



Appropriate nonmycorrhizal controls in arbuscular mycorrhiza research: a microbiome perspective

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Abstract

Establishment of nonmycorrhizal controls is a “classic and recurrent theme” in mycorrhizal research. For decades, authors reported mycorrhizal plant growth/nutrition as compared to various nonmycorrhizal controls. In such studies, uncertainties remain about which nonmycorrhizal controls are most appropriate and, in particular, what effects the control inoculations have on substrate and root microbiomes. Here, different types of control and mycorrhizal inoculations were compared with respect to plant growth and nutrition, as well as the structure of root and substrate microbiomes, assessed by next-generation sequencing. We compared uninoculated (“absolute”) control to inoculation with blank pot culture lacking arbuscular mycorrhizal fungi, filtrate of that blank inoculum, and filtrate of complex pot-produced mycorrhizal inoculum. Those treatments were compared to a standard mycorrhizal treatment, where the previously sterilized substrate was inoculated with complex pot-produced inoculum containing *Rhizophagus irregularis* SYM5. Besides this, monoxenically produced inoculum of the same fungus was applied either alone or in combination with blank inoculum. The results indicate that the presence of mycorrhizal fungus always resulted in stimulation of *Andropogon gerardii* plant biomass as well as in elevated phosphorus content of the plants. The microbial (bacterial and fungal) communities developing in the differently inoculated treatments, however, differed substantially from each other and no control could be obtained comparable with the treatment inoculated with complex mycorrhizal inoculum. Soil microorganisms with significant biological competences that could potentially contribute to the effects of the various inoculants on the plants were detected in roots and in plant cultivation substrate in some of the treatments.

Keywords Appropriate nonmycorrhizal control · Microbiome · Pot experiment · *Rhizophagus irregularis* · *Arthrobotrys* · *Andropogon gerardii*

Introduction

Frequently, research on the (eco-)physiology of arbuscular mycorrhizal symbiosis necessitates establishment of

nonmycorrhizal control treatments, which are then compared to the mycorrhizal plants to demonstrate benefits and/or costs of the symbiosis (Kahiluoto et al. 2000; Řezáčová et al. 2018). Mycorrhizal plants are usually created by exposing them to viable inoculum of arbuscular mycorrhizal fungus (or fungi), most commonly produced in open-pot cultures, containing mycorrhizal fungus and other microbes (e.g., Nazeri et al. 2013). To compare the growth/nutrition of mycorrhizal plants with the control (nonmycorrhizal) plants, mycorrhiza researchers strived for a long time to develop a procedure to establish relevant nonmycorrhizal controls in their experiments, i.e., pots lacking mycorrhizal fungi but otherwise exactly the same as the mycorrhizal pots, with the same nutrient and heavy metal availabilities to plants and with the same composition and diversity of microbial communities (Kahiluoto et al. 2000; Kahiluoto and Vestberg 2000; Slavíková et al. 2017). Such controls are indeed of paramount

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importance with respect to quantification of the effects/contribution of the mycorrhizal fungi (with no other confounding factor) to plant growth/nutrition/health and/or to nutrient/pollutant fluxes in ecosystems (Pizano et al. 2017).

If normal host plant genotypes susceptible to colonization by the arbuscular mycorrhizal fungi are used in the pot trials, experimental substrates (at least in a control nonmycorrhizal treatment) must be free of mycorrhizal fungi at the beginning of such experiments. Typically, the soil/substrate needs to be sterilized/disinfected to reach such a state. This is usually connected with changing physicochemical properties of the soil/substrates (e.g., Shaw et al. 1999) and shifts in important components of microbial communities such as suppression of slowly growing nitrification prokaryotes due to very slow recolonization of the previously sterilized substrate/soil (Veresoglou 2012).

As the arbuscular mycorrhizal fungi are obligate biotrophic organisms, their inoculum cannot be produced on an artificial medium in absence of their host plant and must always be multiplied together with their hosts in microcosm (pot) cultures or in root-organ cultures (Fortin et al. 2002). Instead of pure mycorrhizal fungal biomass, various formulations containing spores, mycelium, and colonized host roots produced under nonsterile conditions are thus most commonly used for inoculation of experimental plants. Such inocula contain, besides mycorrhizal fungi, diverse communities of accompanying microbiota such as bacteria, fungi, protists, algae, and animals. Therefore, interpretation of the results of most of the mycorrhizal experiments remains loaded with uncertainty about possible contribution of such accompanying microorganisms to the observed effects of mycorrhizal inoculation and it remains a challenge to distinguish effects of such accompanying microbiota from the effects of mycorrhizal fungi (Koide and Li 1989).

At this point, a problem of appropriate nonmycorrhizal controls appears. In the best-case scenario, the rooting substrate in mycorrhizal treatment should differ from that in nonmycorrhizal control treatment only by the presence of the mycorrhizal fungus. In a real world, however, accompanying organisms are often introduced into mycorrhizal treatment together with mycorrhizal fungus and even the spores extracted from the crude complex inocula are frequently contaminated by saprotrophs and/or spore parasites. Several possible solutions to address this issue have been used in the past (e.g., Merryweather and Fitter 1996; Kahiluoto et al. 2000; Kahiluoto and Vestberg 2000; Nazeri et al. 2013; Püschel et al. 2017):

- 1) Application of filtrate of pot-cultured mycorrhizal inoculum to the nonmycorrhizal control treatment, which would complement the absence of some accompanying microorganisms present in the mycorrhizal inoculum.
- 2) Application of a substrate from nonmycorrhizal pot inoculum culture, which was cultivated alongside a pot inoculum culture of the mycorrhizal fungus. This substrate represents a kind of “blank” (sometimes referred to as “mock”) inoculum providing accompanying microorganisms and, at the same time, the organic matter and mineral nutrients of the same quality and quantity as present in the mycorrhizal inoculum (e.g., host root fragments, fertilizer residuals).
- 3) Application of monoxenically produced mycorrhizal inoculum lacking accompanying microorganisms and additional organic matter in mycorrhizal treatments. Mycorrhizal and nonmycorrhizal control treatments could then be supplied or not with the same community of accompanying microorganisms (blank or filtrate).
- 4) The use of nonmycorrhizal mutants (genotypes) of host plants in the nonmycorrhizal control treatments with substrate containing the active inoculum of mycorrhizal fungi.
- 5) Inactivation of mycorrhizal fungi in nonsterile substrate/soil in the nonmycorrhizal control treatments by selective fungicides such as benomyl.

The first method of nonmycorrhizal control treatment establishment is the most frequently used approach, but the filtration process obviously discriminates for size/mobility of the different biotic components and thus cannot be regarded as optimal. Mainly, it prevents inclusion into the nonmycorrhizal controls of diaspores of most soil microfauna such as nematodes or mites, reducing the functionally important diversity of larger organisms (Wagg et al. 2014). The presence of soil/inoculum filtrate is thus unlikely to fully support reestablishment of the complex soil/inoculum microbiome even in terms of its effect on the development of mycorrhizal symbiosis: mycorrhizal plants grown in sterilized soil/substrate may show lower colonization levels of their roots by the mycorrhizal fungi than the plants grown in unsterilized soil and plant biomass may be higher in plants grown in sterilized soil without filtrate addition (Manian et al. 1995).

The second method introduces complex microbial community into the inoculated treatment but it is known that the microbiome of mycorrhizal roots may differ from that of nonmycorrhizal roots (Marschner et al. 2001; Viollet et al. 2011). Moreover, the establishment of “blank” cultures themselves suffers from the same problem as the establishment of nonmycorrhizal control treatments. Then, even the second method cannot be accepted without reservations to create an ideal nonmycorrhizal control.

The third method can provide the ideal controls, but it has been relatively infrequently used in mycorrhizal experimentation so far (but see Leigh et al. 2011; Kiers et al. 2011). The reason is that only a limited number of species/genotypes of arbuscular mycorrhizal fungi has hitherto been multiplied on

axenically grown host (for example on the root organ culture) and preparation of pure inocula via the in vitro cultures can be laborious and expensive (but see Rosikiewicz et al. 2017). Hypothetically, surface decontamination of spores extracted from nonsterile pot cultures is also possible but there is a risk of survival of resistant stages of contaminants and intracellular parasites/endosymbionts of the mycorrhizal fungal spores that cannot be efficiently detected or removed (Naumann et al. 2010).

The fourth method is promising but requires tight nonmycorrhizal mutant genotypes of host plants that differ from the wild (mycorrhizal) isolines just by their inability to form mycorrhizae, other physiological properties being exactly the same. Such genotypes are extremely rare and confined to only a handful of model plant species, which limits the use of this experimental strategy (Marsh and Schultze 2001; Cavagnaro et al. 2006; Willmann et al. 2013; Kojima et al. 2014).

The fifth method assumes the existence of a fungicide which is highly selective for arbuscular mycorrhizal fungi. Though benomyl was frequently used in the past and scrutinized also by Kahiluoto et al. (2000), the absolute specificity of this fungicide towards the arbuscular mycorrhizal fungi without affecting other soil biota cannot be expected and its application also does not prevent the establishment of mycorrhizal symbiosis completely (Kahiluoto et al. 2000; Kahiluoto and Vestberg 2000).

Having the recently available high-throughput microbial community profiling tools and axenically produced arbuscular mycorrhizal fungal inoculum at hand, we conducted a pot experiment with a model host plant *Andropogon gerardii*, employing a number of different mycorrhizal inoculation approaches and established also a number of nonmycorrhizal control treatments, so as to identify which of the control treatments would be the most appropriate to the mycorrhizal inoculation with respect to plant growth and/or nutrition and also with respect to the composition/structure of root/substrate microbiome. We expected that the microbial communities in mycorrhizal and nonmycorrhizal treatments inoculated by different materials (e.g., complex open-pot produced mycorrhizal inoculum, inoculum of arbuscular mycorrhizal fungus produced monoxenically in vitro, blank inoculum, and inoculum filtrates) will markedly differ from each other and that this would have pronounced effects on the plant performance in the pots.

The following questions were asked to specifically address which of the different control inoculation treatments would be most appropriate to use in studies on mycorrhiza (eco-)physiology and in future studies addressing effects of mycorrhizal symbiosis on other soil microbiota:

1) Do there exist soil microorganisms which are specifically associated with arbuscular mycorrhizal symbiosis and

which thus appear exclusively or preferentially in mycorrhizal pots regardless of the manner of their establishment, such as inoculation with complex open-pot produced inoculum or inoculation with monoxenically produced spores complemented or not with other soil microbiota?

2) Is the inoculation with filtrates derived from blank or mycorrhizal inocula sufficient to reestablish substrate/root microbiomes in experimental pot cultures which would be similar to that in cultures inoculated with complete (unfiltered) blank or with the mycorrhizal inocula?

3) Is there evidence for gradual adaptation of soil microbiomes to the presence of mycorrhizal symbiosis? The answer to this question is directly connected with following two very practical questions regarding the design of the mycorrhizal pot experiments:

- Is the blank (mock) inoculum, i.e., the inoculum produced in pots in the same manner as the mycorrhizal pot cultures but lacking mycorrhizal fungi, an appropriate control inoculum for experiments with complete (pot-based) mycorrhizal inocula?
- Is the inoculation with monoxenically produced mycorrhizal hyphae and spores combined with blank inoculum equivalent to inoculation with the complete soil inoculum?

Materials and methods

Design of the experiment

The pot experiment involved seven inoculation treatments presented in Table 1 and in the Supplementary Fig. S1. Each treatment involved 12 replicate pots (2 l each, 10 × 10 × 20 cm) filled with mixture of γ -sterilized soil (> 25 kGy; further details in Řezáčová et al. 2018), autoclaved sand, and autoclaved zeolite 1–2.5 mm grain size (10:45:45, by volume). This substrate had the following properties: pH (H₂O) 8.9, 0.22% total organic carbon (C), 0.013% total nitrogen (N), 46.5 mg/kg total phosphorus (P), and 2.6 mg/kg water extractable P. The pots were then supplied with appropriate filtrates, inocula, or blanks (see below), sown with 30 seeds of *Andropogon gerardii* (provided by Jelitto Staudensamen GmbH, Schwarmstedt, Germany, www.jelitto.com) per pot, randomized and incubated in a greenhouse with supplementary lighting (> 200 μ mol photosynthetically active radiation over 14 h daily) for 10 weeks (January 26, 2015 to April 7, 2015). From the fifth week after planting, each pot received weekly 65 ml mineral nutrient solution according to Long Ashton (Mortimer et al. 2008) with phosphorus concentration decreased to 20% of the original recipe.

Table 1 Overview of the inoculation treatments used in this study. For detailed description of preparation of inocula see “Materials and methods” and the online supplementary materials—Supplementary Fig. S1

Treatment code	Inoculation
UI	None; the treatment was subjected to spontaneous colonization by microflora present in greenhouse
BL	Inoculated by pot culture substrate lacking arbuscular mycorrhizal fungi (blank)
BLMX	Inoculated by inoculum used in BL combined with inoculum used in MX treatments
BLF	Inoculated by filtrate of inoculum used in BL treatment
CI	Inoculated by complex mycorrhizal inoculum (pot culture substrate containing <i>Rhizophagus irregularis</i> SYM5)
CIF	Inoculated by filtrate of inoculum used in CI
MX	Inoculated by monoxenically produced inoculum of <i>Rhizophagus irregularis</i> SYM5 only, no other inoculation

Complex mycorrhizal inoculum

Arbuscular mycorrhizal fungus *Rhizophagus irregularis* SYM5 was cultivated in open pot culture in a greenhouse with leek as a host plant for 7 months. The cultures were maintained in the same pots and substrate as used in the greenhouse experiment described above. At the beginning of cultivation, the cultures were inoculated with approximately 20 mg of pure, monoxenically produced biomass (mycelium + spores) of *R. irregularis*, suspended in 1 ml water, obtained as a gift from Symbiom Ltd., Lanškroun, Czech Republic (www.symbiom.cz). The mycelium was released from the medium originally solidified by Phytigel by slow agitation in 10 mM potassium citrate (pH 6.0), washed in 1% MgSO₄·7H₂O, dried briefly on paper towel and resuspended in water. Upon the inoculation, the substrate in each pot was added with 200 ml of filtrate obtained from the same nonmycorrhizal substrate previously planted with leek. The filtrate was prepared by vigorous agitation (20 min) of the substrate suspension in water (1:10, w/w) and filtration through two layers of filter paper (see below for more details). Complex mycorrhizal inoculum, composed of mycorrhizal hyphal fragments, spores, colonized roots (chopped to fragments below 1 cm in length) mixed with the potting substrate, was applied at a rate of 60 g per experimental pot, each pot receiving 8.0 ± 1.0 mg mycelium (that could be picked by fine forceps) and 228 ± 5 mg mycorrhizal root biomass (FW) as infective structures. DNA was extracted from three ~600 mg aliquots of the complete inoculum as described in “DNA extraction” for substrate samples. The concentration of nuclear large ribosomal subunit (LSU) rRNA gene of *R. irregularis* was then quantified (in triplicate) as described in “Quantification of the mycorrhizal fungus by qPCR” and was equal to 4.69 ± 1.22 × 10³ gene copies per mg dry weight of the substrate sample.

Blank inoculum

This control (blank, mock) inoculum was prepared using the same method as for the above complex mycorrhizal inoculum (including addition of the identical filtrate) with the exception

that the substrate was not inoculated with *R. irregularis* and received 1 ml sterile water instead. This inoculum, produced for the same period of time as the complex mycorrhizal inoculum (7 months), was then applied at a rate of 60 g per experimental pot. The concentration of root biomass was assessed visually and corresponded roughly to that in the complex mycorrhizal inoculum. No detectable amounts of nuclear LSU rRNA gene of *R. irregularis*, estimated exactly as described for the complex mycorrhizal inoculum, were found in the blank inoculum.

Filtrates of complex mycorrhizal inoculum or blank inoculum

The filtrates were prepared by vigorous agitation (20 min) of the respective substrate suspension in tap water (1:10, w/w) and filtration through two layers of filter paper. Filtration paper of the type 2R/80g (Merci Ltd., Brno, Czech Republic, product order no. 480622080050) was used for this purpose, producing the filtrate with particle size < 6 μm and with the great majority of the particles being smaller than 2 μm. Sixty milliliters of filtrate was applied per respective experimental pot.

Monoxenically produced inoculum

The mycelium and spores were released from 6-month-old monoxenic cultures (hyphae-only compartments) of *Rhizophagus irregularis* SYM5 provided by Symbiom Ltd. by slow agitation in 10 mM potassium citrate (pH 6.0), washed in 1% MgSO₄·7H₂O, and pre-dried briefly on a paper towel. Three aliquots (3.22, 3.12 and 2.50 mg FW) were collected to characterize the length of mycelium and spore concentration, and one aliquot (40.7 mg FW) was then dried at 40 °C to evaluate the gravimetric water content. The obtained fungal biomass contained 123.0 ± 2.2 spores per mg dry weight, and its mycelial length was 6.55 ± 0.49 m per mg (measured by grid-line intersect method after staining with Trypan blue). The dry mass constituted 56.3% of the fresh biomass pre-dried on a paper towel. DNA was further

extracted from three 5 mg aliquots of dried fungal biomass disintegrated by 3-min agitation in 1.5-ml Eppendorf tubes with three 5-mm zirconium oxide beads, using a cetyltrimethylammonium bromide (CTAB) buffer supplied with internal standard as described below in section “DNA extraction.” The concentration of nuclear LSU rRNA gene of *R. irregularis* was then quantified (in triplicate) as described in “Quantification of the mycorrhizal fungus by qPCR.” The concentration was equal to $3.27 \pm 0.11 \times 10^9$ gene copies per mg dry biomass of the fungus.

A fresh mycelium sample (326 mg FW) was suspended in 250 ml water and used as monoxenically produced inoculum provided at a rate of 10 ml per pot. This inoculum was applied either alone or combined with the blank inoculum.

Plant and substrate analyses

After 10 weeks of cultivation, *A. gerardii* plants were harvested, roots separated from the substrate, and root-free substrate aliquots collected. The root systems were first shaken off the substrate and subsequently washed in tap water. One hundred-milligram aliquot of roots from each pot was stained by trypan blue for visual inspection of mycorrhizal colonization, and remaining root biomass was dried at 65 °C to constant weight. Then, the dry root biomass was pulverized and aliquots were used for DNA extraction (see below) and estimation of total phosphorus and total nitrogen concentrations. The shoot biomass was analyzed using the same procedure as the roots with exception that no DNA was extracted.

Phosphorus concentration in plant biomass was determined using the malachite green spectrophotometric method (Ohno and Zibilske 1991), whereas total nitrogen concentration in the biomass samples was measured by combustion C/N analyzer (Flash EA 2000 elemental analyzer, ThermoFisher Scientific, Waltham MA, USA).

Fifty-gram substrate aliquots were dried at 65 °C, pulverized, and subjected to DNA extraction as detailed below.

DNA extraction

DNA was extracted from ~ 800 mg pulverized substrate samples using NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) according to the supplier’s recommendation using the SL1 extraction buffer and enhancer SX. Before the extraction, 20 µl was added to each sample of the internal DNA standard containing 4.16×10^7 molecules of a linearized plasmid carrying fragment of a cassava mosaic virus (Thonar et al. 2012).

DNA from ~ 100 mg pulverized root samples was extracted using glass milk method with the CTAB extraction buffer as described in Gryndler et al. (2014). Before the extraction, 20 µl of the same internal DNA standard as above was added to each sample.

The DNA extracts from both the root and substrate samples were used as templates in quantitative real-time PCR assays or were amplified with tagged primers and sequenced as described below.

Quantification of the mycorrhizal fungus by qPCR

The qPCR reaction mixture contained 2 µl template, 4 µl 5× HOT FIREPol®Probe qPCR Mix Plus (ROX) (Solis Biodyne, Tartu, Estonia), 0.4 µl forward primer “intra-f” (25 µM, TTCGGGTAATCAGCCTTTCG), 0.4 µl reverse primer “intra-r” (25 µM, TCAGAGATCAGACAGGTAGC), 0.1 µl hydrolysis (TaqMan) probe “intra-probe” (25 µM, fluorescein–TTAACCAACCACACGGGCAAGTACABHQ1 quencher), and 13.1 µl water. With this marker set (described originally by Thonar et al. 2012), the cycling conditions in StepOnePlus™ Real-Time PCR System (Applied Biosystems) were as follows: initial denaturation at 95 °C for 15 min, followed by 60 cycles of denaturation at 95 °C for 10 s, annealing at 52 °C for 60 s, and elongation at 72 °C for 10 s.

The raw qPCR data were corrected for internal standard losses in each sample as described in Thonar et al. (2012).

Illumina MiSeq sequencing of fungal and bacterial communities

A fragment of fungal internal transcribed spacer (ITS) region from each of the root or substrate DNA samples was amplified (in triplicates). To this end, PCR was carried out in 25 µl reaction format, where each reaction mixture contained 12.5 µl 2× Combi PPP Mix (hot start type, Top-Bio, Vestec, Czech Republic), 0.5 µl primer ITS1F (10 µM), 0.5 µl primer ITS4 (10 µM), 1 µl template DNA (5–50 ng/µl), and 10.5 µl water. Primers were adopted from White et al. (1990). The mixture was first denatured at 94 °C for 4 min and then subjected to 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s, and elongation at 72 °C for 90 s. Final elongation at 72 °C lasted for 10 min. The three replicates per sample were then pooled, purified by Qiaquick PCR purification kit (Qiagen) with elution volume 30 µl, diluted 1:1000, and used as a template in a second PCR amplification step. Single PCR amplification was then performed per sample, the reaction mixture being the same as in first amplification described above except that tagged primers gITS7_Txx (Ihrmark et al. 2012) and ITS4_xxx (White et al. 1990), targeting the ITS2 region, were used. DNA fragments originating from different samples were unambiguously distinguished by tag combinations of the primers. Primer sequences are provided in Supplementary Table S1. The reaction mixture was first denatured at 94 °C for 4 min and then subjected to 25 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 30 s. Final elongation at

72 °C lasted for 10 min. The final PCR product was purified by Qiaquick PCR purification kit.

A fragment of V4 region of the small ribosomal subunit rRNA gene of prokaryotic (bacterial) communities was amplified from each of the DNA samples in technical triplicates in a single step, with reaction mixture as in the case of amplification of fungal ITS region, but with a different polymerase mix TPBS DNA-free 2 × Master Mix (certified to be free of bacterial DNA, hot start type, Top-Bio) and modified tagged primers 515Fd_Txxx and 806Rd_Txxx (Aprill et al. 2015). DNA fragments originating from different samples were unambiguously distinguished by tag combinations of the primers. For primer sequences, see Supplementary Table S1. The cycling conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s, and elongation at 72 °C for 75 s. Final elongation at 72 °C lasted for 10 min. Technical replicates per each sample were then pooled and the amplicons purified by Qiaquick PCR purification kit.

Bacterial and fungal amplicons were quantified using Picogreen fluorescence, diluted to concentration of 20 ng/μl, and combined to a single sequencing library, which was then subjected to sequencing at MiSeq Illumina platform (2 × 250 bp).

Sequencing data were treated by Seed software (Větrovský and Baldrian 2013), version 2.0.4 as follows: after removing low-quality sequences and sequences shorter than 150 bp or longer than 400 bp (bacteria) or sequences shorter than 40 bp (fungi), ITS sequences were extracted from the fungal amplicons, the contigs were chimaera-cleaned/clustered by the Usearch tool (version 8.1.1861; Edgar and Flyvbjerg 2015) at the similarity level 97%, and the most abundant sequences were compared (blastn algorithm, Blast tool, version 2.2.26+; Altschul et al. 1990) with GenBank database (environmental sequences, metagenomes, and unidentified organisms excluded).

The sequences originating from amplicons of bacterial communities that were assigned to mitochondria, plastids, or eukaryotes were excluded from further analyses. The same filtration procedure was done for sequences from amplicons of fungal communities assigned to arbuscular mycorrhizal fungi and eukaryotic organisms other than fungi. Remaining sequences were resampled to retain 6000 fungal sequences per root sample, 8000 fungal sequences per substrate sample, 6000 bacterial sequences per substrate sample, and 2000 bacterial sequences per root sample (the root samples returned large numbers of mitochondrial and plastid sequences when amplified with bacterial primers). Resampled sequences were newly clustered and most abundant sequences identified and filtered as described above. Sequences have then been deposited in Sequence Read Archive (NCBI) under accession number SRP116631. Operational taxonomic units (OTUs) corresponding to clusters at the level of genus were named

according to the best GenBank hit genus name, and OTUs with the same genus assignment were summed and further treated as compound OTUs. Final numbers of sequences per sample used in the subsequent statistical analyses are given in supplementary file [SequencingSupplementary.xlsx](#).

The effects of experimental treatments on plant parameters were analyzed using analysis of variance followed by Duncan's multiple range test (software Statistica 7.1, Statsoft, Prague, Czech Republic), whereas the effects of experimental treatments on microbial communities in the roots/substrate were analyzed by redundancy analysis (RDA; software Canoco 5.04; ter Braak and Šmilauer 2012) using log-transformed sequence counts. The results of RDA have been presented in the form of biplots. Bacterial and fungal data were always analyzed separately. The selection of genera shown in the biplot diagrams is based on *t* value biplots, which identify taxa with a significant relation to the tested explanatory variable (ter Braak and Šmilauer 2012). Variation of numbers of sequences used per sample is orthogonally relative to the analyzed factors, and mean numbers of sequences in compared groups are almost the same. This eliminated the need for data standardization.

Data exclusion

Very small plant biomass, not allowing the analyses of plant P and N concentrations, was observed in one replicate of the uninoculated control treatment. This replicate (including sequencing samples RB06, TB06, RH06, and TH06, see supplementary file [SequencingSupplementary.xlsx](#)) has been completely discarded from subsequent microbiome and plant analyses. Further, single replicate of the treatment inoculated with the filtrate of complex mycorrhizal inoculum became mycorrhizal. At the same time, single replicate of the treatment inoculated with the monoxenically produced mycorrhizal inoculum was only weakly colonized—very low rDNA gene copynumbers of *R. irregularis* were detected using the qPCR. These two pots have thus also been discarded from subsequent analyses (including sequencing samples RB72, TB72, RH72, TH72, RB80, TB80, RH80, TH80).

Results

Response of plant parameters to different inoculation treatments

Quantification of mycorrhizal colonization using qPCR revealed that mycorrhizal symbiosis was established in all treatments inoculated with the living mycorrhizal fungus (Fig. 1). Whereas the plants in treatments inoculated with complex inoculum and monoxenically produced inoculum were equally colonized, the plants inoculated with monoxenically

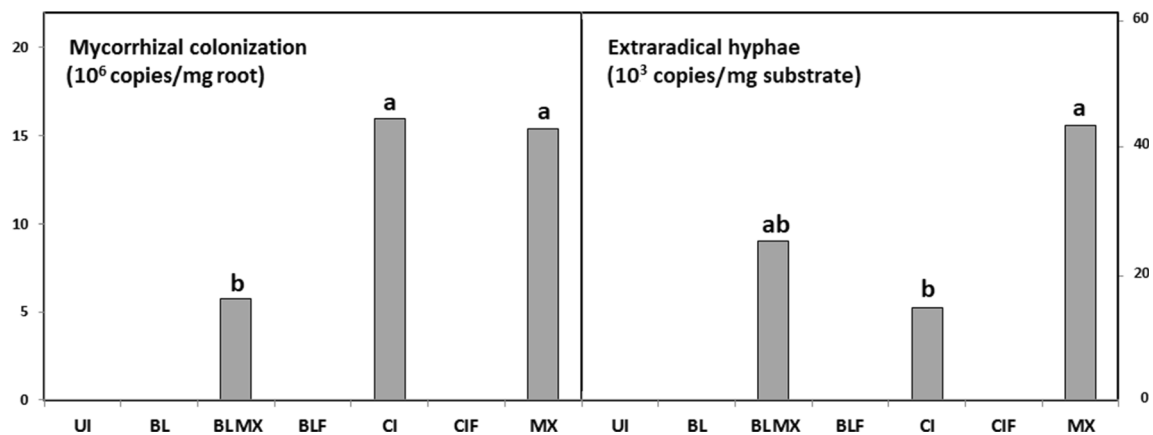


Fig. 1 The extent of root and potting substrate colonization by the fungus *Rhizophagus irregularis*, given as the concentration of rDNA copies of the fungus per milligram dry weight of the respective material in the following treatments: uninoculated (UI), inoculated with blank inoculum (BL), blank inoculum combined with monoxenically produced inoculum (BLMX), blank inoculum filtrate (BLF), complex mycorrhizal inoculum (CI), complex inoculum filtrate (CIF), or with the monoxenically produced mycorrhizal inoculum (MX). Mean values are shown; different letters accompanying treatment means indicate significant differences between the means as per one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$). Only treatments with positive qPCR signal were considered for the statistical analysis

produced inoculum in combination with blank inoculum showed, on average, less than a half the density of *R. irregularis* rDNA copies in the root biomass. Additional microscopic inspection of roots confirmed normal morphology of mycorrhizal colonization in all mycorrhizal treatments. Extraradical mycelium was most dense in the treatment receiving monoxenically produced inoculum and somewhat less developed in the substrate with monoxenically produced mycorrhizal inoculum combined with blank inoculum. It was, however, significantly lower in the substrate inoculated with complex inoculum as compared to the treatment inoculated with monoxenically produced inoculum (Fig. 1).

The analysis of plant parameters revealed that mycorrhizal symbiosis always significantly increased the biomass of both shoots and roots relative to any of the nonmycorrhizal control treatments (Fig. 2) and that uninoculated (“absolute”) control produced far the lowest biomass among all the treatments. Noteworthy, shoot dry weight produced in the control treatment inoculated with the filtrate from the complex mycorrhizal inoculum was significantly higher than that in any other control treatment. Mycorrhizal plant biomass was almost the same in all mycorrhizal treatments, regardless of the type of mycorrhizal inoculum applied (Fig. 2).

The amount of N accumulated in plants was the same in all inoculated treatments, mycorrhizal and nonmycorrhizal, with the exception of the uninoculated (absolute) control treatment that showed significantly lower N content than all the other treatments (Fig. 2). On the other hand, the amount of P accumulated in plants was strongly dependent on the mycorrhizal symbiosis and was the highest in the treatment inoculated with monoxenically produced mycorrhizal inoculum, followed by the two other mycorrhizal treatments (Fig. 2). P content of the plants in the absolute control was the lowest among all the treatments (Fig. 2).

mycorrhizal inoculum (CI), complex inoculum filtrate (CIF), or with the monoxenically produced mycorrhizal inoculum (MX). Mean values are shown; different letters accompanying treatment means indicate significant differences between the means as per one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$). Only treatments with positive qPCR signal were considered for the statistical analysis

Response of root-associated microbiome to experimental treatments

Before resampling, 350,297 sequences belonging to Mucoromycota/Glomeromycotina were discarded from the sequence dataset obtained from fungal amplicons. These sequences constituted 21.4% of sequences in the mycorrhizal treatments and 0.63% of sequences in the nonmycorrhizal treatments.

In total, 650,985 bacterial and 1,155,342 fungal sequences entered the statistical analyses after resampling. Among them, 7182 bacterial and 2473 fungal OTUs were identified including singletons (i.e., the OTUs composed of a single sequence).

The abundance of bacterial phyla and fungal classes in different samples (bacteria and fungi were analyzed separately) is presented in Supplementary Fig. S2. Proteobacteria tended to dominate root samples, whereas Actinobacteria were the most abundant group in majority of the substrate samples. Eurotiomycetes constituted one of the most abundant fungal groups in substrate samples but were almost absent from the roots. Orbiliomycetes were detectable in notable amounts only in the treatments inoculated with complex mycorrhizal inoculum and with the blank inoculum.

The analyses of differences of root and combined root + substrate microbiomes revealed that fungi were always more responsive to the different inoculation treatments than bacteria, which was reflected in generally higher portion of explained variance (Table 2). All the analyses produced statistically highly significant results, the p values being always equal or lower than 0.001 (not shown in Table 2). In most cases, the explained variance was higher in root-associated microbial communities, compared to the complete microbial community in pots (where the microbial communities of roots and substrate were taken together).

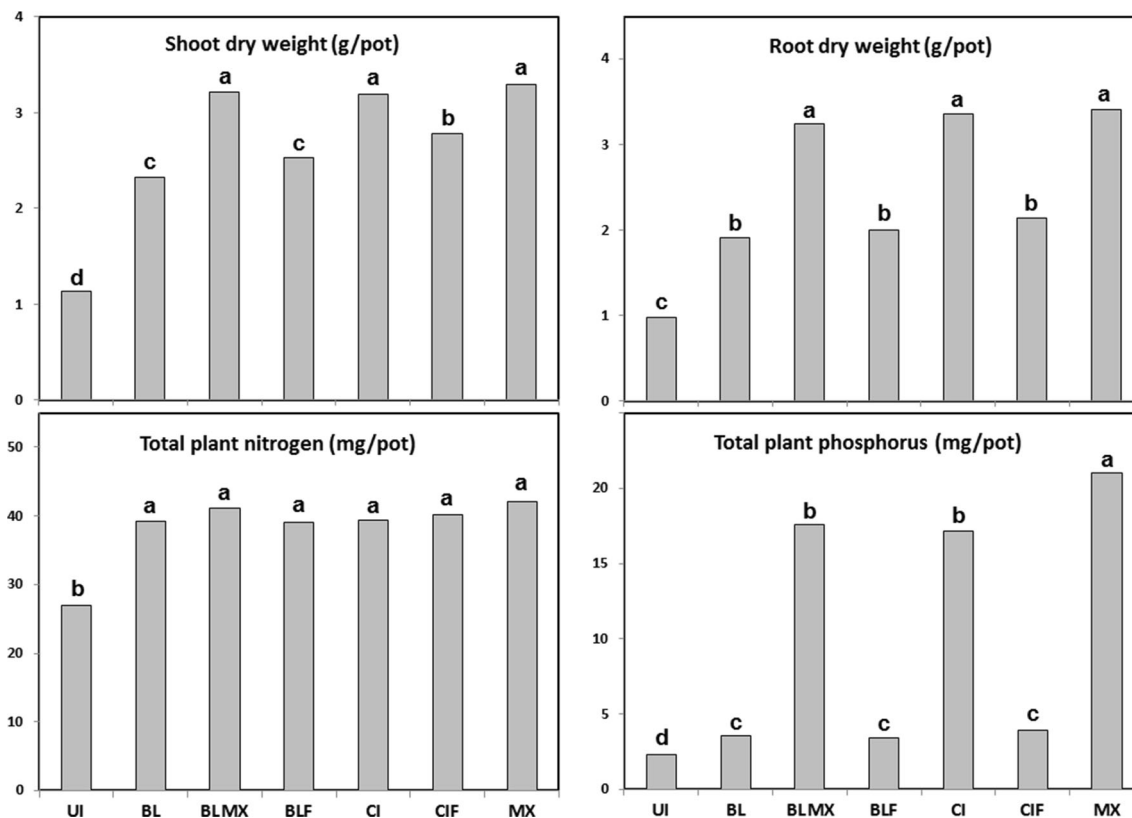


Fig. 2 Shoot dry weight, root dry weight, total plant nitrogen, and total plant phosphorus contained in the experimental plants (*Andropogon gerardii*) as affected by different inoculation treatments. Different letters

accompanying treatment means indicate significant differences between the means as per one-way ANOVA followed by Duncan’s multiple range test ($p < 0.05$). For treatment codes, see legend to Fig. 1

Table 2 The results of redundancy analysis of experimental treatments on the bacterial and fungal component of root and combined root and substrate microflora. Bacterial and fungal data were analyzed separately. Percentage of the total variance explained (%) and pseudo-F value (F) are presented. All effects were statistically significant at $p \leq 0.001$. The sign

“+” in the leftmost column indicates that the treatments were pooled together for the analysis, the sign “×” separates the groups of compared treatments and the sign “/” separates the treatments used in the interaction testing. The analyses defined below were carried out to answer the questions formulated in “Introduction”

Treatments	Bacteria				Fungi			
	Roots + substrate		Roots		Roots + substrate		Roots	
	%	F	%	F	%	F	%	F
Question 1								
(UI + BL) × (CI + MX)	4.5	7.5	8.5	5.1	9.3	14.6	17.5	11.5
(UI + MX) × (BL + CI)	7.2	12.0	20.2	12.1	12.5	19.7	16.8	11.0
UI/BL/CI/MX	3.5	6.1	8.2	5.5	11.8	11.6	11.0	8.5
Question 2								
(BL + BLF) × (CI + CIF)	3.1	5.5	9.1	5.0	6.7	11.4	10.9	7.2
(BL + CI) × (BLF + CIF)	4.5	7.9	10.5	5.7	18.5	31.4	22.0	14.5
BL/BLF/CI/CIF	2.0	3.6	5.8	3.3	5.8	10.8	11.9	9.3
Question 3								
BL × BLMX × CI	5.2	3.6	10.3	3.1	19.8	14.1	34.5	8.7
BL × CI	5.7	5.3	16.5	4.4	20.3	19.1	38.7	13.9
BLMX × CI	3.4	3.1	10.4	2.6	21.3	19.5	33.7	11.2

RDA testing the effects of mycorrhizal inoculation and the origin of accompanying microorganisms revealed that composition of the fungal and bacterial communities of roots was always different between mycorrhizal treatments spontaneously colonized by other microorganisms and those inoculated with the complex mycorrhizal inoculum. On the other hand, perceptible unification was observed for mycorrhizal and nonmycorrhizal treatments inoculated with pot-produced solid materials, i.e., including all the accompanying bacterial communities present in the blank inoculum and in the complex mycorrhizal inoculum (Fig. 3). *T* value biplots (Fig. 4) show that several fungal and bacterial taxa were significantly associated with mycorrhizal symbiosis. However, no exclusive association of any microbial community component with the presence of the mycorrhizal fungus was observed.

When the effects of the inoculation with complete inocula, both the blank and the mycorrhizal ones, on the structure of microbial communities were compared with the effects of their filtrates, great differences were noted for both the bacterial and fungal components (Fig. 5). Taking into account significantly increased shoot biomass in the treatment inoculated with the complex mycorrhizal inoculum filtrate (relative to other inoculated control treatments), it is interesting to note that there does exist a group of several fungi that are preferentially associated with the treatment inoculated with complex mycorrhizal inoculum. Yet, there are no fungi significantly associated with the treatment inoculated with the filtrate of

this kind of inoculum. Similarly, this RDA biplot (Fig. 5) shows contrasting fungal community composition in treatments inoculated with the complete nonmycorrhizal inoculum (blank) and with its filtrate. Bacterial community component was different between all the four above treatments, and the genus *Agrobacterium* (comprising plant growth promoting rhizobacteria) was perceptibly associated with both treatments receiving filtrates.

RDA classified sample diagram displaying the similarity of root bacterial and fungal communities in treatments inoculated with the blank inoculum; blank inoculum combined with monoxenically produced mycorrhizal inoculum and the complex mycorrhizal inoculum (Fig. 6) showed that bacterial communities of these three treatments were all conspicuously different. Similar but somehow weaker trend was observable for the fungal communities. In the latter case, the groups of sample scores representing the treatments inoculated with blank inoculum and inoculated with blank inoculum combined with monoxenically produced mycorrhizal inoculum showed a partial overlap (Fig. 6).

Microbial OTUs with interesting abundance patterns

Some identified OTUs tended to associate with the plant roots and may thus be considered as the rhizosphere/rhizoplane specialists. For example, OTU corresponding to bacterial genus *Cupriavidus* was particularly abundant

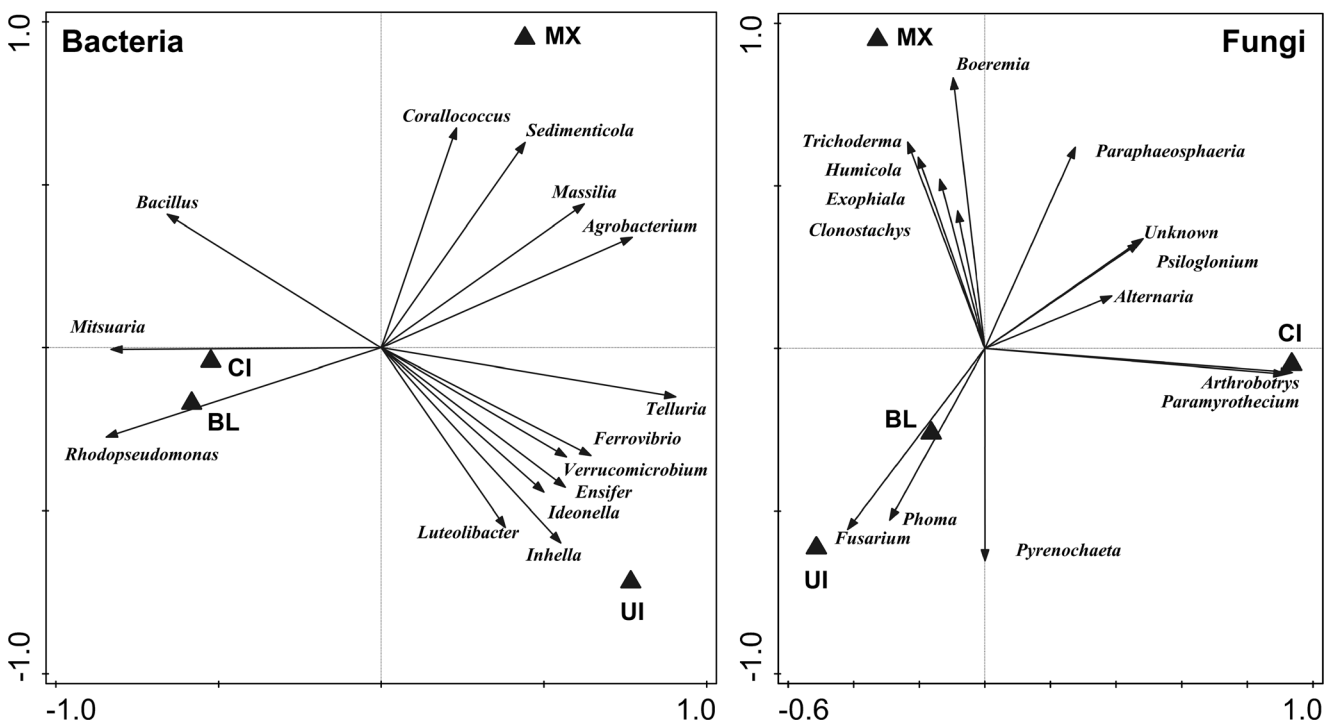


Fig. 3 Ordination biplots corresponding to the RDA of combined effects of inoculation with mycorrhizal fungus and inoculation with accompanying microorganisms on the microbial communities in root samples. The first (horizontal) and second (vertical) axes explain,

respectively, 20.6 and 10.2% of the total variation in bacterial OTU data and 28.4 and 13.3% in the fungal OTU data. For treatment codes, see legend to Fig. 1

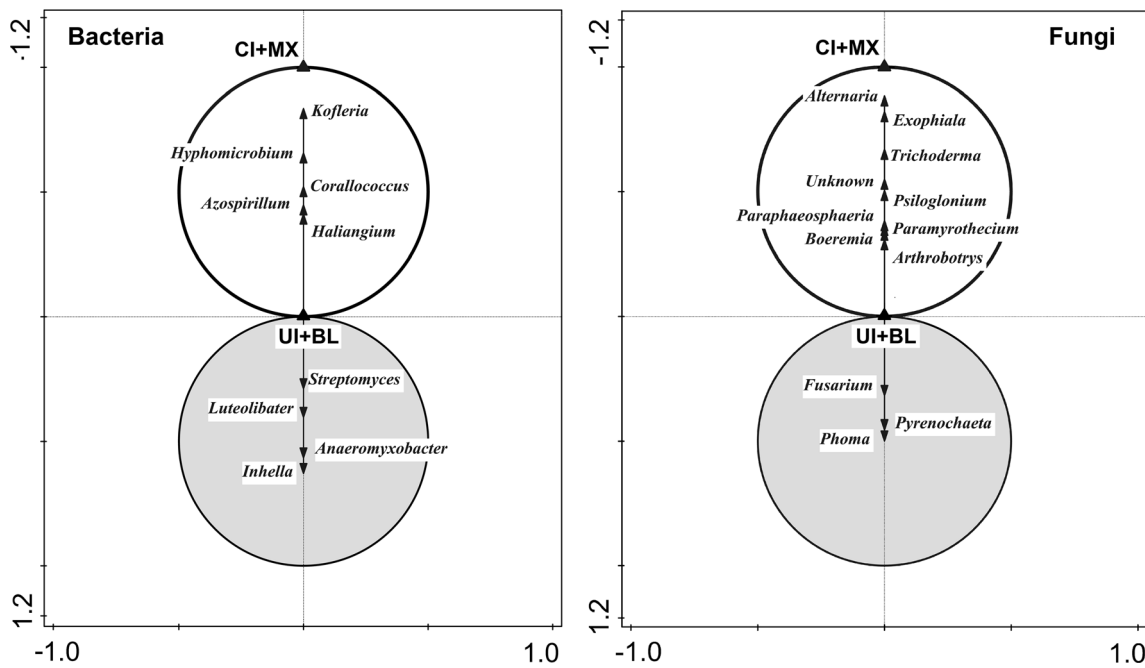


Fig. 4 T value biplots with Van Dobben circles delimiting the scores of root-associated OTU groups significantly interacting with presence of mycorrhizal fungus at $p \leq 0.05$ (redundancy analysis/reduced rank regression). White-filled circles enclose the scores of OTU groups positively

correlating with the mycorrhizal fungus; gray circles contain the scores showing significant negative correlation with the presence of mycorrhizal fungus. For treatment codes, see legend to Fig. 1

in roots of the treatment inoculated with the monoxenically produced mycorrhizal inoculum (Fig. 7). The most abundant sequence was 100% similar (97.9% coverage) to the GenBank sequence KY614187 of *Cupriavidus gilardii*, but

the same similarity was observed to the sequence HQ829834 assigned to *Ralstonia* sp. Thus, the OTU identification at the genus level is ambiguous and must be regarded as provisional.

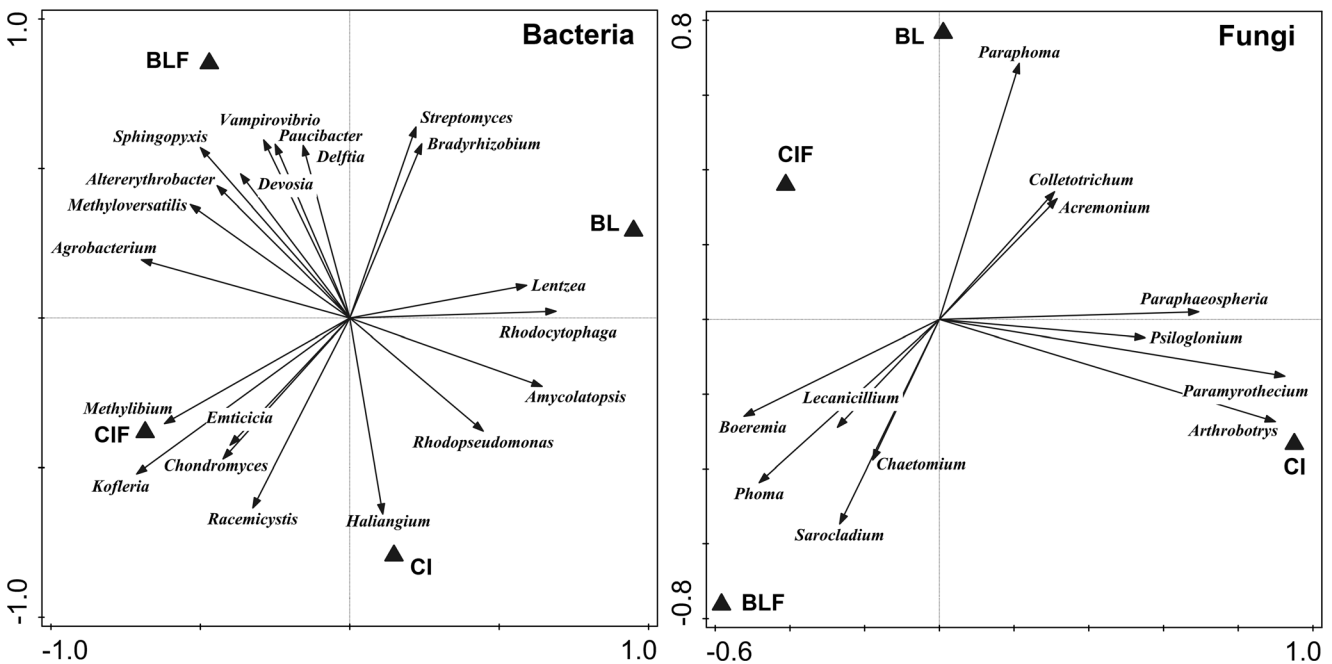


Fig. 5 RDA ordination biplots of the combined effects of inoculation with microbial communities produced in the absence (BL, BLF) or in the presence (CI, CIF) of mycorrhizal fungus, either unfiltered (BL, CI) or filtered (BLF, CIF), on microbial communities developed in/on the

roots of experimental plants. The first (horizontal) and second (vertical) axis explain, respectively, 11.2 and 8.8% of the total variation in bacterial OTU data, and 29.6 and 10.0% of the fungal OTU data. For treatment codes see legend to Fig. 1

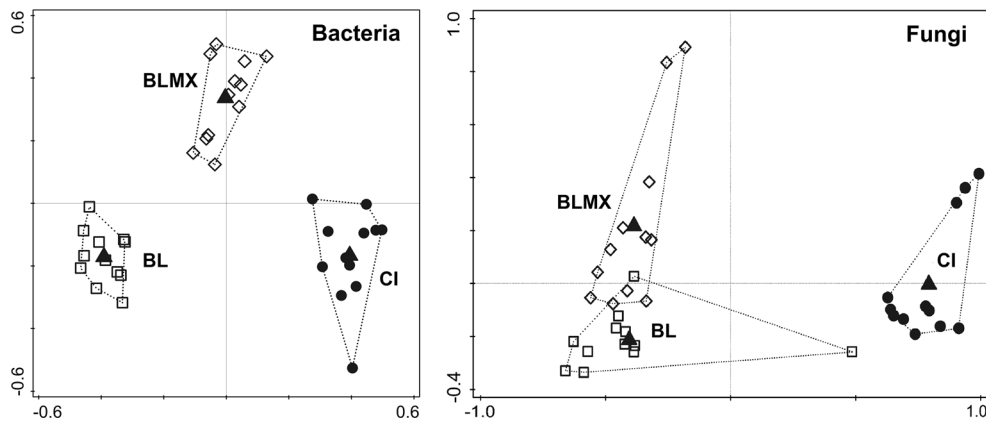


Fig. 6 RDA classified sample diagrams of the similarity of root bacterial and fungal communities developed in treatments inoculated with blank inoculum (BL), blank inoculum combined with monoxenically produced mycorrhizal inoculum (BLMX), and complex mycorrhizal inoculum

(CI). Black triangles in the polygon centers represent the individual treatments, other symbols covered by the polygons being individual samples of the treatment

The fungal genus *Arthrobotrys* (Fig. 8) was mainly represented by very abundant OTU corresponding to *Arthrobotrys scaphoides* with 100% sequence similarity and 100% coverage with the GenBank sequence KF494006. This OTU was dominant (almost 50% of all sequences) in the rhizosphere of the treatment inoculated with the complex mycorrhizal inoculum and was also detected in the substrate of the same treatment. It was not found in the pots inoculated with the blank inoculum.

The most abundant OTU assigned to fungal genus *Boeremia* (Ascomycota, Pezizomycotina, Dothideomycetes, Pleosporales) showed 100% sequence similarity (100% coverage) to several GenBank sequences from the genus *Boeremia*, such as *Boeremia exigua* (KX272600), *Boeremia strasseri* (MF113480), or to sequence of *Phoma multirostrata* (KX099647). This less frequent OTU was present mainly in the treatments inoculated with filtrates of blank inoculum and complex mycorrhizal inoculum and in

Fig. 7 Abundance (expressed as median values of relative abundances of reads within the sample) of selected bacterial OTUs in different experimental treatments. For treatment codes, see legend to Fig. 1. Vertical error bars delimit intervals between 25 and 75% quantiles

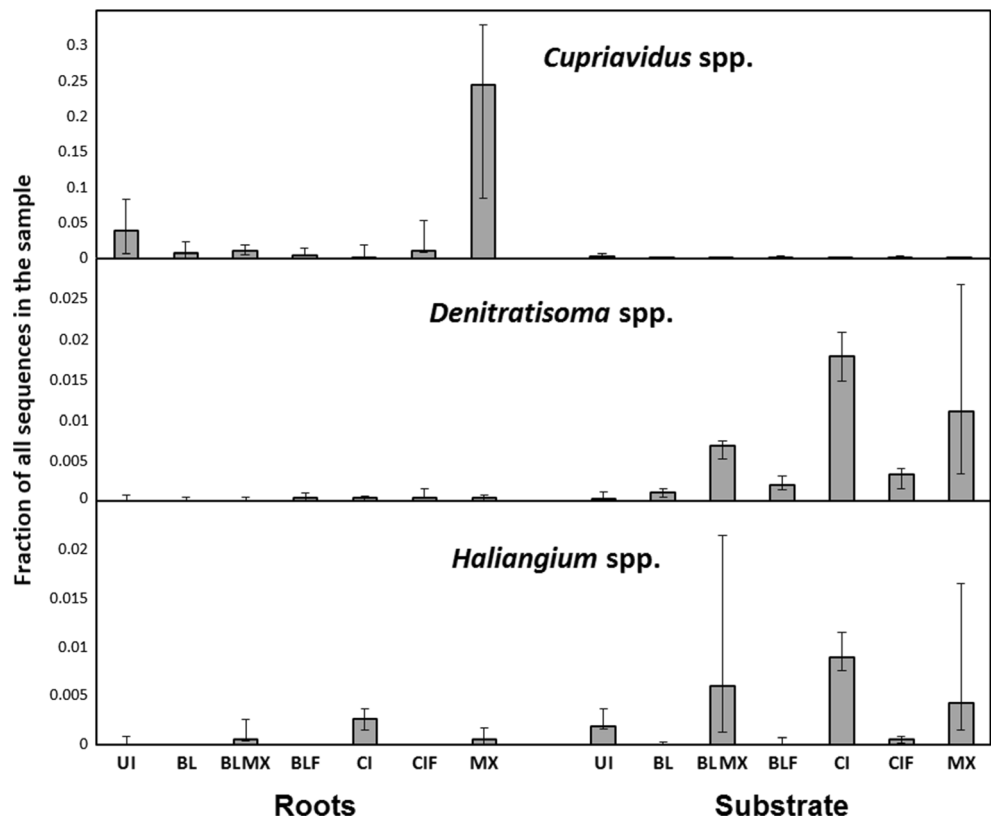
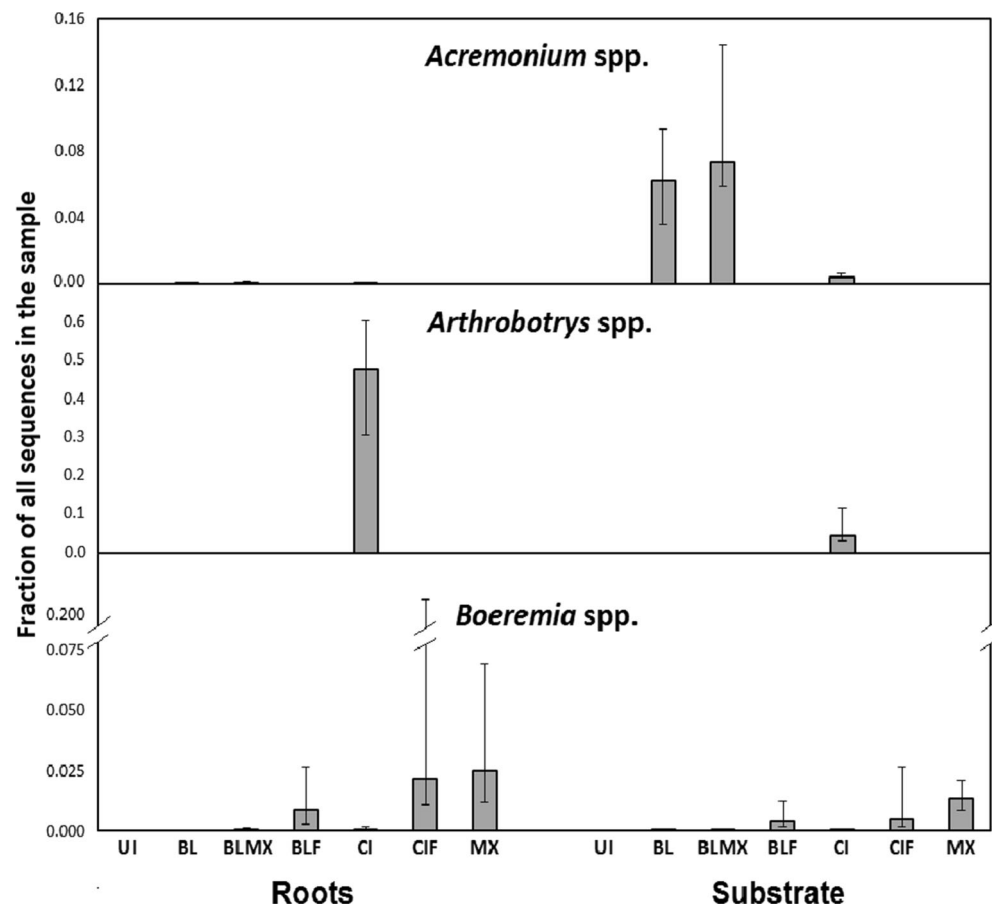


Fig. 8 Abundance of selected fungal OTUs in different experimental treatments. Explanation of details as in Fig. 7



the treatment added with monoxenically produced mycorrhizal inoculum (Fig. 8). It was almost absent from treatments inoculated with the complex mycorrhizal inoculum or the blank inoculum. It is noteworthy that the substrate of the treatment inoculated simultaneously with the blank inoculum and monoxenically produced mycorrhizal inoculum was relatively enriched in *Penicillium smithii* OTU compared to the other mycorrhizal treatments (data not shown).

Several bacterial and fungal OTUs were observed as associated with particular treatments, and some of them were detected almost exclusively in the substrate, without obvious association with roots or rhizosphere. Such interesting but rare bacterial OTU, associated mainly with substrate of mycorrhizal treatments, was *Denitratisoma* (Fig. 7). The most abundant sequence was similar to the GenBank sequence of *Denitratisoma oestradiolicum* (NR_04324, 98.6% similarity, 99.7% coverage).

Two rare OTUs corresponding to *Haliangium* spp. bacteria have been detected mainly in the substrate of mycorrhizal plants (Fig. 7). Most abundant sequences of these OTUs are similar to the GenBank sequences of *Haliangium tepidum* (NR_02478) and *Haliangium ochraceum* (EF108312). However, the similarity was rather low, close to 90%. The low similarity of most abundant sequences indicates that the

analyzed OTUs are not close relatives of the above *Haliangium* species but rather correspond to hitherto unsequenced/unidentified bacteria.

Acremonium-like most abundant OTU showed 100% sequence similarity and coverage with GenBank sequences of several *Acremonium* spp., such as *A. furcatum* (NR_145349) and *A. persicinum* (KX674656). This fungal OTU was present almost exclusively in the substrate inoculated with the blank inoculum (Fig. 8). It was also found in the treatment added with the complex mycorrhizal inoculum, indicating the origin (previous open-pot cultures used to produce mycorrhizal and blank inocula) of this particular taxon in our experimental samples.

Discussion

Our large data indicate that creating appropriate nonmycorrhizal controls in mycorrhizal research is a delicate task, particularly if the root and/or substrate microbiomes are under examination. The positive effects of blank inoculum or mycorrhizal inoculum filtrate on plant biomass, as compared with the uninoculated (absolute) control, indicate the importance of soil microbiome for plant performance. There is,

however, still a big question if any unfilterable biotic components, other than mycorrhizal fungi, are responsible for any of the effects observed. Our results indicate that soil filtrate (in our case containing potentially plant growth promoting *Agrobacterium* spp.) can compensate for so called “boomerang effect,” a strong inhibition of plant growth in sterilized substrates induced by “unbalanced” microbial community (Kreutzer 1960). However, the filtrate quality may be determining the final composition of microbial community and variable effects due to the mode of filtrate application should thus be anticipated. For example, unlike our results, it has been observed that soil filtrate addition does not necessarily result in changes of plant growth, compared to uninoculated control with pasteurized soil (Fisher and Jayachandran 2002).

It is very important to note at this point that the “boomerang effect” was compensated even if the monoxenically produced inoculum was applied alone, which originally does not contain any accompanying saprotrophic microbes and is thus subject of virtually the same “random contamination” as the uninoculated (absolute) control. This observation calls for an alternative explanation—e.g., mediation of substrate microbiome through biological activity of chemical compounds contained in mycorrhizal mycelium. Arbuscular mycorrhizal fungal biomass, such as spores or exudates from germinating spores, is namely capable to substantially change gene expression in mycorrhizal hosts and elicit alkaloid biosynthesis in tobacco if injected into the base of stems. It has been shown in experiment where material obtained from 1250 surface-disinfected spores of *Glomus etunicatum* was applied per plant (de Andrade et al. 2013). It is also known that mycorrhizal roots contain different concentrations of secondary metabolites than the nonmycorrhizal roots (Schliemann et al. 2008; Rivero et al. 2015). Some of them may possess important biological activities, may be even produced by the mycorrhizal fungus itself, and may help explaining the strong effect of monoxenically produced inoculum on substrate microbiome and the plants in our experiment. The concentration of such metabolites in the substrate supplied by the monoxenically produced inoculum was negligible relative to the substrate amount present in the pot (13 mg fresh inoculum versus 2 L substrate volume) and must thus have been effective at very low concentrations.

The above observation accords with our main finding: mycorrhizal symbiosis strongly modifies microbial communities in plant cultivation substrates (and most probably also in soils) so that it is virtually impossible to establish a nonsterile cultivation experiment in which the treatments differ only by the presence of mycorrhizal fungus, the other components of saprotrophic microbiome being identical. On the other hand, the addition of a filtrate from a pot-cultured mycorrhizal inoculum may considerably suppress AM hyphal growth and reduce the root P content, supporting earlier view that specific bacterial populations can reduce effectiveness of the

mycorrhizal symbiosis in sterilized microcosms (Leigh et al. 2011). This is consistent with our observation of considerable decrease in mycorrhizal colonization levels in the treatment inoculated with the blank inoculum combined with monoxenically produced inoculum of the mycorrhizal fungus as compared to the other mycorrhizal treatments. This phenomenon might be caused by high abundance of potentially toxinogenic *Penicillium smithii* OTU in this particular inoculation treatment.

In our experiment, the plants inoculated with the complex mycorrhizal inoculum received 4600× larger amount of the inoculation material than the plants receiving monoxenic inoculum. However, the concentration of the mycorrhizal fungus, expressed in terms of nuclear LSU rRNA gene copynumbers, was 700,000× higher in the monoxenic inoculum. Then, the pots inoculated with the monoxenic inoculum received by approx. 150× more biomass of the mycorrhizal fungus detectable as nuclear LSU rRNA gene copynumbers compared to pots inoculated with the complex mycorrhizal inoculum. Interestingly, considerable differences in the amounts of the inoculum used in the two mentioned experimental treatments did result in fully comparable levels of colonization of the host plant roots. This may indicate that relatively small amounts of the infectious material, common in complex inocula used in mycorrhizal research and also in practical applications such as horticulture or open field agriculture, are sufficient to ensure “normal” mycorrhiza development.

The development of extraradical mycelium was, however, different among the three mycorrhizal treatments, which may either indicate that development of extraradical fungal structures was more dependent on inoculum density than root colonization or this parameter was more affected by the associated microbiomes than the root colonization. It is also important to note that the observed pattern of plant P supply was approximately consistent with abundance of mycorrhizal mycelium in the substrate, which agrees with common view on the mycorrhizal symbiosis functioning, where the development of extraradical mycelium is usually the bottleneck for P supply to plants via the mycorrhizal hyphae (Smith et al. 2003).

The most striking result of this entire experiment comparing various inoculation treatments is the magnitude of the dissimilarity in composition of microbial communities between the individual treatments. Whereas the plant parameters including mycorrhizal colonization and P contents responded to mycorrhizal inoculation fairly homogeneously (with few notable exceptions) and did not depend strongly on the type of the mycorrhizal inoculum used, no similar patterns in bacterial and fungal community composition among those treatments were observed.

The formation of contrasting microbiomes in the different mycorrhizal treatments that was not reflected in plant

performance, together with less vigorous plant growth in the nonmycorrhizal treatments, indicated a kind of functional redundancy in substrate/root microbiomes within the mycorrhizal treatments. Functional redundancy of soil microorganisms has been clearly demonstrated for soil microorganisms inhabiting differently treated soils for example by Souza et al. (2015). It is thus possible that the microbiomes lacking mycorrhizal fungi are incomplete in their ability to maintain microbial functional redundancy.

To summarize, we can respond to the questions regarding the appropriate control treatments posed in the “Introduction” as follows:

- 1) Yes, there are indeed some rarer bacterial taxa tending to increase their abundance in mycorrhizal treatments as compared to the nonmycorrhizal controls.
- 2) The inoculation with pot-produced blank inoculum or pot-produced complex inoculum filtrates is not sufficient to establish substrate/rhizosphere microbiomes of the quality comparable with that in the pots inoculated with unfiltered blank inoculum or unfiltered complex mycorrhizal inoculum.
- 3) Yes, a gradual adaptation of rhizosphere microbiomes to the presence of mycorrhizal symbiosis has been confirmed in terms of a difference in composition of microbial community developed in treatments inoculated with the blank inoculum and with the blank inoculum combined with monoxenically produced mycorrhizal inoculum. Further, the application of complex mycorrhizal inoculum resulted in development of different bacterial and fungal communities as compared with the treatment inoculated with blank inoculum. It is important to note at this point that both kinds of pot-produced inocula initially received the same soil filtrate as a source of accompanying microbial communities.

It follows from the above statements that the blank inoculum (produced in pots in the same manner as mycorrhizal pot cultures, but lacking the mycorrhizal fungi) is not fully appropriate control inoculum for experiments with complete soil inocula. Further, strictly speaking, the inoculation with monoxenically produced mycorrhizal biomass combined with blank inoculum cannot be taken as an equivalent to inoculation with complete soil inoculum. Virtually, there is obviously no perfect solution among those tested in our experiment as all control treatments differed substantially in their microbiome from the complex mycorrhizal inoculum-treated pots. Thus, accompanying microbial communities present in complex inocula of arbuscular mycorrhizal fungi should be seriously taken into consideration and thoroughly analyzed if other than purely physiological effects of mycorrhizal symbiosis are being addressed. But even in physiological studies, the type of nonmycorrhizal control treatment may matter. This is

demonstrated by significantly different values of shoot dry weight between the different control treatments in our experiment—possibly due to presence/absence of some of the microorganisms detected in the particular treatments.

Mainly, highly abundant fungal OTU corresponding to *Arthrobotrys* spp. (best hit: *A. scaphoides*) was dominant in the rhizosphere of the treatment inoculated with the complex mycorrhizal inoculum. This OTU was responsible for almost 50% of all fungal sequences from that particular inoculation treatment. The abundance of this OTU in other treatments was much lower. Fungal genus *Arthrobotrys* (Ascomycota, Pezizomycotina, Orbiliomycetes) contains predatory fungi, including *A. scaphoides*, capturing plant pathogenic nematodes (Den Belder and Jansen 1994). If these fungi prefer to develop in the presence of arbuscular mycorrhizal fungi (for example, in pot cultures maintained in a greenhouse), it might at least partially explain voluminous data on antagonism between arbuscular mycorrhizal fungi and phytopathogenic nematodes (e.g., Schouteden et al. 2015; Campos et al. 2017). On the other hand, plant beneficial microbial (bacterial) community may still be present in the filtrate of complex mycorrhizal inoculum which may explain the highest shoot biomass in this particular treatment among all the other control treatments tested here.

Other interesting fungal OTU corresponding to the genus *Boeremia* was present mainly in the treatments inoculated with the inoculum filtrates and in the treatment inoculated with the monoxenically produced mycorrhizal inoculum. There are plant pathogenic fungi within the genus *Boeremia*, including *B. exigua*, which may produce phytotoxins and might be potentially used as a mycoherbicide (Cimmino et al. 2008). This means that, though our experiment was grown under relatively “clean” conditions in an experimental greenhouse, some potentially pathogenic fungi may still accumulate in the pots. If such a potentially pathogenic fungus is produced in a pot culture together with arbuscular mycorrhizal fungus, it may represent a menace for plants inoculated with such a material.

Acremonium-like OTU, for unknown reason, was present almost exclusively in the substrate of treatments inoculated with the blank inoculum. The fungal genus *Acremonium* (Ascomycota, Pezizomycotina, Sordariomycetes) comprises saprotrophic species but also the species that coexist with grasses as endophytes (Christensen et al. 1993) and may interact with mycorrhizal fungi, decreasing production of their propagules (Chu-Chou et al. 1992). Thus, the members of this genus are probably not dangerous for plants directly but may hamper development and/or functioning of the mycorrhizal symbiosis and thus, indirectly, affect the plants.

OTU corresponding to bacterial genus *Cupriavidus* (best hit: *C. gilardii*) was surprisingly abundant in roots of the treatment inoculated with the monoxenically produced mycorrhizal inoculum but was relatively rare in the other treatments. *C.*

gildardii is primarily a soil species known to degrade phenoxyalkanoic acid herbicides such as 2,4-D (Wu et al. 2017) but can also tolerate very high concentrations of heavy metals (copper) in the environment and possess efficient cell detoxification mechanisms (Wang et al. 2015). It is unclear why its abundance reached about 25% of all bacterial sequences in this particular treatment.

Two less abundant bacterial OTUs, *Denitratisoma* and *Haliangium*, tended to associate with substrate of mycorrhizal treatments. While *Haliangium* OTU most abundant sequence is only weakly similar to its best GenBank hit and cannot be properly linked to any previously known bacterial species, *Denitratisoma* OTU probably corresponds to *D. oestradiolicum*. This bacterium was first isolated from activated sludge of a municipal wastewater treatment plant using 17 β -oestradiol as a sole source of C and energy and is able to exploit a variety of organic acids and fatty acids (Fahrbach et al. 2006). In our experiment, this bacterium might exploit lipidic compounds stored in the arbuscular mycorrhizal fungi, and this may explain their preference for mycorrhizal treatments.

Here we showed that there were dramatic differences in the composition of microbial communities between the different inoculation treatments. Interestingly, the performance of the host plants in all mycorrhizal treatments was nearly the same, not really reflecting the differences in communities of accompanying microbes among the treatments. The same cannot, however, be claimed for the various nonmycorrhizal control treatments. Further, there were large differences in the composition of microbial (both bacterial and fungal) communities among the individual nonmycorrhizal treatments, and this was reflected in couple of differences in plant growth, but not necessarily in the P and/or N uptake by the plants. The differences in plant growth parameters between the different nonmycorrhizal inoculated controls may be less conspicuous when looking at plant growth/nutrition under ambient conditions but may be probably very different if exposed to nematode grazing (Schouteden et al. 2015), other pathogen pressure (Wehner et al. 2010), or a range of abiotic stresses (drought, salinity, nutrient deficiency, etc.). It is very unlikely to produce nonmycorrhizal controls with similar composition of microbial communities as in the mycorrhizal treatments. Our recent results thus represent an important and rare contribution to the knowledge on the mycorrhizal and nonmycorrhizal microbiomes and designing appropriate controls in mycorrhizal research. Our results also indicate that at least some of the inconsistencies in the published literature may be explainable by uncertainties with respect to the composition of microbial communities in the nonmycorrhizal controls. We believe that our work will stimulate further research in this field and help to understand the microbial factors that can influence plant growth/nutrition in model experiments with arbuscular mycorrhizal fungi.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Mol Biol* 215:403–410
- Apprill A, McNally S, Parsons R, Laura Weber L (2015) Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb Ecol* 75:129–137
- Campos MAD, da Silva FSB, Yano-Melo AM, de Melo NF, Maia LC (2017) Application of arbuscular mycorrhizal fungi during the acclimatization of *Alpinia purpurata* to induce tolerance to *Meloidogyne arenaria*. *Plant Pathol J* 33:329–336
- Cavagnaro TR, Jackson LE, Six J, Ferris H, Goyal S, Asami D, Scow KM (2006) Arbuscular mycorrhizas, microbial communities, nutrient availability, and soil aggregates in organic tomato production. *Plant Soil* 282:209–225
- Christensen MJ, Leuchtman A, Rowan DD, Tapper BA (1993) Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and perennial ryegrass (*Lolium perenne*). *Mycol Res* 97:1083–1092
- Chu-Chou M, Guo B, An ZQ, Hendrix JW, Ferriss RS, Siegel MR, Dougherty CT, Burrus PB (1992) Suppression of mycorrhizal fungi in fescue by the *Acremonium coenophialum* endophyte. *Soil Biol Biochem* 24:633–637
- Cimmino A, Andolfi A, Berestetskiy A, Evidente A (2008) Production of phytotoxins by *Phoma exigua* var. *exigua*, a potential mycoherbicide against perennial thistles. *J Agric Food Chem* 56: 6304–6309
- de Andrade S, Malik S, Sawaya ACHF, Bottcher A, Mazzafera P (2013) Elicitation of tobacco alkaloid biosynthesis by disrupted spores and filtrate of germinating spores of the arbuscular mycorrhizal fungi *Glomus etunicatum*. *J Plant Interact* 8:162–169
- Den Belder E, Jansen E (1994) Capture of plant-parasitic nematodes by an adhesive hyphae forming isolate of *Arthrobotrys oligospora* and some other nematode-trapping fungi. *Nematologica* 40:423–437
- Edgar RC, Flyvbjerg H (2015) Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* 31: 3476–3482
- Fahrbach M, Kuever J, Meinke R, Kämpfer P, Hollender J (2006) *Denitratisoma oestradiolicum* gen. nov., sp. nov., a 17 β -oestradiol-degrading, denitrifying betaproteobacterium. *Int J Syst Evol Microbiol* 56:1547–1552
- Fisher JB, Jayachandran K (2002) Arbuscular mycorrhizal fungi enhance seedling growth in two endangered plant species from south Florida. *Int J Plant Sci* 163:559–566
- Fortin JA, Becard G, Declercq S, Dalpe Y, St-Arnaud M, Coughlan AP, Piche Y (2002) Arbuscular mycorrhiza on root-organ cultures. *Can J Bot* 80:1–20
- Gryndler M, Černá L, Bukovská P, Hřšelová H, Jansa J, (2014) *Tuber aestivum* association with non-host roots. *Mycorrhiza* 24 (8):603–610

- Ihrmark K, Bödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandström-Durling M, Clemmensen KE, Lindahl BD (2012) New primers to amplify the fungal ITS2 region—evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol Ecol* 82:666–677
- Kahiluoto H, Vestberg M (2000) Creation of a non-mycorrhizal control for a bioassay of AM effectiveness. 2. Benomyl application and soil sampling time. *Mycorrhiza* 9:259–270
- Kahiluoto H, Ketoja E, Vestberg M (2000) Creation of a non-mycorrhizal control for a bioassay of AM effectiveness. 1. Comparison of methods. *Mycorrhiza* 9:241–258
- Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A, Palmer TM, West SA, Vandenkoornhuyse P, Jansa J, Bücking H (2011) Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* 333:880–882
- Koide R, Li M (1989) Appropriate controls for vesicular-arbuscular mycorrhiza research. *New Phytol* 111:35–44
- Kojima T, Saito K, Oba H, Yoshida Y, Terasawa J, Umehara Y, Suganuma N, Kawaguchi M, Ohtomo R (2014) Isolation and phenotypic characterization of *Lotus japonicus* mutants specifically defective in arbuscular mycorrhizal formation. *Plant Cell Physiol* 55:928–941
- Kreutzer WA (1960) Soil treatment. In: Horsfall JG, Dimond AE (eds) *Plant pathology III—the diseased population epidemics and control*. Academic Press, New York, pp 431–476
- Leigh J, Fitter AH, Hodge A (2011) Growth and symbiotic effectiveness of an arbuscular mycorrhizal fungus in organic matter in competition with soil bacteria. *FEMS Microbiol Ecol* 76:428–438
- Manian S, Edathil TT, Udayan K (1995) Vesicular-arbuscular mycorrhizal colonization and growth of tomato (*Lycopersicon esculentum*) in autoclaved soil. *Pertanika J Trop Agric Sci* 18:95–101
- Marschner P, Crowley DE, Lieberei R (2001) Arbuscular mycorrhizal infection changes the bacterial 16S rDNA community composition in the rhizosphere of maize. *Mycorrhiza* 11:297–302
- Marsh JF, Schultze M (2001) Analysis of arbuscular mycorrhizas using symbiosis-defective plant mutants. *New Phytol* 150:525–532
- Merryweather J, Fitter A (1996) Phosphorus nutrition of an obligately mycorrhizal plant treated with the fungicide benomyl in the field. *New Phytol* 132:307–311
- Mortimer PE, Perez-Fernandez MA, Valentine AJ (2008) The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of the tripartite symbiosis with nodulated *Phaseolus vulgaris*. *Soil Biol Biochem* 40:1019–1027
- Naumann M, Schübler A, Bonfante P (2010) The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the *Mollicutes*. *ISME J* 4:862–871
- Nazeri NK, Lambers H, Tibbett M, Ryan MH (2013) Do arbuscular mycorrhizas or heterotrophic soil microbes contribute toward plant acquisition of a pulse of mineral phosphate? *Plant Soil* 373:699–710
- Ohno T, Zibilske LM (1991) Determination of low concentrations of phosphorus in soil extracts using malachite green. *Soil Sci Soc Am J* 55:892–895
- Pizano C, Mangan SA, Graham JH, Kitajima K (2017) Host-specific effects of soil microbial filtrates prevail over those of arbuscular mycorrhizae in a fragmented landscape. *Ecol Appl* 27:1946–1957
- Püschel D, Janoušková M, Voříšková A, Gryndlerová H, Vosátka M, Jansa J (2017) Arbuscular mycorrhiza stimulates biological nitrogen fixation in two *Medicago* spp. through improved phosphorus acquisition. *Front Plant Sci* 8:390
- Řezáčová V, Slavíková R, Sochorová L, Konvalinková T, Procházková V, Šťovíček V, Hršelová H, Beskid O, Hujšlová M, Gryndlerová H, Gryndler M, Püschel D, Jansa J (2018) Mycorrhizal symbiosis induces plant carbon re-allocation differently in C₃ and C₄ *Panicum* grasses. *Plant Soil* 425:441–456. <https://doi.org/10.1007/s11104-018-3606-9>
- Rivero J, Gamir J, Aroca R, Pozo MJ, Flors V (2015) Metabolic transition in mycorrhizal tomato roots. *Front Microbiol* 6:598
- Rosikiewicz P, Bonvin J, Sanders IR (2017) Cost-efficient production of in vitro *Rhizophagus irregularis*. *Mycorrhiza* 27:477–486
- Schliemann W, Ammer C, Strack D (2008) Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. *Phytochemistry* 69:112–146
- Schouteden N, De Waele D, Panis B, Vos CM (2015) Arbuscular mycorrhizal fungi for the biocontrol of plant-parasitic nematodes: a review of the mechanisms involved. *Front Microbiol* 6:1280
- Shaw LJ, Beaton Y, Glover LA, Killham K, Meharg AA (1999) Re-inoculation of autoclaved soil as a non-sterile treatment for xenobiotic sorption and biodegradation studies. *Appl Soil Ecol* 11:217–226
- Slavíková R, Püschel D, Janoušková M, Hujšlová M, Konvalinková T, Gryndlerová H, Gryndler M, Weiser M, Jansa J (2017) Monitoring CO₂ emissions to gain a dynamic view of carbon allocation to arbuscular mycorrhizal fungi. *Mycorrhiza* 27:35–51
- Smith SE, Smith FA, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiol* 133:16–20
- Souza RC, Hungria M, Cantao ME, Vasconcelos ATR, Nogueira MA, Vicente VA (2015) Metagenomic analysis reveals microbial functional redundancies and specificities in a soil under different tillage and crop-management regimes. *Appl Soil Ecol* 86:106–112
- Ter Braak CJF, Šmilauer P (2012) *Canoco reference manual and user's guide: software for ordination (version 5)*. Microcomputer Power, Ithaca 496 pp
- Thonar C, Erb A, Jansa J (2012) Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities—marker design, verification, calibration and field validation. *Mol Ecol Resour* 12:219–232
- Veresoglou SD (2012) Arbuscular mycorrhiza prevents suppression of actual nitrification rates in the (myco-)rhizosphere of *Plantago lanceolata*. *Pedosphere* 22:225–229
- Větrovský T, Baldrian P (2013) Analysis of soil fungal communities by amplicon pyrosequencing: current approaches to data analysis and the introduction of the pipeline SEED. *Biol Fertil Soils* 49:1027–1037
- Violet A, Corberand T, Mougél C, Robin A, Lemanceau P, Mazurier S (2011) Fluorescent pseudomonads harboring type III secretion genes are enriched in the mycorrhizosphere of *Medicago truncatula*. *FEMS Microbiol Ecol* 75:457–467
- Wagg C, Bender SF, Widmer F, van der Heijden MGA (2014) Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proc Natl Acad Sci U S A* 111:5266–5270
- Wang X, Chen M, Xiao J, Hao L, Crowley DE, Zhang Z, Yu J, Huang N, Huo M, Wu J (2015) Genome sequence analysis of the naphthenic acid degrading and metal resistant bacterium *Cupriavidus gilardii* CR3. *PLoS One* 10:e0132881
- Wehner J, Antunes PM, Powell JR, Mazukatow J, Rillig MC (2010) Plant pathogen protection by arbuscular mycorrhizas: a role for fungal diversity? *Pedobiologia* 53:197–201
- White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR protocols: a guide to methods and applications*. Academic Press, San Diego, pp 315–322
- Willmann M, Gerlach N, Buer B, Polatajko A, Nagy R, Koebe E, Jansa J, Flisch R, Bucher M (2013) Mycorrhizal phosphate uptake pathway in maize: vital for growth and cob development on nutrient poor agricultural and greenhouse soils. *Front Plant Sci* 4:533
- Wu X, Wang W, Liu J, Pan D, Tu X, Lv P, Wang Y, Cao H, Wang Y, Rimao Hua R (2017) Rapid biodegradation of the herbicide 2,4-dichlorophenoxyacetic acid by *Cupriavidus gilardii* T-1. *J Agric Food Chem* 65:3711–3720