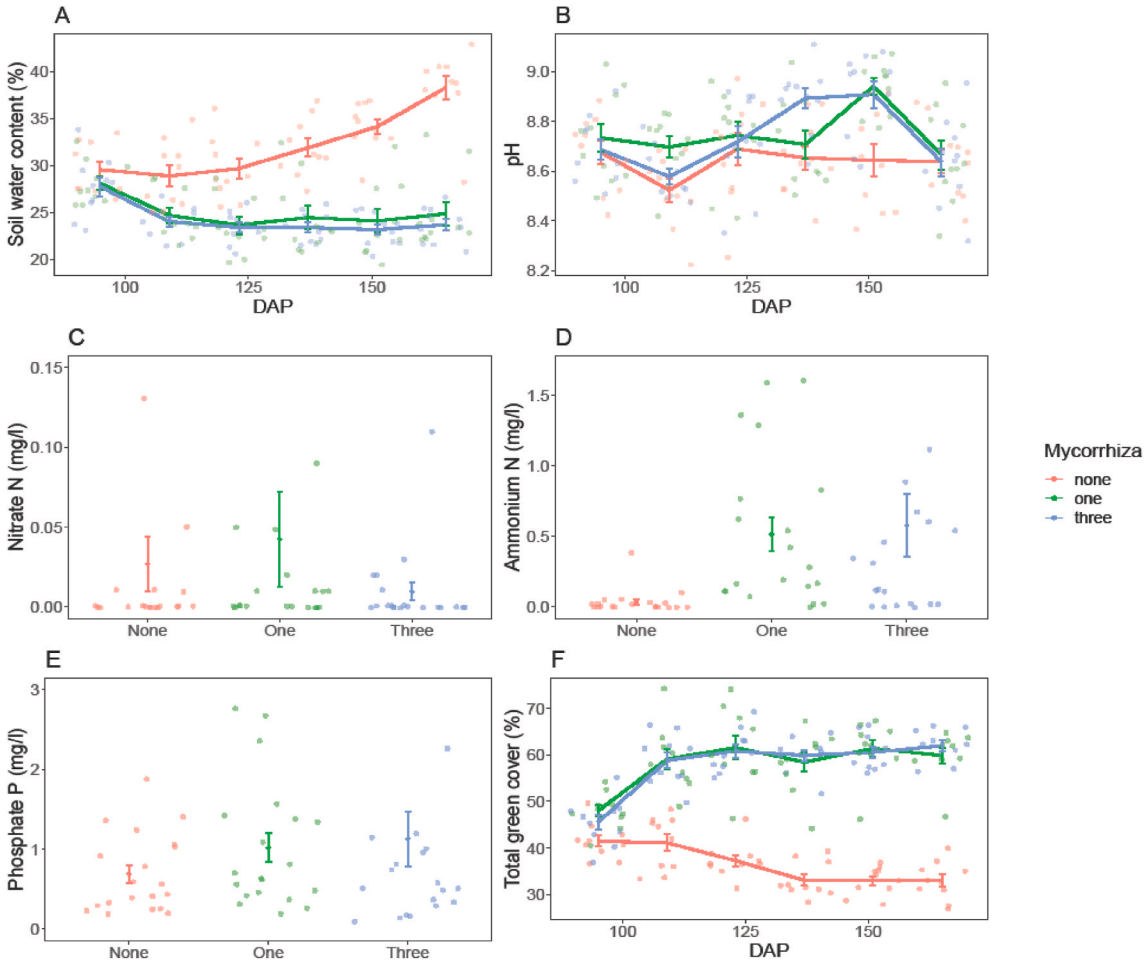

Fig. 5. Effect of inoculation treatments on total community biotic and abiotic parameters. A: the effect of mycorrhizal inoculation on soil water content over time. B: the effect of mycorrhizal inoculation on pH over time, C: the overall effect of mycorrhizal inoculation on plant-available nitrate-N, two outliers (nonmycorrhizal: 0.33, and with one AM species: 0.6) were omitted for visual purposes, D: the overall effect of mycorrhizal inoculation on ammonium-N, two outliers (treatment with three AM species: 2.14 and 4.09) were omitted for visual purposes, E: the overall effect of mycorrhizal inoculation on phosphate-P, two outliers (treatment with three AM species: 4.75 and 6.03) were omitted for clarity and F: overall effect of mycorrhizal inoculation on total green cover over time. Significant differences were found for soil water content, pH, ammonium-N and total green cover. Lines represent average values with standard error bars, different colors represent different inoculation treatments. Points represent individual mesocosm measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

variation contained within the axis. Again, as with the eukaryotes, the effect of inoculation treatment is also captured within soil water content as this correlated significantly with AM fungal inoculation (Table 1).

4. Discussion

Although the importance of AM fungi for plants has already been illustrated numerous times on a variety of plant species (Chaudhary et al., 2016), and on other individual guilds of the soil microbial community, the effect of AM fungi on the whole soil community (Bacteria and Eukaryotes), and how this effect propagates through time, has not yet been thoroughly examined under field conditions. Our study used a mesocosm experiment, sampled through time, to examine the influence of AM fungi on both plants, the soil environment and the whole soil microbial community. This is a very novel approach to look at the role AM fungi play in the soil food web, as no other study has measured all these parameters together in a time series. As the growing season progresses, changes in the soil microbial community can be

Table 2
Effect of inoculation treatments and time on measured *H. lanatus* and *T. pratense* parameters. Significant p-values are highlighted in bold, biomass was only determined at the end. See also Fig. 6 for graphical representation of these variables according to inoculation treatments, split by species.

		Time			Inoculation treatment			Interaction		
		DF	*F* statistic	*p*-value	DF	*F* statistic	*p*-value	DF	*F* statistic	*p*-value
T. pratense	Biomass	*NA*	*NA*	*NA*	2	30.26	**< 0.0001**	*NA*	*NA*	*NA*
	Mortality	1147	45.7	**< 0.0001**	2,27	13.19	**0.0001**	2147	11.3	**< 0.0001**
	Leaf area	1,87	124.72	**< 0.0001**	2,27	34.5	**< 0.0001**	2,87	25.74	**< 0.0001**
	Stomatal conductivity	1,87	8.04	**0.0057**	2,27	27.96	**< 0.0001**	2,87	2.1	0.13
H. lanatus	Biomass	*NA*	*NA*	*NA*	2	0.12	0.88	*NA*	*NA*	*NA*
	Mortality	1147	403.17	**< 0.0001**	2,27	10.74	**0.0004**	2147	23.05	**< 0.0001**
	Leaf area	1,87	38.81	**< 0.0001**	2,27	0.55	0.59	2,87	1.13	0.33
	Stomatal conductivity	1,87	0.0016	0.97	2,27	0.34	0.71	2,87	1.14	0.33

correlated to changes in biotic (plant) and abiotic (soil) parameters as well as to AM fungal status.

The intensive sampling over time and addition of inoculum containing a developed microbial community as well as a microbial wash at the start of the experiment, allowed us to reveal the dynamic temporal changes of bacterial and especially eukaryotic communities throughout the growing season. Time had by far the largest influence on communities, independent of inoculation treatment, showing that the drivers are similar in all mesocosms. Indeed, plant growth stage has a large effect on the associated fungal and protist communities (Gao et al., 2020; Xiong et al., 2020). It is unknown whether the time influence we observed was solely due to the progression through the growing season, the relative immaturity of the mesocosm soil communities, or turnover of residual DNA after pasteurization. We would expect the timespan between the setup and measurements to be sufficient for most residual DNA to degrade, but still some of this DNA could still have been picked up by the HTS process. To be able to separate residual DNA turnover, development and normal growing season progression, future research could use mesocosms that are older and have had a chance to develop a stable soil community over several years.

After accounting for temporal changes, inoculation with AM fungi had a particularly large influence on the eukaryote soil microbial community structure and richness in the developing mesocosms, and to a lesser extent on the bacterial community structure. This corresponds to the findings of Fierer (2017), who suggested that bacteria are more affected by abiotic variables, and less by biotic interactions compared to eukaryotes. Given that AM fungi serve as a potential habitat for bacteria (Scheublin et al., 2010), and also release exudates which can serve as additional food sources for surrounding soil bacteria (Miller and Jastrow, 2000; Singh, 2012; Toljander et al., 2007), it is surprising that we did not observe any significant influence on bacterial richness. Conversely, the release of exudates could provide a potential explanation for the observed changes in soil eukaryotic community structure and richness. Furthermore the presence of AM fungi and associated increase in hyphal biomass could potentially alter eukaryotic abundance and richness by acting as an additional food source (Caravaca and Ruess, 2014). The higher eukaryotic richness observed in the nonmycorrhizal inoculation treatment could potentially be explained by the more oligotrophic conditions created in those mesocosms by the lower plant productivity, supporting a higher diversity but also likely lower abundances.

We did not detect changes in specific eukaryotic OTUs known to be affected by AM fungi, such as pathogenic nematodes (Veresoglou and Rillig, 2012) and root rot fungi (Trabelsi and Mhamdi, 2013). However, we did find a decrease in relative abundance of several nematode and Ciliophora OTUs in the mycorrhizal inoculation treatments, although these were all bacteria, protist and yeast feeding taxa (Joubert et al., 2006; Sheldon et al., 1986). Finally we recovered one OTU of Gastrotricha which greatly increased in mycorrhizal inoculation treatments. Terrestrial gastrotrichans are also known consumers of detritus, bacteria and protists (Kisielewska et al., 2015). The observed effect of AM fungi causing a shift in the bacterial community composition was expected and supports current knowledge (Marschner and Baumann, 2003). However only a small number of OTUs responded significantly to AM inoculation, which contrasts with some previous findings where much greater numbers of bacterial taxa responded to mycorrhizal inoculation (Rodríguez-Caballero et al., 2017). We did find an increase in *Rhizobium* (OTU 7155, see Fig. 3), which suggests that AM fungi are promoting the formation of root nodules, which are necessary for nitrogen fixation and legume growth. The significant increase of ammonium in the mycorrhizal inoculation treatments further corroborates higher nitrogen fixation and/or OM mineralization rates mediated by AM fungi. Indeed, colonization by AM fungi is a crucial step in the early formation of nodules because of their high phosphate costs (Li et al., 2009; Mortimer et al., 2008). In general, the OTUs we recovered do not appear to be directly related to plant growth, with the exception of *Rhizobium*. However most of the responding eukaryotic OTUs point to a potential increase in carbon fluxes through the microbial loop, as the recovered taxa were mainly bacteria, fungal and protist feeders. Given that our soil was alkaline and very poor in organic material, and that these conditions decrease availability of some nutrients and increase the importance of AM fungi (Aarle et al., 2002; Marschner, 1995), it makes sense that carbon fluxes through the microbial loop also increase in importance. Future studies could repeat our approach with different soil types and over several years and examine which taxa respond to AM fungi, and how persistent these effects are.

Several plant and soil parameters that were changed by mycorrhizal inoculation affected both the bacterial and eukaryotic community variation. Particularly measures of soil water content, *T. pratense* stomatal conductance, *T. pratense* mortality and total green cover were most strongly correlated with community variation. Soil water content can influence both bacterial and eukaryotic soil communities (Ochoa-Hueso et al., 2018; Schimel, 2018). The necessity of AM fungi for legume health and growth is also very well-documented (Chalk et al., 2006). The significant increase in *T. pratense* stomatal conductance in particular shows that AM fungi increased potential plant photosynthetic rate, which is corroborated by previous findings of AM fungal colonization increasing water and nutrient uptake, allowing plants to maintain open stomata for longer, which in turn increases their photosynthetic potential (Allen, 2007; Augé, 2000; Huang et al., 1985). This increase in photosynthetic potential likely results in higher carbon inputs into the soil, which can lead to changing soil communities of basal groups such as bacteria (Baudoin et al., 2003; Eilers et al., 2010) and fungi (De Graaff et al., 2010), which are then consumed by other soil organisms, leading to potential changes in the soil meso- and macrofauna (Albers et al., 2006). Furthermore, larger plants also support larger root systems, which in turn may increase hyphal abundance in the soil. Both roots and hyphae act as food sources for several soil eukaryotic groups (Caravaca and Ruess, 2014; Högberg and Read, 2006). Our results show a clear separation in the eukaryotic soil community based on inoculation treatment. It is important to keep in mind that the introduction of different inoculants, which differ in their composition of soil microbes, can inherently lead to a bias in the final observed community. Although we did observe differences in initial inoculant community composition,

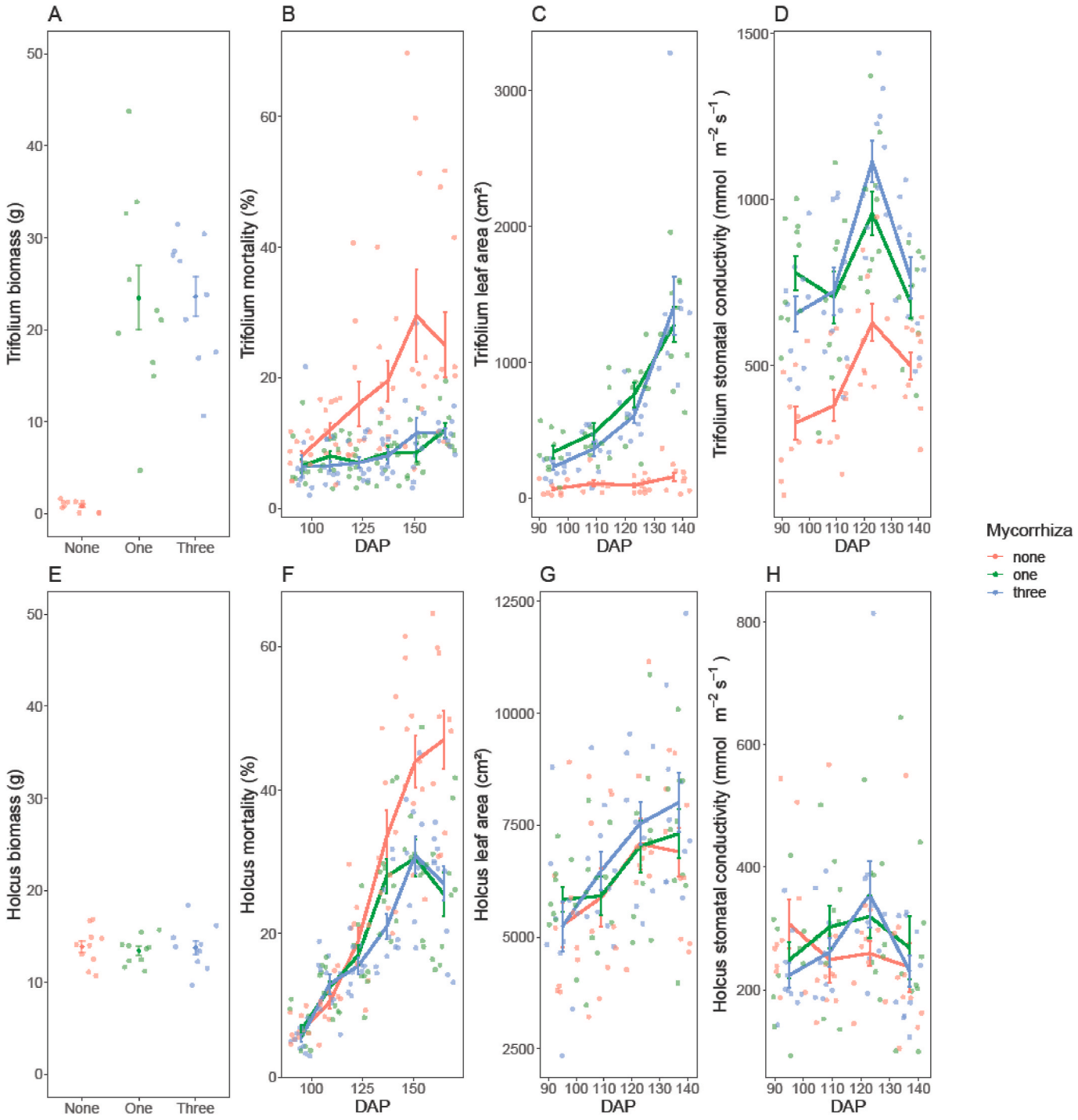

Fig. 6. Effect of inoculation treatments on plant parameters per species. A to D: the effect of mycorrhizal inoculation on *T. pratense* biomass (measured in grams) at the end of the experiment, mortality, leaf area and stomatal conductivity through time. Significant differences were found for all parameters. E to H: the effect of mycorrhizal inoculation on *H. lanatus* biomass at the end of the experiment, mortality, leaf area and stomatal conductivity through time. Significant differences were only found for mortality. Lines represent average values with standard error bars, different colors represent different inoculation treatments. Points represent individual mesocosm measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

specifically in eukaryotes, we did not find evidence for a bias throughout the experiment (see Supplementary Information for more details). Despite the clear observed separation in the eukaryotic community, the variance explained by the inoculation treatment in our model was only modest. A potential explanation for this could be the heterogeneity of the substrate. Although the used soil was mixed thoroughly before pasteurization and use, there will inherently still be heterogeneity at the spatial level of soil microbes, potentially causing more variation and obscuring pure mycorrhizal effects (Nunan et al., 2020; Seaton et al., 2020). Most of the observed variation can be explained by the plant growth parameters and soil water content. These in turn were influenced greatly by the presence of AM fungi. This leads us to speculate that for eukaryotes in particular, there is a very large indirect influence of AM fungi on other soil organisms through enhanced plant growth. We have summarised these mechanisms, as well as the potential direct mechanisms, in a conceptual graph (Fig. 7).

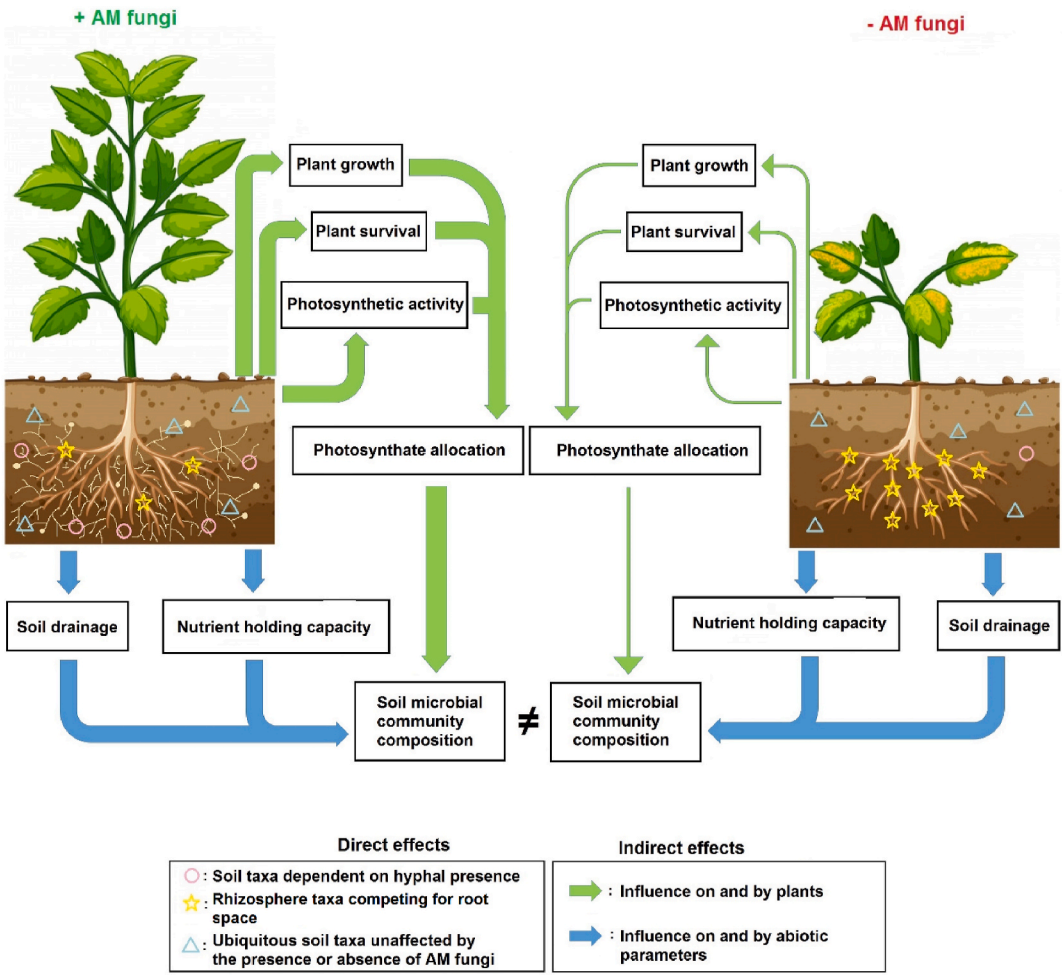

Fig. 7. Conceptual model describing potential direct and indirect effects of the presence of AM fungal inoculum on the soil community. Arbuscular mycorrhizal fungi directly influence other soil taxa by competing for root space, excreting exudates and serving as food and habitat. As AM fungi colonize roots and increase nutrient and water status of the host, growth is increased as well as photosynthetic activity, whilst simultaneously decreasing mortality. This increases the amount of photosynthate that can be allocated to roots and associated AM fungi, which finds its way into the soil either by excretion by the roots and/or hyphae, or through consumption of root and/or hyphal mass. AM fungi simultaneously affect soil communities by modifying the soil environment. In our case this was most prominent for water and nutrient holding capacity of the soil, which are both considered important factors for many soil taxa.

The ways in which AM fungi influence soil communities through plants may depend on the identity of plant species and their associated physiology. Our grass species, *H. lanatus* did not show many plant growth-related changes due to mycorrhizal inoculation, which is typical for C3 grass species (Hetrick et al., 1988). However, mortality was significantly decreased in the mycorrhizal inoculation treatments. A potential explanation for this is root stress caused by the increasing soil water content in the nonmycorrhizal inoculation treatment. In the mycorrhizal inoculation treatments, the increasing leaf area and stomatal conductivity of *T. pratense* likely leads to higher evapotranspiration potential. Furthermore, the increase in plant roots together with the presence of exudates from the AM fungi may have caused increased drainage due to the formation of aggregates (Miller and Jastrow, 2000; Siddiky et al., 2012). This interpretation is supported by the observation that the nonmycorrhizal inoculation treatment showed more slumping of the soil and increasingly poor drainage throughout the experimental period (personal observation). Together this illustrates that even plants that are less dependent on AM fungal colonization can still greatly benefit from their presence, as AM fungi generally create more favourable soil conditions.

This experiment clearly illustrates the overall effect of AM fungal inoculation on plants and soil microbial communities. Both eukaryotic and bacterial soil community structure change with time as a result of mycorrhizal inoculation, and plant growth is generally enhanced. However several questions regarding AM fungi still remain unresolved. We did not measure carbon allocation into the soil, which leaves our suggested pathway of indirect effects through plant productivity untested. Further studies should focus on the quantification of photosynthate transfer into the soil, and how this is affected by AM fungi under different soil conditions. Secondly, although our experiment was not performed in a laboratory, it still represents a study under highly controlled conditions. The pasteurization of the soil reduces the size of soil microbial populations, which gradually recolonized the soil-plant system during the experiment. This perturbation is probably more extreme than perturbations commonly experienced in the field, which affects our interpretation of the findings. Additionally the introduction of both mycorrhizal and nonmycorrhizal inoculants, each containing their own distinct set of microbes, could lead to variation in the soil microbial community, even though we did not find any evidence of this happening in our case. Similar experiments, performed over several years or on fully stabilized soils should help resolve these issues and provide further insights into the effect of AM fungi on soil microbial food webs. This further research is important, since in the light of the ever increasing pressure on natural and agricultural plant and soil communities due to changing climates, the stability of plants and their associated soil microbial communities to disturbances in the presence and

absence of AM fungi remains one of the research priorities in plant and soil ecology.

Data accessibility

The raw sequences were deposited in the National Centre for Biotechnology Information's (NCBI) Sequence Read Archive (SRA) database under the bioproject with ID PRJNA791953. Additional information is available from the corresponding author on reasonable request.

Author contributions

JDG, EV and JTW conceived the idea and designed the experiment and subsequent analyses. SG provided advice on eukaryotic sequencing and MGAH provided advice on the use of AM fungi in experimental setups. JDG and EME established and maintained the experiment setup and conducted measurements and sampling. JDG performed lab work, bioinformatics and statistical analyses with guidance from EV and JTW. All authors contributed to interpretation of the results, and read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108524.

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