



Arbuscular Mycorrhizal Fungi Alter the Community Structure of Ammonia Oxidizers at High Fertility via Competition for Soil NH_4^+

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Abstract

Nitrification represents a central process in the cycling of nitrogen (N) which in high-fertility habitats can occasionally be undesirable. Here, we explore how arbuscular mycorrhiza (AM) impacts nitrification when N availability is not limiting to plant growth. We wanted to test which of the mechanisms that have been proposed in the literature best describes how AM influences nitrification. We manipulated the growth settings of *Plantago lanceolata* so that we could control the mycorrhizal state of our plants. AM induced no changes in the potential nitrification rates or the estimates of ammonium oxidizing (AO) bacteria. However, we could observe a moderate shift in the community of ammonia-oxidizers, which matched the shift we saw when comparing hyphosphere to rhizosphere soil samples and mirrored well changes in the availability of ammonium in soil. We interpret our results as support that it is competition for N that drives the interaction between AM and AO. Our experiment sheds light on an understudied interaction which is pertinent to typical management practices in agricultural systems.

Keywords Ammonium oxidizers · Arbuscular mycorrhiza · Glomeromycota · Mycorrhizal hyphosphere · Nitrification potential

Introduction

Most of the inorganic exchangeable nitrogen (N) in soil is in the forms of ammonium (NH_4^+) and nitrate (NO_3^-). Because plants differ in their relative investment in NH_4^+ and NO_3^- transporters, some plants specialize in NH_4^+ whereas others in NO_3^- uptake [1, 10, 30], which allows for a further partitioning of the plant niche and promotes plant coexistence

[11, 62]. This is not the only reason, however, why the relative availability of NH_4^+ over NO_3^- in soil matters. NH_4^+ and NO_3^- represent the start and end products of nitrification, a central process in the cycling of N [50]. Whereas NH_4^+ , the form of N which most microbes utilize for nutrition [35, 65], diffuses only moderately in the soil environment, NO_3^- molecules can easily leach and under anaerobic conditions are converted to dinitrogen through denitrification [49]. Losses of N from the soil are undesirable not only because they compromise soil fertility but also because leaching of N drives eutrophication of aquatic habitats whereas incomplete denitrification generates greenhouse gases such as NO and N_2O [17, 59]. Losses of N should be most detrimental in fertile habitats, and in particular in agriculture. The costs associated with eutrophication in the USA alone exceed two billion dollars per year [21] and agriculture accounts for almost 70% of total greenhouse gas emissions [32]. A possible way to mitigate problems that arise from N losses in agriculture is through slowing down nitrification (e.g. [38, 57]).

The rate-limiting step of nitrification, the oxidation of NH_4^+ to NO_3^- , is a biological process that is carried out by a relatively small group of autotrophic microbes that oxidize ammonia (AO): ammonia oxidizing bacteria (AOB) and archaea (AOA). Evidence is accruing to suggest that the ecological significance of AOB is high in disturbed environments such as arable soil and improved grasslands, whereas AOA

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almost exclusively drive nitrification in low-pH environments [5, 20, 31, 51]. There is a broad range of parameters that influence rates of terrestrial nitrification that includes substrate availability, pH, soil aeration, soil moisture and temperature [18, 7, 37, 24, 4]. An additional factor that can impact nitrification rates and the ammonia oxidizing community, either directly or through altering substrate availability, is the structure of the plant community [66, 68, 72]. We know that the differences in the potential of different plant species to contribute to nitrification can be partially explained by the degree to which they form mycorrhizal associations, which has so far been shown for low-fertility terrestrial habitats [72]. In particular, plants that are colonized extensively by arbuscular mycorrhizal (AM) fungi support lower potential nitrification rates (i.e. a proxy of the size of the ammonia oxidizing community) than those that do not [16, 72].

It is unclear whether AM can drive a comparable suppression of nitrification rates under conditions of high fertility that resemble agricultural settings (Fig. 1c—proposition one). When the availability of NH_4^+ is high, any likely gains of either AM fungi or ammonia oxidizers out of investing into allelopathic compounds should be trivial and competition for available NH_4^+ would be of little ecological importance. If inoculation with AM fungi suppresses nitrification irrespective of soil fertility, via promoting mycorrhizal technology in agriculture we may also be slowing down nitrification rates; this should represent a compelling reason to further support AM-biotechnology in agriculture [55, 64]. We propose three main mechanisms that can explain the negative effect of AM on nitrification: competition of AM fungi with ammonia oxidizers for NH_4^+ ; allelopathy (both mechanisms are direct); and a priming effect of increased C exudation leading to microbial N-mining in the absence of mycorrhiza which stimulates N-mineralization (this mechanism is indirect) [72, 74]. It is desirable to address which of these mechanisms underlie AM fungal-induced suppressions of AO in the rhizosphere, and experimenting under high fertility could shed light on this question. For example, a suppression of AO at an NH_4^+ availability that is non-limiting for either group of organisms, could rule out that the suppression of AO is because of interference competition between AO and AM fungi. To address proposition one, we formulate our first hypothesis, that addition of mycorrhiza under high fertility settings suppresses potential nitrification rates and densities of AOB (Fig. 1c).

Changes in nitrification rates are likely to occur even when the potential nitrification rates remain steady through changes in community structure of AO (Fig. 1b—proposition two). In other words, two AO communities may nitrify at comparable rates under idealized conditions but differences in the way they cope with abiotic stresses can induce different nitrification trajectories over time. A community

shift in ammonia oxidizers could thus result in more subtle effects, such as a higher temporal and spatial stability of nitrification rates if the richness of the AO community increases [42, 76] or an altered resilience to biotic and abiotic stresses resulting from better or worse stress tolerance of the new community members [2, 28]. To address proposition two, we formulate our second hypothesis that mycorrhiza induces community shifts via direct mechanisms acting on the AOB community.

The degree to which AM fungi might promote specific members of the AO community either in the form of endobacteria or as prokaryotes highly specific to AM [9, 69] is unclear (proposition three). To the best of our understanding there has been no attempt so far to identify AO that associate closely with AM and we know very little about whether AM mediates shifts in the community structure of AO. If we were to find AO taxa that benefit from AM fungi, this would imply that the influence of AM fungi on AO community structure is of such high ecological significance that induces a considerable AO community turnover. To address proposition three, we formulate our third hypothesis that there are several AOB taxa that proliferate only when the plant associates with AM fungi and that benefit from similar mechanisms that favour other AM-specific prokaryotes.

Moreover, the changes that AM induces in nitrification could be divided into direct effects that are induced by the hyphae of AM fungi such as exploitation competition and allelopathy and indirect effects that describe AM-induced changes in the physiology of the host plant impacting nitrification mainly through modifying the quantity and composition of rhizodeposits in the rhizosphere (proposition four). With the possible exception of Chen et al. [16] who used compartmentalized mesocosms to disentangle these two effects there have been no other attempts to quantify the relative importance of these direct vs indirect changes in the AO community that are induced by the inoculation with AM fungi. As an example, indirect changes in the AO community might mostly influence AO taxa that depend heavily on rhizodeposits and can effectively compete for mineralized NH_4^+ with other rhizosphere organisms. By contrast, direct changes could be more subtle and influence equally all AO. To address proposition four, we formulate our fourth hypothesis that plants can partially mask any AM-induced shifts in the bacterial community of AO.

Here, we report on a controlled experiment that aimed at addressing the above-mentioned hypotheses on the way AM impact nitrification. We manipulated the mycorrhizal state and induced extensive disturbance to the AO community in compartmentalized high-fertility mesocosms so as to establish mycorrhizal—non-mycorrhizal *Plantago lanceolata* contrasts on soil that did not contain any AOA.

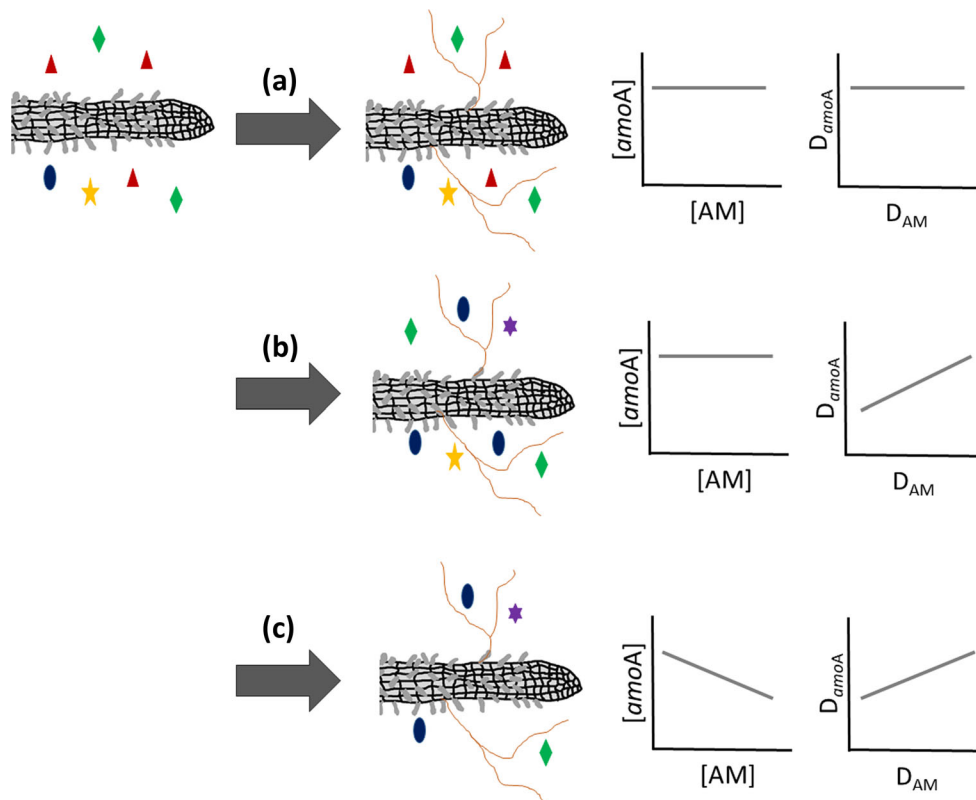


Fig. 1 Conceptual diagram highlighting the possible outcomes that manipulations of arbuscular mycorrhiza (AM; shown as red filaments; different taxa not distinguished for clarity) can have on the community structure of ammonium oxidizing bacteria (AOB; shapes surrounding roots of various colours, representing different taxa) at conditions of moderate/high N availability. **a** AM has no effect on AOB. AM abundance $[AM]$ does not affect densities of AOB which is here highlighted by the expression of the gene *amoA*, $[amoA]$. Moreover, there is no relationship between community distances of AM (D_{AM}) and community distance of AOB (D_{amoA}). **b** AM only influences the

community structure of AOB (hypothesis two). There is no relationship between AM abundance $[AM]$ and densities of AOB $[amoA]$. However, AOB communities change with changes in AM abundance or community structure which is manifested by a positive relationship between community distances of AM (D_{AM}) and community distance of AOB (D_{amoA}). **c** In this example, AM influences both densities and community structure of AOB (hypothesis one). In addition to the relationship between community distances of AM and AOB, we have a negative relationship between densities of AM, $[AM]$, and AOB, $[amoA]$. This relationship could also be positive

Materials and Methods

Experimental Design

The mesocosm-based experimental setup was a two by two split plot design with six replicates. The two main factors described (i) the time the soil had been incubated with microbes prior to the initiation (i.e. transplanting) of the experiment (either 14 days or 97 days so that the resulting AO communities differed in maturity); and (ii) addition or no-addition of spore inoculum of a common and well-studied AM fungus *Rhizophagus irregularis*. An 80 mg aliquot (approximately 1240 spores) of axenic spore inoculum (SYMPLANTA-001, SYMPLANTA, Germany) was added to the rhizosphere compartment of each mesocosm which was inoculated with mycorrhiza. The split plot factor related to whether the soil was assayed from the root (rhizosphere) compartment or the root exclusion hyphosphere compartment where fungal hyphae gained access through a fine

mesh (30 μm) but roots were excluded (e.g. [73]). Earlier experiments (e.g. [73]) where we had added concentrated mycorrhizal inoculum in the proximity of the seedling in compartmentalized systems resulted in successful AM colonization of both compartments.

Soil, Mesocosm Setup and Harvest

Cylindrical mesocosms (9-cm internal diameter, 20-cm height) which had been perpendicularly divided into two halves that were separated by a mesh of 30 μm were constructed as described by Veresoglou et al. [73]. The mesh delineated two compartments within the mesocosm, only one of which (rhizosphere compartment) was accessible to plant roots.

The plant growth substrate was an Albic Luvisol soil (73.6% sand, 18.8% silt, 7.6% clay) with 1.9% organic carbon, 150 mg/kg exchangeable N and a pH (CaCl_2) of 7.1. In the past, the lab has successfully used the soil for experiments to manipulate mycorrhiza [53, 61]. A common practice to

promote mycorrhizal colonization in mycorrhizal studies is to mix soil with sand. In order to preserve the high nutrient availability in the growth substrate, we did not mix the soil with any other growth substrate. Moreover, we did not add any nutrients to the soil throughout the experiment; as a result, at harvest, we recovered a considerably lower nutrient availability, which we attributed to the assimilation of nutrients from the plant. The soil was sieved (2 mm) and homogenized prior to initiating the experiment as follows: soil was steam heated to 99 °C for an hour, thrice over three consecutive days. Steam-treated soil was randomly divided into two 30 kg batches and brought up to 60% water holding capacity with a soil microbial filtrate produced by filtering (20- μ m sieve) a 1:9 (*w:v*) solution of soil:sterile water after incubation (horizontal shaker 200 rotations per minute) for 1 h as modified from Veresoglou et al. [72]. Following inoculation, the soil was incubated at room temperature for 83 days in autoclave bags which were aerated once per week for 10 min. One of the two autoclaved bags was then steam-sterilized again at 99 °C for an hour (i.e. at this stage, the AM fungal community was already inactive and a single steam-sterilization was deemed enough to suppress AO densities) and approximately 8 kg of soil were used to fill the mesocosms we describe earlier. To maximize the chances of an even distribution of microbes, each of the two compartments per mesocosm received 20 ml of a freshly made microbial filtrate which was generated as described earlier. The mesocosms were further incubated for 14 days at room temperature resulting in two incubation treatments: Assuming a cell division rate of one fission per 12 h (i.e. descriptive of the subset of AOB with fast growth rates under idealized growth conditions), 14 days is equivalent to less than 28 generations for fast growing AOB and 97 days, which allowed over 100 generations for fast growing AOB [49].

At day 97, three *Plantago lanceolata* seedlings were transplanted to the ‘rhizosphere’ compartments of each mesocosm. Seeds had been surface sterilized and left to germinate on a sterile filter paper a week prior the initiation of the experiment as described in Veresoglou et al. [72, 73]. Plants grew in a growth chamber at 16-h light; 60% moisture; 15 °C and 25 °C night and day temperature, respectively and were watered daily gravimetrically to 60% water holding capacity. Ninety-four days later (i.e. day 191) plants were harvested. At harvest, the aboveground plant biomass of each plant was dried at 60 °C for a week for dry weight determination. Extracted roots were similarly dried and weighed after representative subsamples of extracted roots were taken for staining and microscopic detection of mycorrhizal structures).

From each rhizosphere and hyphosphere compartment per microcosm, 2 g of soil cored at a depth of 3 cm at the mesocosm, was transferred to 15-ml tubes that were amended with 5 ml of an RNA preservation solution (MoBio LifeGuard Soil Preservation Solution), and immediately snap frozen in

liquid nitrogen before storage at –80 °C (for approximately 2 months). Two further 5 g subsamples of soil per compartment were frozen at –80 °C, one to assess inorganic N (NH_4^+ : [12]; NO_3^- : [43]) and one to assess hyphal densities [52]. The thawing time before extracting inorganic N with 1 M KCl did not exceed an hour. The criteria for discriminating between AM and non-AM hyphae were as in [13]. A further 10 g of soil was stored in a fridge overnight for assessment of potential nitrification rates the following day [72]. The assay involves the incubation, under aerobic conditions, of 10 g of soil in a potassium phosphate buffer (pH = 7.2), following the addition of ammonium sulfate and a nitrite reduction inhibitor, sodium chlorate. The solution is assayed at 2, 8, 14 and 26 h after initiation of the assay and the change of nitrite concentrations over time expresses the respective potential nitrification rate.

Molecular Analyses

RNA was extracted from soil samples using the PowerSoil Total RNA Isolation Kit (MoBio Laboratories INC) in conjunction with the On-Spin Column DNase I Kit (Diavora GmbH) to remove genomic DNA following manufacturer’s instructions. RNA quality and quantity of extracted RNA was confirmed via electrophoresis and subsequently reverse transcribed (14 μ l RNA in a 20- μ l reaction which was subsequently diluted with dd H_2O 100-fold) to cDNA with the High Capacity RNA-to-cDNA Kit (Applied Biosystems Inc). As RNA from a single rhizosphere sample was accidentally discarded, our molecular analyses were carried out on a total of 47 samples.

To quantify microbial taxa we implemented an absolute quantification via RT-qPCR as follows: we used three target genes: eubacterial 16S; bacterial *amoA* and archaeal *amoA* genes. To generate standard curves, DNA was extracted from pure cultures of an AOA *Nitrosotalea devanatterra* (Nd1) and an AOB *Nitrospira briensis* C-128, amplified with the primer sets described below and cloned in *Escherichia coli* using the pGEM-T Easy cloning kit (pGEM-T Cloning Systems; Promega, Madison, WI). Extracted plasmid DNA via a plasmid extraction kit (DNeasy, QIAGEN) of known concentrations (five 10-fold dilutions: AOB, 10^2 – 10^6 ; AOA, 10^3 – 10^7 ; 16S, 10^5 – 10^9) were used in triplicates as standards for RT-qPCR reactions. Reactions volumes (10 μ l RT-qPCR) contained 1 \times SYBR-based PCR master mix (Kapa SYBR Fast, ABI Prism) 10 μ M of each primer) and 2 μ l template cDNA/DNA. The following primer pairs were used: Arch-*amoA*F and Arch-*amoA*R [27] for AO-archaea; *amoA*-1F and *amoA*-2R [56] for AO-bacteria; Muyzer-F and Muyzer-R [44] for general eubacteria. The default amplification settings (2 min at 94 °C, and 40 cycles of 20 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C followed by a melt curve step 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C) were applied. Each

assay was performed in triplicates. The following quality control criteria were achieved: no amplification in the negative control; standard deviation across technical replicates was below 0.3; and R^2 value for the standard curve was above 0.99. Abundances were expressed as gene copies per g of soil. The Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) guidelines-compliant checklist was fulfilled.

To assess the active AO-bacterial community structure in our samples tag-encoded FLX amplicon sequencing was carried out on a 454 FLX Roche pyrosequencer targeting the ammonia monooxygenase (*amoA*) gene. The forward *amoA*-1F primer was constructed with a 10-bp bar code to the primer *amoA*-1F in combination with the primer *amoA*-2R. The 25 μ l master reactions consisted of 0.3 μ l of a proof reading polymerase—Pfu DNA polymerase (Thermo Fisher Scientific Biosciences), 2.5 μ l 10 \times buffer, 1 μ l of a 10 μ M working solution of each of the forward and reverse tagged and linked primers, 0.5 μ l dNTPs, 2 μ l BSA, 17.7 μ l water and 1 μ l template cDNA. Amplifications settings were as follows: 94 $^{\circ}$ C for 7 min followed by 35 cycles consisting of 94 $^{\circ}$ C for 60s, 55 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 60s and a 10-min elongation step at 72 $^{\circ}$ C. Amplified PCR products were purified with a NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel) and sequencing performed by Macrogen (Macrogen Inc., Netherlands).

Bioinformatics

We used the pipeline from Schloss et al. [60] as implemented in Mothur v.1.32.1 to quality control raw sequences. To delineate AOB taxa an unsupervised Bayesian clustering approach was implemented in CROP [29]. We then normalized sequencing depth to 2500 sequences through resampling with replacement [75]. Remaining taxa were blasted (BlastN) against GenBank for AOB verification. Representative AOB sequences from the literature together with sequences representative of each OTU in our study were included to generate a phylogenetic tree of AOB in BEAST version 1.5.3 [22].

Statistical Analysis

To assess how inoculation treatment and AM state impacted biotic and abiotic parameters we used repeated-measures ANOVAs with the ID of each separate mesocosm representing an experimental unit. Repeated-measures designs were used to correct for the fact that corresponding rhizosphere and hyphosphere compartments were part of the same experimental unit. The assumptions of these tests were examined on the residuals and whenever necessary we transformed our data.

To assess whether AO-bacterial taxa showed an affinity to specific manipulations, we used an indicator species analysis

[23] as implemented in the package *indicspecies* [19]. We further used Faith's PD index [25] to assess phylogenetic diversity in our samples. To assess N-priming effects, we calculated the ratios of bacterial activity (i.e. 16S gene copies g^{-1} soil in a log₁₀ scale) over total inorganic N availability.

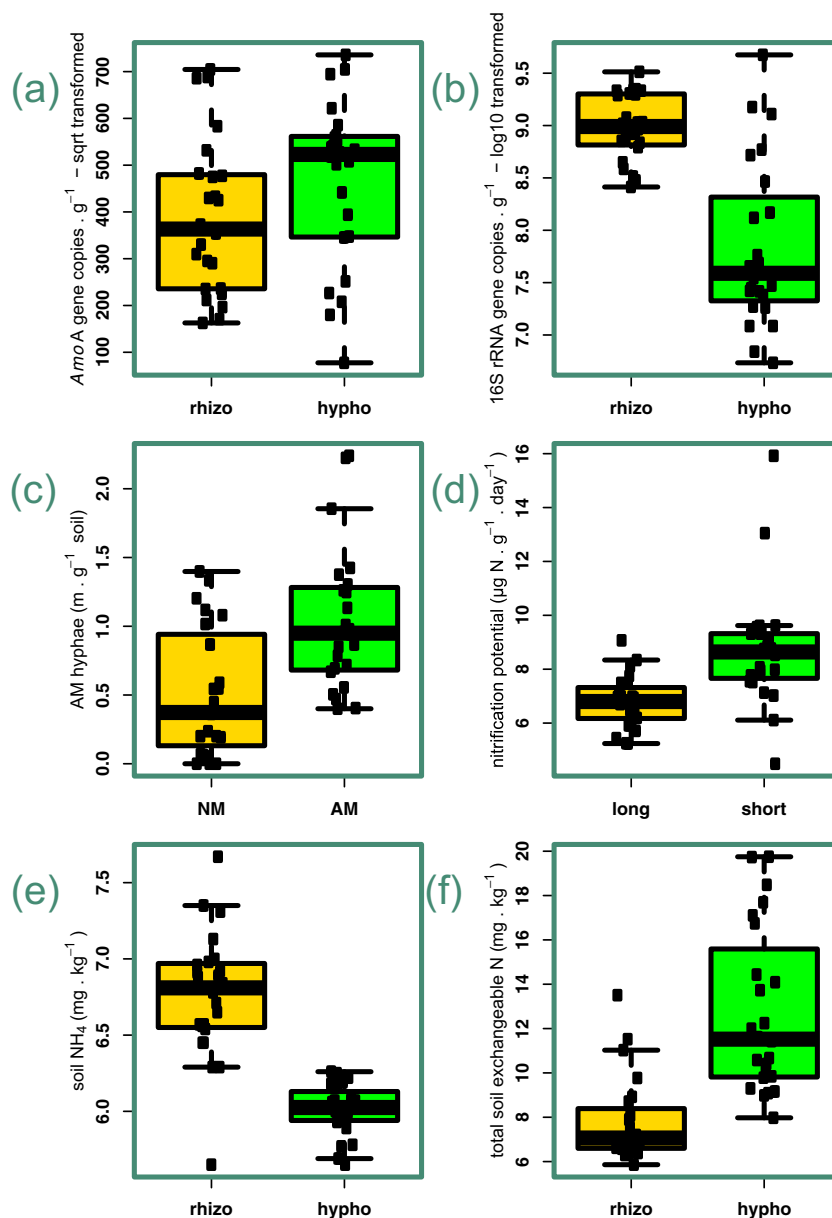
To assess how the community structure of AO-bacteria changed in response to our manipulations, we used a redundancy analysis on Hellinger transformed relative abundance data of AO-bacteria [39]. To examine which of our predictors were significant, we applied a test of significance of terms developed by Legendre et al. [40] and implemented through the command *anova.cca* in the R package *vegan* [47]. Based on the test of Legendre et al. [40], significance of terms is evaluated after comparing the observed F values with those that are obtained following a randomization procedure (i.e. permutations). To account for the split plot nature of our experimental design, we introduced a constraint in our permutations so that rhizosphere and hyphosphere compartments were always paired [48]. We report P values both without (P_{naive}) and with ($P_{splitplot}$), this permutation constraint. We were additionally interested in the way the AO community changes while interacting with plant roots and AM fungi (i.e. realistic settings). This is why we repeated the ordination with the subset of rhizosphere samples.

We address *amoA* community co-occurrence patterns with a null model analysis as implemented with the *cooc_null_model* command in the R package *EcoSimR* v 3.1.3. We discarded abundance information of our community matrix and worked with a presence-absence table. To generate the simulated values, we used the *sim9* randomization algorithm (i.e. we fixed both row and column sums in our permutations) and worked with a C -score (i.e. checkerboard score) metric. We analysed separately community data from short- and long-incubation treatments to control for the differences in the microbial community structure which were already present when we initiated the experiment. We further assembled a table of forbidden species combinations and assessed the probability of them not co-occurring at each subset of the dataset.

Results

Following staining, we observed mycorrhizal structures in all plants that had been inoculated with mycorrhiza (but none in plants that had not received mycorrhizal inoculum) and the densities of AM hyphae in soil were higher in these mesocosms (Fig. 2c). Long-incubation times resulted in high densities of non-mycorrhizal hyphae, whereas AM hyphae were denser in the AM compartments in the long incubation treatment (Fig. 2c; Fig. S1c). There were no differences in biomass between AM-inoculated and non-mycorrhizal plants (Fig. S3a).

Fig. 2 Bee swarm plots illustrating how some key parameters we assayed varied with regard to the most influential experimental predictor. With the exception of *AmoA* gene transcript numbers in panel (a) displayed differences were significant. Specifically: **a** there were no difference in *amoA* gene transcript numbers or their variance irrespective of predictor used (see hypothesis one and hypothesis four); **b** rhizosphere compartments contained approximately 14.5 times as many bacteria as hyphosphere compartment; **c** we could detect significantly more AM hyphae in mycorrhizal (AM) samples—note that identifying a hypha as mycorrhizal involves a certain degree of uncertainty; **d** the only experimental factor that explained potential nitrification rates was incubation time; **e** we found considerably higher availability of NH_4^+ in the rhizosphere compartments; **f** by contrast, total exchangeable N availability (here calculated as the sum of NH_4^+ and NO_3^- concentrations) was highest in the hyphosphere compartments. More specific information on the relationship between the manipulations and the response variables is presented at Fig. S1 and Fig. S2



Archaeal *amoA* gene transcript densities in soil were below the RT-qPCR detection limit (data not shown). Bacterial *amoA* gene transcript numbers did not change with incubation time (Fig. S3b), addition of AM fungi (Fig. S3c) or in the presence of plant roots (Fig. 2a; Fig. S1a) and averaged (across all treatments) 2.1×10^6 copies per g of dry soil. Based on 16S rRNA gene transcripts, there were approximately 14.5 times more bacteria in rhizosphere soil than in hyphosphere soil; however, the addition of AM fungi or incubation time resulted in no further differences (Fig. 2b); Fig. S1b. Potential nitrification rates were only influenced by incubation time and were higher in the soil that had only been incubated for 2 weeks (short incubation—Fig. 2d; Fig. S2a). N availability at harvest was considerably lower than in the

beginning of the experiment (Fig. 2e, f; Fig. S2 compared to inorganic N concentrations reported at mesocosm setup); however, it remained high compared to most comparable controlled experiments in the literature (e.g. [26]). We observed higher concentrations of exchangeable N in the soil that was incubated for 2 weeks (Fig. 2f); however, incubation time did not explain differences in soil NH_4^+ concentrations which were higher in rhizosphere soil (Fig. 2e). We observed no differences across the two mycorrhizal treatments in the ratio of 16S gene copies over N availability, with the only significant factor being microcosm compartment (Fig. S4).

We obtained 378,917 quality controlled reads ranging (with the exception of two samples with 1118 and 1194 reads respectively) between 3546 and 11,448 per sample. After

normalizing to 2500 sequences per sample, we counted 31 different AO-bacterial taxa. Representative sequences of each taxon have been deposited to GenBank—accession numbers MF671777–MF671794 (in the supplement we present information on their occurrence across samples). Between 99% and 100% of the reads per soil sample belonged to the *Nitrospira*-like cluster (Fig. 3). The three most abundant taxa, E01ATU4N, E03CWDEP and E03C629G were related to *Nitrosospira* sp. Nsp5 (AY123834, 98% identity, bitscore 737), *Nitrosospira* sp. Nsp17 (AJ298717.1, 98% identity, 516 total score) and *Nitrosospira* sp. APG3 (KC477403, 93% identity, 383 total score) and together accounted for a median of 91.3% (1st quartile 86.5%; 3rd quartile 95.05%) of the *amoA* reads across all samples. Our indicator species analysis yielded a single taxon that was specific to rhizosphere

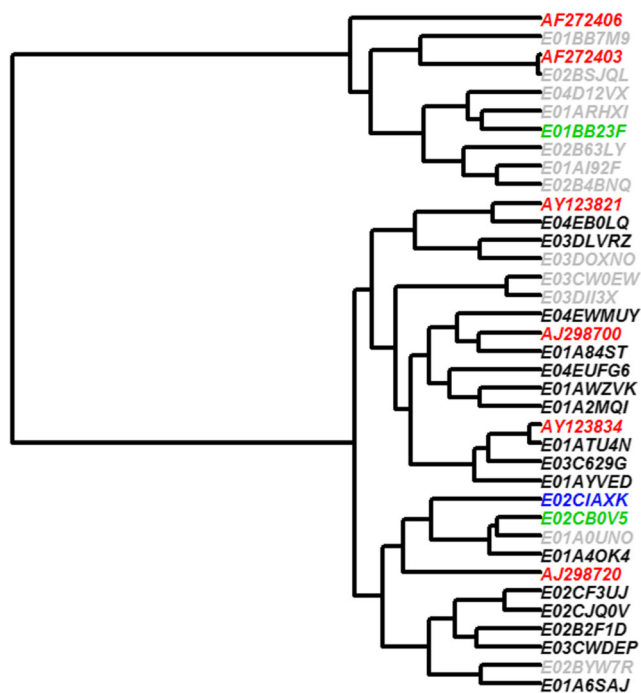


Fig. 3 Phylogenetic tree of reference AO-bacterial taxa (in red) plotted with the taxa remaining after quality control in this study (see hypothesis 3). Taxa in grey were found in fewer than six samples. Taxa in green (the best blast hit of the significant at $P=0.01$ level, E01BB23F was *Nitrosomonas* sp. Nm84 whereas the best blast hit of E02CB0V5 was *Nitrosovibrio* sp. RY3C) were specific of non-mycorrhizal samples whereas the taxon in blue (the best blast hit of E02CIAXK was *Nitrosospira* sp. N15) was specific to hyphosphere soil. Delineation of the AO taxa was based on a 97% similarity threshold as implemented in CROP ([29] Bioinformatics 5: 611–618). The two major clusters in the tree correspond to the *Nitrosomonas* group (top) and the *Nitrospira* group (bottom). The reference taxa were as follows: AF272406: *Nitrosomonas oligotropha*, AF272403: *Nitrosomonas urea*, AY123821: *Nitrospira briensis*, AJ298700: *Nitrosococcus oceani*, AY123834: *Nitrospira* sp. Nsp5, AJ298720: *Nitrospira tenuis*. The tree was rooted with the *amoA* sequence of a gamma-proteobacterium, *Nitrosococcus halophilus* (AF272521) that was cropped from the tree for a better visualization. Note the high frequency of indicator species and their more or less even spread over the clades of the tree

compartments (E02CIAXK, best blast hit: *Nitrosospira* sp. N15 AY123832, identity 92% total score 420) and two indicator species of the absence of mycorrhiza (E02CB0V5, E01BB23F, best blast hits *Nitrosovibrio* sp. RY3C, DQ228466, identity 96% total score 651 and *Nitrosomonas* sp. Nm84, AY123818, identity 93% total score 656, respectively). Based on our indicator species analysis, we found higher (ten species vs two and one for the other treatments, $\chi^2=11.2$, $P=0.003$) AO specificity to the different incubation treatments with three species being characteristic of a low incubation time (E01A6SAJ, E02CIAXK, E01BB23F) and seven species of an extensive incubation time (E04EWMUY, E02CJQ0V, E02CF3UJ, E02CB0V5, E01A84ST, E01A2MQI, E01A4OK4). We found no relationship between either Shannon diversity, richness or Faith's PD index and potential nitrification rates (Fig. S5). We detected, however, a higher evenness in the rhizosphere rather than the hyphosphere compartments (Fig. S3d).

Before carrying out a constrained ordination analysis, we further scaled our Hellinger transformed community matrix to control for the fact that the three most abundant AO taxa dominated the AO community. This is a procedure where the relative abundances of each taxon are rescaled to a zero mean and a unit standard deviation so that each taxon has an equal influence to the correlation matrix and is also known as a Z-score transformation. Incubation time ($F=4.80$, $P_{\text{naive}} < 0.001$, $P_{\text{splitplot}} < 0.001$), competition with plant roots ($F=1.64$, $P_{\text{naive}} = 0.010$, $P_{\text{splitplot}} < 0.001$) and inoculation with AM fungi ($F=1.50$, $P_{\text{naive}} = 0.023$, $P_{\text{splitplot}} = 0.026$) were all important predictors of the community structure and the result was robust to the order in which we introduced the three predictors (Fig. 4a). The act of scaling (through Z-score transforming) a community matrix before a constrained ordination divides variance equally across all taxa present in the community matrix (e.g. [46]). To ensure that our results were not driven by taxa that were only present in a few samples, we repeated the analysis after filtering out taxa with low occurrence frequencies (Fig. S6) and found that the F values for AM fungi (and consequently significance) consistently increased. Through excluding taxa that were only present in five or fewer samples in the dataset, for example, we found the following F values (incubation: 7.46, plant compartment: 2.24, AM status: 2.00); we focus our results, however, on the analysis of the full community matrix, so as not to make arbitrary decisions on the exclusion threshold. We noticed that the predictor 'AM fungi' was no longer significant when we did not scale the community matrix suggesting that AM fungi had little influence on the three dominant species. We found two significant RDA axes in our analysis (Fig. 4a). The first RDA axis explained 9.5% of variance and described the effect of incubation time, whereas the

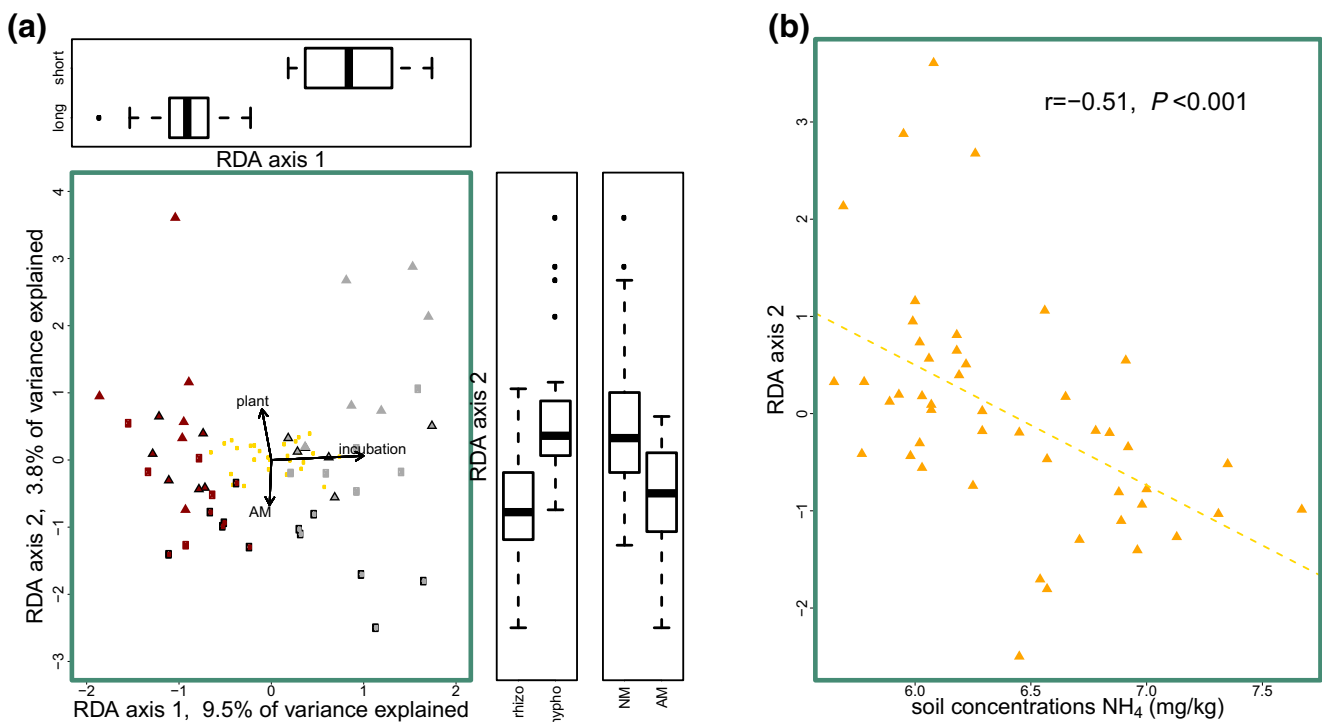


Fig. 4 **a** Redundancy analysis (RDA) ordination plot produced after Hellinger-transforming the AO-bacterial community matrix and fitting three predictors: incubation time; mycorrhizal status and plant compartment (see hypothesis two). The three predictors explained together (adjusted R^2 value) 9.73% of total variance. The loading vector for incubation time was parallel to RDA axis 1 whereas those of AM state and root compartment vertical. We generated boxplots for each of these predictors (incubation time—RDA axis 1; AM state and root compartment—RDA axis 2) to highlight the spread of scores in those axes. Rhizosphere samples are depicted as circles whereas hyphosphere

as triangles; non-mycorrhizal samples were uniformly colored whereas AM-inoculated samples have a black outline. Grey samples had been incubated with a microbial filtrate before initiating of the experiment for 14 days whereas red ones for 97. There were no significant RDA axes other than the two displayed. We plotted categorical predictors as continuous variables for better visualization. **(b)** We plotted the scores of RDA axis 2 against respective residual soil concentrations of NH_4^+ to highlight the strength of the relationship of the two variables. This suggests that both AM state and root compartment factors influenced AO community structure through altering the availability of NH_4^+

second axis explained 3.8% of the variance and was characterized by high loadings of the predictors AM-addition and root compartment (Fig. 4a). The addition of mycorrhiza shifted the AO community in RDA space in an opposite direction than the transition from the rhizosphere to the hyphosphere. We extracted the scores of the second axis and regressed it against residual soil NH_4^+ concentrations and found a strong relationship ($r = -0.51$, $P < 0.0001$, Fig. 4b). We repeated the ordination analysis with the subset of rhizosphere-only samples and despite the lower statistical power, we found that both incubation time ($F = 3.24$, $P = 0.001$) and AM state ($F = 1.48$, $P = 0.038$) were significant.

In the subset of samples that had been incubated for a short time (Fig. S7—right) as well as the full dataset (not shown), we found evidence for non-random co-occurrence patterns. In both cases, the observed C-score was higher than the simulated suggesting community matrices more segregated than expected by chance. We also report on the 20 most characteristic instances where OTUs did not co-occur in our dataset (Table S1).

Discussion

We carried out a controlled experiment and addressed how mycorrhiza impacts nitrification in a naturally fertile soil. We asked if the addition of mycorrhiza under high-fertility settings suppresses potential nitrification rates and densities of AOB (Fig. 1c); (ii) mycorrhiza induces community shifts via direct mechanisms to the AOB community; (iii) there are several AOB taxa that proliferate only when the plant associates with AM fungi and benefit from comparable mechanisms that favour other AM-specific prokaryotes; (iv) plants can partially mask any AM-induced shifts in the bacterial community of AO. In our experiment, we steam-sterilized the soil which allowed us to manipulate the mycorrhizal state of *P. lanceolata*. The absence of AOA resulted in a more tractable system and granted us a high mechanistic resolution. We worked with cDNA, which allowed us to exclude non-active microbes from our measurements.

Our null model analysis showed that the AO communities did not represent random assemblages but that they

were structured by biotic and abiotic interactions which we tried to determine. The potential nitrification rates and densities of *amoA* gene transcripts did not change with either the inoculation with AM or the manipulation of root access (i.e. evidence against hypothesis one). We observed, however, that both AM inoculation and access of roots mediated small (~4% of variance) but significant shifts in the community structure of AOB (i.e. scenario in Fig. 1b; evidence in support of hypothesis two). Adding AM propagules to the soil resulted in AOB community shifts in the opposite direction in RDA space (which we later show that depicts oligotrophic growth conditions) of those we observed while transitioning from rhizosphere soil in our mesocosms to hyphosphere/bulk soil. The AOB community shifts correlated well with declines in the availability of residual NH_4^+ in soil that had been either inoculated with AM fungi or was located in the rhizosphere compartments. We found more bacteria in the rhizosphere than in the hyphosphere of our mesocosms, and we believe that both heterotrophic bacteria (more abundant in the rhizosphere—Fig. S8) and AM fungi (AM-treatments) immobilized soil NH_4^+ which was then unavailable to microbial taxa that cannot compete equally well such as AOB [8]. We found no evidence for an N-priming effect in the rhizosphere, i.e. that we would detect higher availability of NH_4^+ and nitrification rates in the rhizosphere because of higher carbon availability (Fig. 2a, f). Since we also observed no suppression of AOB in AM-inoculated mesocosms, we find it unlikely that either N-priming effects or allelopathy (i.e. if there were allelopathic effects, we would expect that AM fungi reduce rates of potential nitrification even under fertile setting which was untrue) explained AOB dynamics. We thus think that competition with other microbes for soil NH_4^+ (i.e. the first of the three postulated mechanisms) mainly explains how mycorrhiza shapes the community structure of AO in high-fertility habitats.

Our key result here was that AM fungi induced shifts in the community structure of AOB communities in our mesocosms via exploitative competition for NH_4^+ . It is important to carefully evaluate the degree to which our data support this claim. In our ordination analysis, the effect of AM fungi on AOB was captured exclusively by the second RDA axis, which only explained 3.8% of variability. Nevertheless, this was a significant RDA axis (one of the two), the loading of the AM fungal vector in the RDA coincided with that of the rhizosphere compartment which has a plausible explanation, and the scores of the second RDA axis reflected well the residual NH_4^+ status of the soil. These three arguments together suggest that the AOB community shifts were non-random (Fig. 1b). Moreover, via RDA, we visualized the responses of the entire community of AOB and not of single taxa. Obviously, some taxa should have been less sensitive

to NH_4^+ availability than others, particularly given that even at the end of the experiment NH_4^+ availability was high. We believe that using state-of-the-art techniques, we could capture a weak (given the high soil fertility) but real relationship in our system. Competition between AOB and AM fungi might have only taken place at a microscale in depletion zones [58] close to the root and AM hyphae and we find it remarkable that it was of sufficient strength to alter the structure of AOB at a much larger scale. Because AOB grow so slowly, the AOB community structure should be less responsive to changes to the abiotic environment and resemble better earlier growth stages than communities of faster growing microbes do. This unavoidably leads to *amoA* communities capturing better average conditions at large spatial scales than transient fluctuations in NH_4^+ availability at a microscale. In agreement with this argument, we only found indicator species that specialized in rhizosphere soil or absence of AM fungi. Soil NH_4^+ was higher in rhizosphere compared to hyphosphere soil and there was a trend for NH_4^+ to be higher in the absence of AM fungi (Fig. 2e; Fig. S2b; also consult Fig. 4b). Most likely these indicator species were not actually specific to the rhizosphere or the absence of mycorrhiza but appeared as such because they could not tolerate an unusually low NH_4^+ availability (i.e. evidence in support of hypothesis four). This finding, which we could not attribute to differences in the water availability between mycorrhizal and non-mycorrhizal mesocosms, as we carefully watered the mesocosms on a daily basis, was incongruent with hypothesis three, that there are several AOB taxa that proliferate only when the plant associates with AM fungi and benefit from comparable mechanisms that favour other AM-specific prokaryotes.

In our experiment, the incubation time of the soil with the bacterial filtrate was the manipulation that had the strongest effects on both AO community structure and potential nitrification rates. As a matter of fact, incubation time impacted AO and nitrification in a way that resembled experimenting with two entirely different soils. AO often require weeks for cell division which makes them some of the slowest-growing known microbes [49]. Amora-Lazcano et al. [3] represents one of the earliest studies that focused on the interplay between AO and AM. The authors of that time-course experiment demonstrated how strongly the dynamics of non-equilibrium-AO communities change over time under the combined influence of AO growth, growing plants and mycorrhizal fungi and drew attention to how important it is to consider AM in nitrification studies [3]. To a certain degree, limitations of studying AO under non-equilibrium conditions have been discussed in Veresoglou [71]. Nevertheless, it came as a surprise to realize that incubation time had so strong an influence that overshadowed those of major sinks of soil NH_4^+ such as

plant roots and AM. Nonetheless, we still observed very consistent responses of AO to plant roots and AM across the two incubation treatments (Fig. 4). To induce greater maturity in our microbial community, we waited for over 3 months in our long-incubation treatment; this should have been sufficient time for AOB to reach densities that were comparable to those found under field settings. For example in Veresoglou et al. [72], we report nitrification potential rates in unsterilized grassland soils between 1 and 10 $\mu\text{g N g}^{-1} \text{day}^{-1}$ compared to mean endpoint measurements exceeding 7.5 $\mu\text{g N g}^{-1} \text{day}^{-1}$ that we found in this study. Unfortunately, we did not assay *amoA* gene expression numbers at the beginning of the experiment, but we could observe pronounced differences between the two incubation treatments at the end of the experiment (Fig. 2d; Fig. 4). We believe that via applying these two treatments, we captured well the two extremes of AOB maturity settings and we can generalize our results beyond the state settings of AOB when initiating experiments.

Our result that in fertile soils mycorrhiza does not alter soil densities of AO-bacteria supports another pioneering study by Cavagnaro et al. [14]. Cavagnaro et al. [14] grew mycorrhizal and mycorrhizal-defective-mutant tomato plants grown under agricultural settings and found no AM-state-induced differences in RT-qPCR-assessed *amoA* gene transcript densities. Negative results can always bring into question whether the absence of the relationship is a robust result or reflects a limited statistical power. It appears to us that the relationship between potential nitrification rates and AM state, which is strong in low-fertility soils [72], are weakened enough under conditions of high fertility to remain undetectable even in well-executed studies. We believe that under settings of high-N-availability, the ecological importance of AM fungi in reducing potential nitrification rates and suppressing AO declines, and AM fungi have less conspicuous effects on the nitrifying community because under such conditions AM fungi mainly support the nutrition of their plant-hosts through providing P [33, 34, 63, 74]. Because of their shift towards plant P-nutrition, AM fungi should no longer represent major sinks of soil NH_4^+ , but instead only assimilate soil NH_4^+ to meet their growth demands which should be of little ecosystem importance. Given that the interaction mechanism between AM fungi and nitrification is probably competition for NH_4^+ , this should prevent them from having strong direct effects on nitrification.

Concurrent shifts in the structure of the AO-community, however, probably remain important from an ecological point of view. As we argue in the introduction, shifts in the AO community should alter the temporal and spatial stability of actual nitrification rates and the resilience of the nitrifying community to abiotic stresses. Nitrification represents an indispensable constituent of ecosystem functioning and

any fluctuations in process rates over time can be ecologically meaningful. Moreover, we are convinced that our experimental settings may have underestimated how AM influences nitrifiers because we did not assess the full spectrum of ecosystem functions that arise from mycorrhiza. We are becoming increasingly aware, for example, that mycorrhizal plants reduce nutrient leaching from soil compared to non-mycorrhizal counterparts notwithstanding NO_3^- availability [15, 36, 67]. Additionally, mycorrhiza through improving soil aggregation [41, 54] promotes aeration in the soil environment and lowers potential rates of denitrification [6]. Most importantly, we could not test the full spectrum of mycorrhizal functions to the plant-host such as pathogen protection and better water relationships which should further improve the relative fitness of AM-plants [45, 70]. Currently, we cannot fully understand how these additional considerations may alter our understanding of nitrification and mycorrhiza in the long-term but represent interesting topics for future experimental work.

In the introduction, we claimed that through studying the interplay between mycorrhiza and nitrification, we could address an important topic from an agricultural point of view. There are definitely numerous reasons for farmers to manage for mycorrhiza, some of which are discussed in Rillig et al. [55]. Here, we did not observe any changes in the rates of potential nitrification that we could attribute to mycorrhiza. A question that remains open, however, relates to the degree to which the shifts we observed in the community structure of AO after adding mycorrhiza might mitigate nitrification rates under conditions of drought or pathogen stress. Could small but predictable changes in *amoA* community composition such as those we detected in our experiment alter resilience and stability of nitrification rates to stress factors to a degree that they are of ecological significance?

Conclusion

We presented here possibly the most comprehensive study to date on how mycorrhiza impacts nitrification under conditions of high N availability. We formulated four hypotheses. We found no evidence that mycorrhiza alters potential nitrification rates or that there are AO taxa that exhibit a high affinity to AM fungal treatments (hypotheses One and Three). We did find support for AM fungal-induced shifts in the community structure of AO-bacteria and that plant roots overwhelm to a large degree the impact of mycorrhiza. Taken together, our results further illuminate indirect effects of mycorrhiza on ecosystem functioning which could be of high applied value in farming and the conservation of ecosystems.

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Author Contributions Conceived the study, run and harvested the experiment and assayed biochemical parameters: SDV; shared the molecular work: SDV, EV and OM; extracted hyphae from soil: IM; did the statistical analysis and bioinformatics: SDV; SDV wrote the manuscript with the help of EV, RS and MCR and everybody provided comments.

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