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ORIGINAL ARTICLE

Dead *Rhizophagus irregularis* biomass mysteriously stimulates plant growth

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Abstract

Arbuscular mycorrhizal (AM) fungi establish symbiotic associations with many plant species, transferring significant amounts of soil nutrients such as phosphorus to plants and receiving photosynthetically fixed carbon in return. Functioning of AM symbiosis is thus based on interaction between two living partners. The importance of dead AM fungal biomass (necromass) in ecosystem processes remains unclear. Here, we applied either living biomass or necromass (0.0004 potting substrate weight percent) of monoxenically produced AM fungus (*Rhizophagus irregularis*) into previously sterilized potting substrate planted with *Andropogon gerardii*. Plant biomass production significantly improved in both treatments as compared to non-amended controls. Living AM fungus, in contrast to the necromass, specifically improved plant acquisition of nutrients normally supplied to the plants by AM fungal networks, such as phosphorus and zinc. There was, however, no difference between the two amendment treatments with respect to plant uptake of other nutrients such as nitrogen and/or magnesium, indicating that the effect on plants of the AM fungal necromass was not primarily nutritional. Plant growth stimulation by the necromass could thus be either due to AM fungal metabolites directly affecting the plants, indirectly due to changes in soil/root microbiomes or due to physicochemical modifications of the potting substrate. In the necromass, we identified several potentially bioactive molecules. We also provide experimental evidence for significant differences in underground microbiomes depending on the amendment with living or dead AM fungal biomass. This research thus provides the first glimpse into possible mechanisms responsible for observed plant growth stimulation by the AM fungal necromass.

Keywords Arbuscular mycorrhiza (AM) · Mass spectrometry (MS) · Metabolites · Microbiome · Necromass · Signal

Introduction

Arbuscular mycorrhizal (AM) symbiosis is one of the most widespread inter-kingdom associations on Earth, heavily implicated in the nutrition, carbon economy, tolerance to drought and biotic stresses of many plant species, as well as in stabilization of co-existence of different species in terrestrial ecosystems (van der Heijden et al. 2015; Mariotte et al. 2018).

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Mycorrhizal fungi colonize roots of their host plants and the surrounding soil, directly interconnecting these two different niches (Vályi et al. 2016). Various active and highly controlled processes are involved in the functioning of AM symbiosis such as a specific molecular dialogue between the plants and the fungi (Oldroyd et al. 2009) and metabolically active exchange of resources through specific molecular channels (Rausch et al. 2001; Kiers et al. 2011; Garcia et al. 2016). Therefore, the AM symbiosis has always been regarded as a particular case of strictly biotrophic interactions, based on coexistence and finely tuned co-operation between two living entities.

The role of dead AM fungal biomass (necromass) in the soil is much less known, particularly because of methodological limitations in its production in the absence of other organisms (be it roots or microbes). Yet its role may be important, given the extensive hyphal networks formed by AM fungi (Leake et al. 2004) and their fast turnover times. Both microscopic observations and carbon isotopic tracing collectively suggest that it is probably more than 50% of AM fungal hyphae (or at least their carbon) that turns over in less than 1-week time (Bago et al. 1998; Atkinson and Watson 2000; Johnson et al. 2002; Staddon et al. 2003). Hyphal turnover could possibly be even faster when considering fungal grazers and/or mechanical soil disturbance such as tillage (Klironomos and Ursic 1998; Kabir 2005; de Vries et al. 2009). Limitations of availability of sufficient amounts of monoxenically produced AM fungal biomass recently have been overcome (Rosikiewicz et al. 2017), opening new avenues to investigate the pathways and functional significance of AM fungal necromass in soil, as well as the identity and functioning of microbes involved in its recycling. Likewise, thanks to the availability of significant amounts of pure AM fungal biomass, it now will be possible to conduct direct and detailed biochemical/metabolomic analyses of the AM fungi (at least those amenable to grow under monoxenic culture conditions). This was completely unavailable several years ago when researchers had to rely on qualified guesses when estimating metabolic costs of biosynthesis of AM fungal tissues (Kaschuk et al. 2009), and to-date only a handful of published papers have reported biochemical analyses of pure AM fungal biomass (Wewer et al. 2014; Dearth et al. 2018). Along similar lines, there is a high level of uncertainty about the composition of AM fungal cell walls. Although it generally is accepted that some chitin is present in cell walls of AM fungi (Sbrana et al. 1995; Lanfranco et al. 1999; Bonfante and Genre 2015; Zeng et al. 2019), these fungi can obviously withstand higher chitin-synthase inhibitor concentrations than most other fungi (Bago et al. 1996), which may mean that chitin/chitosan is not the only or not the principal component of their cell walls. Quantitative analyses of the composition of AM fungal cell walls are not yet available in published literature to the best of our knowledge.

Here, we report results of a pot experiment in which we compared the growth and mineral nutrition of a mycorrhiza-responsive model plant, *Andropogon gerardii* Vitman, as affected by addition of living or dead AM fungal biomass (devoid of other microbes or host plant roots) into previously sterilized potting substrate, and compared those two treatments with a non-amended control. This research was part of a larger experiment of which results already have been partially published (Gryndler et al. 2018). In the previous publication, plant treatments with living AM fungal biomass (i.e. monoxenically produced mycorrhizal inoculum) and the non-amended control were both included, among several other treatments, aiming mainly at deciphering consequences for the host plants and the underground microbiome of different mycorrhizal and non-mycorrhizal inoculation approaches including soil microbial washes or not. The novel aspect of the current paper is the focus on AM fungal necromass (which has not been reported previously) as compared to the living AM fungal inoculum and the non-amended control. Further, we now elaborate plant uptake of several macro- and micronutrients other than only the phosphorus and nitrogen reported previously, and we also provide novel insights into the chemistry of the AM fungal biomass, focusing on identification of potentially bioactive molecules.

The overall aim of this research was to test whether AM fungal metabolites added with AM fungal necromass could have triggered any detectable plant/underground microbiome responses as compared to living AM fungal inoculum or whether such inputs can be ignored when setting up mycorrhiza-inoculation trials with monoxenically produced AM fungi. This is because it is generally considered to be a good practice to add a mock inoculum to nonmycorrhizal control treatments to offset for possible confounding effects due to inputs of inoculum carrier material, mineral nutrients, water sorbents, environmental contaminants and/or accompanying microbes contained in most AM fungal inoculants. Monoxenically produced AM fungal inocula contain no other microbes, but still might contain bioactive metabolites and/or mineral nutrients exerting measurable effects on plants. Thus, here we examined what is the effect of addition of AM fungal necromass to the same conditions as control (nonmycorrhizal) pots and whether it would be advisable to use AM fungal necromass as a control amendment when running pot experiments with monoxenically produced AM fungal inoculants.

Material and methods

Design of the experiment, plant cultivation and fertilization

The pot experiment constituting this research involved three treatments: (1) non-amended control without application of

any inoculum, nutrients or microbial filtrate into the substrate (this treatment is subsequently called 'Control'); (2) inoculated with monoxenically produced mycorrhizal inoculum (see below; subsequently called 'Living'); and (3) including homogenate of monoxenically produced mycorrhizal inoculum (subsequently called 'Dead'). Each treatment involved 12 pots (2 L each, dimensions 10:10:20 cm, w:d:h) filled with a potting substrate made of γ-sterilized (25 kGy) soil from Litoměřice (described in Řezáčová et al. 2016) and autoclaved sand and zeolite (mixed in proportions 2:9:9, respectively, by volume). This mixture (i.e. potting substrate) had the following properties: pH(H_2O) 8.9, 0.22% total organic C, 0.013% total nitrogen (N), 46.5 mg kg^{-1} total phosphorus (P) and 2.6 mg kg^{-1} water extractable P. The pots were first filled with the substrate, supplemented or not by the AM fungal inoculum (living or dead) in a layer approximately 2 cm below the seeds, and each of the pots was then sown with 30–35 seeds of *Andropogon gerardii* provided by Jelitto Staudensamen GmbH (Schwarmstedt, Germany), and the seeds were covered with a 0.5 cm layer of the substrate. We used *Andropogon gerardii* as a model host plant because of its high responsiveness to AM symbiosis by growth and P uptake in our experimental settings (Püschel et al. 2016), and also because we have extensive experience with molecular analyses of underground (root and substrate) microbiomes of this particular plant species (Bukovská et al. 2018; Gryndler et al. 2018). We did not eliminate any seedlings after emergence (hence there may have been slightly uneven numbers of plant individuals per pot, but this was not quantified). We conducted all measurements on a per-pot basis (rather than for individual plants), thus effectively looking at plant population responses.

Positions of the pots were randomized in an illuminated greenhouse equipped with high pressure metal halide lamps providing at least 200 μmol photosynthetically active radiation flux at the plant level over a 14-h photoperiod. Pots were incubated there for 10 weeks (26 January 2015 through to 7 April 2015). From the fifth week after planting, each pot weekly received 65 mL Long Ashton mineral nutrient solution (Mortimer et al. 2008) with its P concentration decreased to 20% of the original recipe.

Each pot was filled with 2.2 kg dry weight of the potting substrate, containing 4.84 g organic C, 286 mg total N, 102 mg total P and 5.72 mg water-extractable P. Phosphorus and nitrogen inputs with the nutrient solution were (per pot throughout the entire experiment) 65.5 mg N, predominantly as nitrate, and 3.13 mg P provided as orthophosphate. Nutrient inputs from seeds were not accounted in this experiment.

Pots were watered every second day with deionized water to maintain a range between 50 and 75% of the water holding capacity of the potting substrate throughout the experiment. Especial care was taken to prevent any liquid leaching from the pots during watering, thus preventing leaching losses of nutrients from the substrate.

Monoxenically produced AM fungal inoculum

The mycelium and spores of the AM fungus *Rhizophagus irregularis* SYM5 were obtained from 6-month-old monoxenic cultures kindly provided by Symbiom Ltd. (Lanškroun, Czech Republic, www.symbiom.cz). In total, six vessels were used to produce AM fungal biomass. Each vessel contained 250 mL of Phytagel-solidified MSR medium (Cranenbrouck et al. 2005) and the AM fungus. Host roots were separated from the medium by a 42 μm mesh, and were discarded before AM fungal biomass preparation. A piece of approx. 5 mm^3 of the AM fungal biomass was axenically extracted from each vessel by fine forceps, put on water agar enriched with 200 mg L^{-1} yeast extract and incubated for 6 months at 25 °C in the dark to confirm the absence of culturable microbial contaminants. No contaminant microbes were detected (details not shown).

The content of all six AM fungal culture vessels (i.e. the gel containing hyphae and spores, devoid of roots) was pooled and subjected to slow agitation in 10 mM potassium citrate buffer (pH 6.0). The AM fungal biomass was then collected on a 40 μm sieve, washed with 1% $MgSO_4$ $7H_2O$ and briefly dried on a paper towel. Then, 163 mg of the AM fungal biomass (fresh weight) were suspended in 125 mL water. Ten millilitres of this suspension was then provided per pot of the Living treatment. Another batch of the AM fungal biomass weighing 163 mg was then used to prepare AM fungal necromass as follows. The AM fungal biomass was first suspended in 2 mL water and then was homogenized with a ceramic mortar and pestle for 20 min. Material recovered from the mortar was further homogenized in a glass homogenizer for 5 min, diluted with water to 10 mL and subjected to five freeze/thaw cycles (− 80 °C/+ 25 °C). Finally, the homogenate was diluted to 125 mL (using sterile water) and applied at a rate of 10 mL per pot of the Dead treatment. Microphotographs of the inoculants are available as supplementary Fig. S1. Dry weight content of the AM fungal biomass was 56.3% of the fresh weight, and the amount of AM fungus added per pot corresponded approximately to 6630 spores and 48 m hyphae (Gryndler et al. 2018). Carbon, nitrogen and phosphorus inputs with the AM fungal biomass/necromass were 3.96 mg C, 109 μg total N and 12.3 μg P per pot as per elemental analyses conducted either using a CN elemental analyser (Flash EA 2000, ThermoFisher Scientific, Waltham MA, USA), or P colorimetry with HNO_3 extracts of previously ashed AM fungal biomass at 550 °C for 12 h (Ohno and Zibilske 1991; Püschel et al. 2017).

Harvest and analyses of the plants

After 10 weeks of growth, plants were harvested. All individuals from each pot were processed as a single sample. The shoots were weighed fresh and after drying at 65 °C for 3 days.

The roots were separated from the substrate, and a root-free substrate sample was collected and dried at 65 °C for 3 days, pulverized in a ball mill MM200 (Retsch, Haan, Germany) and subjected to DNA extraction using a NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany), spiking every sample with 2×10^7 copies of an internal DNA standard as specified earlier (Thonar et al. 2012). Root biomass was weighed fresh and then again after drying at 65 °C for 3 days. Dried root samples were pulverized in a ball mill, and DNA extracted using the CTAB protocol (Gryndler et al. 2014), spiking every sample with 2×10^7 copies of an internal DNA standard (Thonar et al. 2012). Colonization of roots and of the substrate by the AM fungus was measured by quantitative real-time PCR (qPCR) as described previously (Gryndler et al. 2018), correcting the measured AM fungal abundance in DNA extracts with internal DNA standard recoveries assessed for each sample (Thonar et al. 2012). One root sample (pot ID 6) was too small to allow for DNA extraction and, therefore, was not included in the qPCR analyses.

Phosphorus concentration in plant biomass samples (shoots and roots from each pot analysed separately) was measured by Malachite green colorimetry according to Ohno and Zibilske (1991) after incineration of the plant biomass aliquots (70–100 mg per sample) in a muffle furnace at 550 °C for 12 h. The ashes were extracted with hot concentrated HNO_3 as detailed previously (Püschel et al. 2017; Slavíková et al. 2017). One root sample (pot ID 6) was too small to allow for P analyses. The N and C concentrations in the plant biomass samples were measured with a Flash EA 2000 elemental analyser (ThermoFisher Scientific, Waltham, MA, USA) using samples weighing between 1.5 and 2.5 mg each.

For six randomly selected pots per treatment, analysis of other macro- (K, Ca and Mg) and microelement (Cu, Mn, Fe and Zn) concentrations in plant shoots and roots was conducted. Digestion procedure for those analyses was carried out in a HEPA filtered air environment in a class-100 laminar flow hood. Deionized water with a resistivity of 18.2 MΩ cm (Milli-Q Element, Merck, Kenilworth NJ, USA) was used throughout the sample preparation. Samples of biomass (approximately 100 mg each) were weighed and digested using 5 mL of HNO_3 (≥ 65%, J.T. Baker, supplied via ThermoFischer Scientific, Waltham MA, USA, double-distilled in-house) and 1 mL of HF (47–51%, ultrapure, VWR, Radnor PA, USA) in perfluoroalkoxy-teflon digestion vessels (Savillex, Eden Prairie MN, USA) at 180 °C on a hot plate (overnight), and then evaporated to near dryness. Evaporates were further dissolved in 2% HNO_3, adjusted to 25 mL in polymethylpentene volumetric flasks and appropriately diluted for concentration analysis. For quality control, four procedural blanks and two biological standard reference materials (SRMs) were processed with the samples: SRM 1575a (pine needles, NIST, Gaithersburg MD, USA) and SRM 1570a (spinach leaves, NIST, Gaithersburg MD, USA), results shown in supplementary Table S1 (sheet 'ICPMS references'). Elemental concentrations in the digested samples were determined by an Element2 magnetic sector field inductively coupled plasma mass spectrometer (ICPSFMS, Thermo Fisher Scientific, Waltham MA, USA) via external calibration.

Fungal and prokaryotic microbiome analyses of the roots and substrate samples

Fungal community composition in each root and substrate sample was profiled using ITS2 amplicons. DNA extracts from both roots and substrate samples were used as templates in a semi-nested PCR protocol. In the first step, fungal DNA was amplified using a combination of ITS1F and ITS4 primers (White et al. 1990). Every reaction (25 μL) was carried out in triplicate. Each reaction contained 12.5 μL 2× Combi PPP Mix (hot start Taq, 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^{-1}) and 10.5 μL molecular grade water. The PCR cycling programme was as follows: initial denaturation at 94 °C for 4 min, followed by 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 technical replicates per each biological sample were then mixed, purified from the PCR mix by using a Qiaquick PCR purification kit (QIAGEN, Hilden, Germany) with elution volume 30 μL, diluted 1:1000 with water and used as template in the second PCR amplification step. Another PCR amplification reaction was then set up per each biological sample, with the reaction mixture being the same as in the first PCR except that tagged primers gITS7_Txx (Ihrmark et al. 2012) and ITS4_xxx (White et al. 1990), targeting the ITS2 region of the rDNA operon, were used. The tagged (_Txx and _xxx) primer sequences have been published previously (Gryndler et al. 2018). The PCR cycling programme was as follows: initial denaturation at 94 °C for 4 min followed by 25 cycles including denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 30 s. Final elongation lasted for 10 min at 72 °C. The final PCR product was purified by using a Qiaquick PCR purification kit (Qiagen, Hilden, Germany). DNA fragments originating from different samples were unambiguously distinguished by tag (index) combinations of the primers from the second PCR reaction.

Prokaryotic communities in the DNA samples were profiled by using amplicons of the V4 region of the 16S rRNA gene. The amplicons were generated in a single PCR step, with reaction mixture identical to that described above, but with a different polymerase mix, namely the TPHS DNA-free 2× Master Mix (hot start Taq, Top-Bio, Vestec, Czech Republic), containing no prokaryotic DNA residues. For the PCR, we used modified tagged primers 515Fd_Txxx

and 806Rd_Txxx (Apprill et al. 2015). Every reaction (25 μL) was carried out in triplicate to reduce the risk of PCR bias. The cycling conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles including denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s, and elongation at 72 °C for 75 s. Final elongation lasted for 10 min at 72 °C. Post PCR, the three technical replicates per biological sample were mixed and purified by using a Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA fragments originating from different samples were unambiguously distinguished by tag (index) combinations of the primers. Products of amplification of prokaryotic and fungal DNA from each biological sample were then quantified using Picogreen fluorescence (using a Quant-iT PicoGreen dsDNA Assay Kit, ThermoFisher Scientific, Waltham MA, USA), diluted to a concentration of 20 ng μL^{-1} and combined in a single sequencing library that was sequenced with the MiSeq Illumina platform (2 × 300). Because one of the root samples from the control treatment (pot ID 6) was too small for extracting DNA, and because we detected very little AM fungal biomass in the roots from pot ID 80 (see Table S1), we removed those two pots from the molecular analyses entirely, resulting in 11 full replicates for the Control and Living treatments, and 12 full replicates for the Dead treatment.

Sequencing data were treated by Seed software (Větrovský and Baldrian 2013), version 2.0.3. The software is a Windows-based interface for amplicon high-throughput sequencing data analyses employing external packages such as mafft, mothur, usearch and blastn in a user-friendly environment. For more information, please see the following website: http://www.biomed.cas.cz/mbu/lbwrf/seed/. The sequences were first filtered to remove low-quality sequences (quality score below 30) and sequences shorter than 150 bp or longer than 400 bp from the prokaryotic amplicons. From the fungal sequences, the ITS region was extracted (i.e. effectively leaving out 5.8S and 28S sequences) using the ITSx software package (Bengtsson-Palme et al. 2013) embedded in the Seed software, and ITS sequences shorter than 40 bp were removed from subsequent analyses. The sequences were then chimera-cleaned/clustered with the Usearch tool (version 8.1. 1861, Edgar and Flyvbjerg (2015)) at the similarity level 97% (fungi) or 95% (prokaryotes), and the most abundant representative sequences were compared (blastn algorithm, Blast tool, Altschul et al. (1990), version 2.2.26+) with the GenBank database (environmental sequences, metagenomes and unidentified organisms excluded). Operational taxonomic units (OTUs) corresponding to the different clusters were named according to the best GenBank hit as described previously (Gryndler et al. 2018). From the prokaryotic sequences, those sequences assigned to mitochondria, plastids or eukaryotes were excluded from further analyses. Remaining sequences were resampled to retain 6000 fungal sequences per root sample, 8000 fungal sequences per substrate sample, 6000 prokaryotic sequences per substrate sample and 2000 prokaryotic sequences per root sample. The sequences were then clustered anew (prokaryotes and fungi separately); OTUs merged per taxid (a unique numerical identifier of a stable and persistent taxon based on sequencing evidence, Federhen (2012), http://www.ncbi.nlm.nih.gov/taxonomy), genus, order and phylum for prokaryotes; and per taxid, genus and class for fungi (see Table S1 for data). The microbiome data relevant to the work described here were deposited in the Sequence Read Archive (NCBI, Bethesda MD, USA) under accession number PRJNA407304.

Analysis of AM fungal biomass composition

After detecting a significant effect of AM fungal necromass on plant growth and mineral nutrition, we carried out various biochemical analyses of the AM fungal biomass in order to identify potentially bioactive compounds possibly responsible for the observed effects. First, we separated the fungal metabolites to methanol-soluble (lipidic) and methanol-insoluble fractions as follows: fresh biomass of *Rhizophagus irregularis* obtained from 7-month-old monoxenic cultures (170 mg fresh weight containing hyphae and spores; for more details, please see supplementary methods) was ground in a mortar with 2 mL methanol for 5 min, and then centrifuged at 17,000 g for 20 min at room temperature. The liquid organic phase was used directly for high-resolution electrospray ionization-mass spectrometry. The solid-phase remaining after the extraction with methanol (see above) or methanol-chloroform mixture (see online supplement for details) was used to further purify and analyse cell walls of the AM fungus with particular attention to the presence of chitin. For further details of the methodology and results, please see the online supplementary information.

Calculations and statistics

Nutrient contents in the plant biomass (roots or shoots, per pot) were calculated by multiplying respective nutrient concentrations by the mass of roots or shoots. Biomass, nutrient concentration and nutrient contents data were then tested for homoscedasticity using Bartlett's test. Subsequently, the differences between the inoculation treatments with respect to plant biomass production and nutrient concentrations/contents were tested by one-way analysis of variance (ANOVA). Following significant ($p < 0.05$) ANOVAs, treatment means were compared by Tukey's honestly significant difference (HSD) test with Statgraphics Plus for Windows v. 3.1. Graphs were created with Microsoft Excel 2016 or SigmaPlot v. 13.

The effects of experimental treatments on microbial communities in the roots and the substrate were analysed by

redundancy analysis (RDA), using log-transformed sequence counts and CANOCO 5.1 software (ter Braak and Šmilauer 2018). Since the variation of numbers of sequences used per sample was orthogonal to the analysed factors, and mean numbers of sequences in compared groups were nearly the same (Table S1), there was no need for further data standardization. Microbial community analyses for substrate and root samples were conducted separately, and samples from each pot permuted together in the RDA analysis, using a covariate to remove systematic difference between the two sample sources (substrate and roots). Identification of microbial taxa significantly associated with a specific experimental treatment was carried out using *t* value biplots with CANOCO 5.1 software (ter Braak and Šmilauer 2018).

Results

Plant growth was comparably stimulated by living or dead AM fungal inoculum

There was a significant difference between the Control and the other two (Living and Dead) treatments with respect to shoot biomass. Biomass of shoots in the Control treatment was, on average, less than a half of that in the Living or Dead treatments, with the latter two treatments not significantly different from each other (Fig. 1). Root biomass was largest in the Living treatment, followed by the Dead and Control treatments, which all significantly differed. Root biomass of the Control treatment was only about a third of that in the Dead treatment, and about a quarter of that in the Living treatment (Fig. 1, see also Table S1 for the primary data).

The mycorrhizal fungus only grew in the Living treatment

Rhizophagus was easily detectable by qPCR in both roots and substrate samples from the Living treatment, with exception of roots from one pot (ID 80) belonging to the Living treatment (see Table S1 for details). In contrast, no single sample from the Dead and Control treatments (either roots or substrate) yielded a positive qPCR detection of the AM fungus (Table S1). Absence of root colonization by the AM fungus in the Control and Dead treatments was further confirmed using traditional microscopy of root samples stained with Trypan blue (details not shown).

The living AM fungus stimulated P but not N uptake compared to the dead AM fungal inoculum

The P concentration in both shoots and roots was highest in the Living treatment, and similarly low in the Dead and Control treatments, whereas N concentrations in both shoots

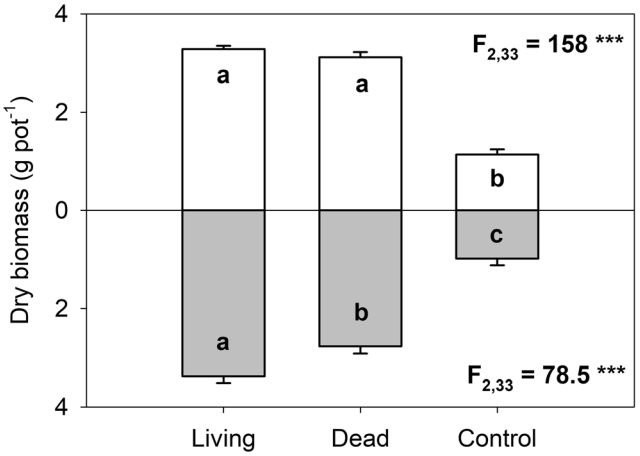

Fig. 1 Dry biomass of shoots (empty bars) and roots (grey bars; shown as positive values below the abscissa) as affected by inoculation of experimental plants with a living arbuscular mycorrhizal (AM) fungus, *Rhizophagus irregularis* (Living), dead biomass of the AM fungus (Dead) and compared to the non-inoculated control (Control). Bars represent mean values per pot (+ 1 standard errors), calculated from 12 true replicates per treatment. Bars sharing the same letter correspond to means not statistically different among the respective treatments ($p \geq 0.05$), as per Tukey's HSD test following significant ANOVA, conducted separately for the shoots and the roots. *F* values for the individual ANOVA analyses are displayed. ***$p < 0.001$

and roots were highest in the plants belonging to the Control treatment (Fig. 2). The amount of P in the plant biomass (i.e. the P content) was highest in the Living treatment, whereas the Dead and Control treatments showed several-fold lower values of P content in the plant biomass, although the values were still higher in the Dead than in the Control treatment (Fig. 2). Nitrogen contents of the plants belonging to the Living and Dead treatments were not significantly different (although the allocation to roots differed between the two treatments), whereas the plants belonging to the Control treatment had significantly lower N contents than the plants in the other two treatments (Fig. 2 and Table S1). Examination of the relationship between concentrations of P and N in shoots or roots and the total amount of plant shoots or roots biomass produced per pot indicated that P was possibly a growth-limiting nutrient under our experimental setting, whereas N was not (supplementary Fig. S2).

Zinc and manganese concentrations were enhanced by the living and dead AM fungus, respectively

There were no strong effects of the experimental treatments on the other macronutrient (K, Ca and Mg) concentrations in the plants (Fig. 3), neither was there a strong indication for any other macronutrient except P (see above) being growth-limiting, possibly with the exception of Mg in the shoots (Fig. S2). For micronutrients, there were only a few strong effects of the experimental treatments on the concentrations of those nutrients in plant biomass. Particularly, shoots from the Living

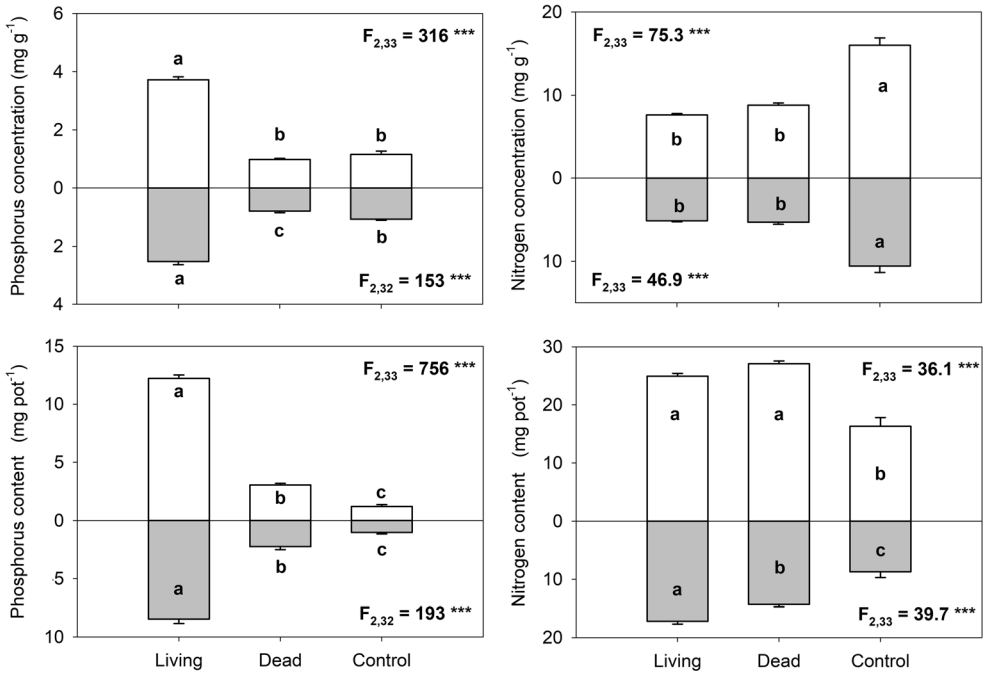

Fig. 2 Phosphorus (P) and nitrogen (N) concentrations and P and N contents in the shoots (empty bars) and roots (grey bars; shown as positive values below the abscissa) as affected by inoculation of the experimental plants with a living arbuscular mycorrhizal (AM) fungus, *Rhizophagus irregularis* (Living), dead biomass of the AM fungus (Dead) and compared to the non-inoculated control (Control). Bars represent mean values per pot (+ 1 standard errors), calculated from 11 (for P in roots) or 12 (for all other situations) true replicates per treatment. Bars sharing the same letter correspond to means not statistically different among the respective treatments ($p \geq 0.05$), as per Tukey's HSD test following significant ANOVA, conducted separately for the shoots and the roots. *F* values for the individual ANOVA analyses are displayed. ***$p < 0.001$

treatment showed significantly higher concentrations of Zn than shoots from the other treatments (Fig. 4), whereas for Cu and Fe, their highest concentrations in the shoots were recorded in the Control treatment (Fig. 4). Our analyses also showed that the concentrations of Fe and Mn in the roots were higher in the plants belonging to the Dead treatment versus the Living treatment, whereas the concentrations of those micronutrients recorded in the roots from the Control treatment were not significantly different from either inoculation treatment (Fig. 4). Examination of the relationship between micronutrient concentrations in plant biomass and total plant biomass produced per pot indicated than Mn was possibly the only growth-limiting micronutrient out of those measured, whereas iron and copper were not (supplementary Fig. S3).

Specific prokaryotes but not fungi were stimulated by the AM fungal necromass

There were systematic differences in the structure of underground (substrate or root) microbiomes (Figs. S4 and S5 and Table S1 for raw data). The microbiomes in the Control treatment showed almost no overlap with the other two treatments, particularly so with the Living treatment (Fig. S6). Despite overlap between the Dead and Living treatments in terms of microbiome structure, there were numerous prokaryotic taxids and also phyla specifically associated with the Dead or with the Living treatments, when comparing only those two treatments, i.e. disregarding the Control (Table 1). Excluding the inoculant AM fungus, there were specific fungal taxids associated with both Living and Dead treatments, whereas no fungal class was specifically associated with the Dead treatment (Table 2). Two fungal classes (Tremellomycetes and Malasseziomycetes) were specifically associated with the Living treatment, however (Table 2).

Phosphatidyl-myo-inositol mannosides—abundant glycolipids in the AM fungal biomass

Mass spectrometry analysis of methanolic extracts of *Rhizophagus irregularis* resulted in identification of various phosphatidyl-myo-inositol mannosides (PIMs), of which the most abundant molecular species contained exclusively stearic acid residues (Table 3). Altogether, this class of compounds represented between 2 and 3% of all compounds extracted from the AM fungal biomass (containing both spores and hyphae) with methanol.

Cell walls of the AM fungus contained chitin

Analysis of solid residues of *Rhizophagus irregularis* biomass after extraction with organic solvents resulted in unequivocal identification of chitin in the cell walls through chitinase

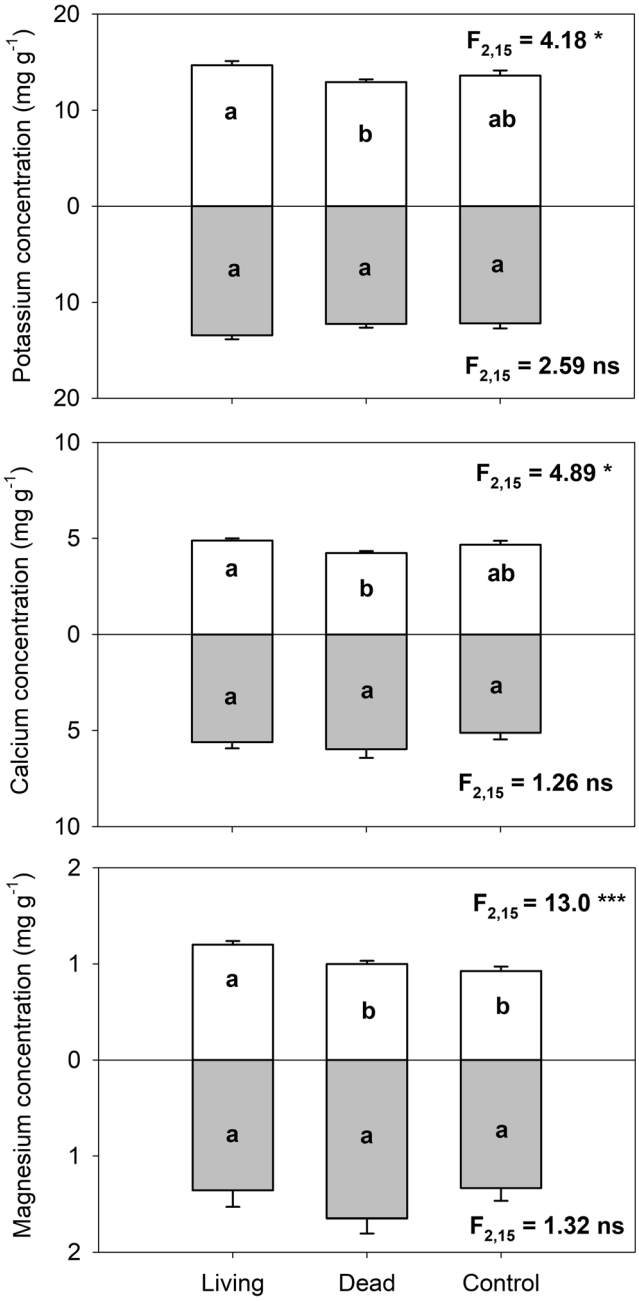

Fig. 3 Concentrations of selected macronutrients (K, Ca, Mg) in plant biomass as affected by inoculation of the experimental plants with a living arbuscular mycorrhizal (AM) fungus (Living), dead biomass of the AM fungus (Dead) and compared to the non-inoculated control (Control). Empty bars represent shoots and grey bars (shown as positive values below the abscissa) represent the roots. Bars show mean values per pot (+ 1 standard errors) calculated from six true replicates per treatment. Bars sharing the same letter correspond to means not statistically different among the respective treatments ($p \geq 0.05$), as per Tukey's HSD test following significant ANOVA, conducted separately for the shoots and the roots. *F* values for the individual ANOVA analyses are displayed. ***$p < 0.001$, *$0.05 > p \geq 0.01$, ns $p \geq 0.05$

digestion assay and subsequent detection of chitin oligomers (see online supplementary information for details). Yet, chitin is probably not the only component of the cell walls of *Rhizophagus irregularis*, as indicated by relatively low N concentration and lack of specific infrared spectral features of the solid residues particularly before alkaline lysis (see online supplementary information for details).

Discussion

In this study, we observed a strong growth stimulation of our model plant by addition of AM fungal necromass to the potting substrate, as compared to non-amended control treatment. The magnitude of the necromass effect was unexpected and possibly important for design of future experiments with monoxenically produced AM fungal inoculants, particularly for producing relevant nonmycorrhizal controls in such future studies. The necromass contained mineral nutrients (presumably in different forms such as inorganic ions and macromolecules) and a range of organic molecules. Although the exact biochemical composition of AM fungal biomass is still largely unknown, it is very likely that at least some of the compounds contained in it have important signalling and/or other biological functions (e.g. serving as source of energy for specific microbes). Other molecules (such as those denoted collectively as 'glomalin') might have a capacity to affect physicochemical properties of the potting substrate such as aggregate stability, aeration, and/or water relations. Because the functioning of AM symbiosis as interaction of two living entities based on mutually exchanged goods and services is well known (Parniske 2008; Garcia et al. 2016), we focus here mainly on explaining the observed stimulation of plant growth by the AM fungal necromass. We compare this treatment with the non-amended controls on the one hand and with addition of living AM fungal inoculum on the other hand, to stress parallels and differences in plant responses, and to speculate about possible underlying mechanisms.

We acknowledge that the AM fungal necromass prepared in our study has only limited relevance to recycling AM fungal necromass in nature, where the AM fungi often retract cytoplasm from old/dying hyphae (Requena et al. 2007; Lee 2011). However, there is at least one situation for which our case study is directly relevant—and that is experiments with rotated in-growth cores (Johnson et al. 2001). Such an approach currently is used often to experimentally control establishment of AM hyphal networks (both in the field and in glasshouse experiments) by repeated severing of hyphal connections through a mesh wall (e.g. Wu et al. 2016; Sánchez-Galindo et al. 2019). Frequent mechanical disturbance of the AM fungal hyphae might cause significant amounts of their cytoplasm (containing various metabolites) to be released into the soil environment. Understanding consequences of such processes may be needed for unbiased interpretation of results of such experiments.

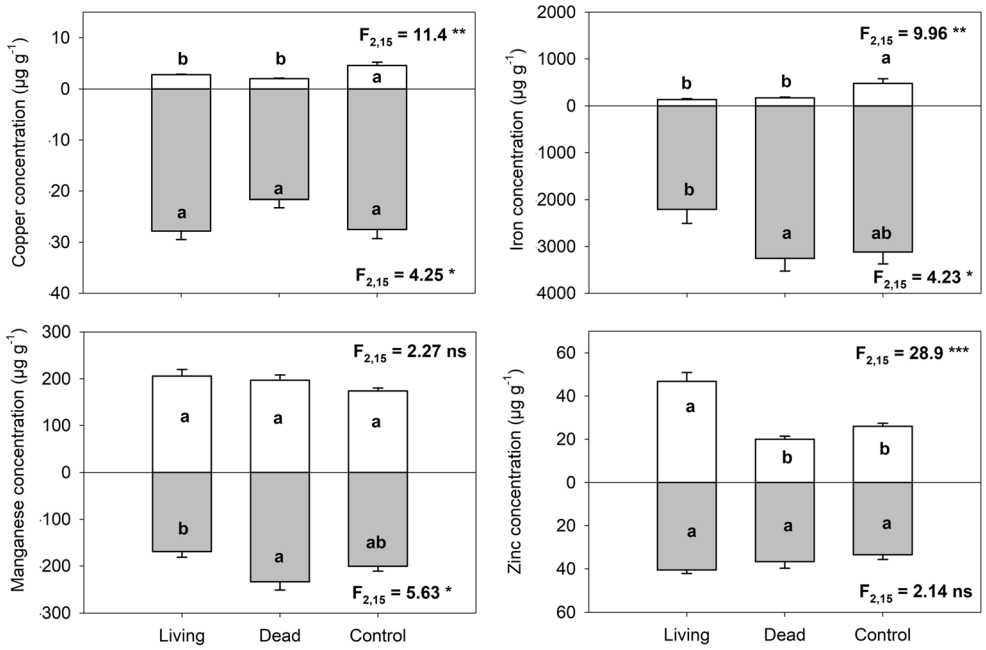

Fig. 4 Concentrations of selected micronutrients (Cu, Fe, Mn, Zn) in plant biomass as affected by inoculation of the experimental plants with a living arbuscular mycorrhizal (AM) fungus (Living), dead biomass of the AM fungus (Dead) and compared to the non-inoculated control (Control). Empty bars represent shoots and grey bars (shown as positive values below the abscissa) represent the roots. Bars show mean values per pot (+ 1 standard errors) calculated from six true replicates per treatment. Bars sharing the same letter correspond to means not statistically different among the respective treatments ($p \geq 0.05$), as per Tukey's HSD test following significant ANOVA, conducted separately for the shoots and the roots. *F* values for the individual ANOVA analyses are displayed. ***$p < 0.001$, **$0.01 > p \geq 0.001$, *$0.05 > p \geq 0.01$, ns $p \geq 0.05$

AM fungi—mineral nutrient uptake mediators vs. a source of such nutrients?

Here, we quantified P and N inputs into the substrate with the AM fungal biomass, applied either living or dead, per pot (0.012 mg P and 0.11 mg N) and compared those with the plant nutrient contents in the Dead treatment (on average and per pot, 5.31 mg P and 41.4 mg N), with the nutrients contained in the substrate (5.72 mg water-extractable P and 286 mg N) and those supplied with the liquid fertilizer (3.13 mg P and 65.5 mg N). It is obvious that the nutrient inputs with the AM fungal necromass were negligible, in fact being less than 1% of the P and N supplied with the nutrient solution, i.e. in fully plant-accessible form, besides the other pools (substrate and seeds). We did not analyse concentrations of other macro- and micronutrients in AM fungal biomass. However, we theoretically calculated how much Mn would have to be contained in the AM fungal biomass to explain the difference in plant Mn uptake between the Dead and the Control treatments by a direct fertilization effect. The focus on Mn is justified because Mn may be particularly important for *Andropogon gerardii* growth, and mycorrhizal symbiosis is supposed to play an important role in Mn supply to this plant species (Weremijewicz and Janos 2013; Weremijewicz et al. 2018). Our calculation revealed that the AM fungal necromass would have to contain more than 11% Mn (by weight), and all this would have to be fully available to plant uptake throughout the 10 weeks of plant growth, if the AM fungal biomass inputs alone were to explain the observed effect— which truly is not a realistic scenario.

In contrast, the living AM fungal inoculum, or rather the functional AM symbiosis resulting from inoculation with such material, supplied the plants with significantly more P and Zn than the amounts encountered in the other plants (belonging to the Dead or Control treatments). Such results are consistent with the well-established knowledge that AM fungi generally improve acquisition by their host plants of nutrients with low mobility in soil such as P and Zn (e.g. Smith et al. 2001; Jansa et al. 2003)—yet these nutrients originate from the soil/substrate rather than from the AM fungal biomass itself. These nutrients are more effectively channelled to the plants via the mycorrhizal (indirect) pathway than via the direct (root/root hairs) pathway (Smith et al. 2011). This often, but not always, leads to improvement of plant growth under P-limiting conditions (Smith et al. 2011; Konvalinková et al. 2015). Because there was a very large difference in P concentrations in the biomass of plants in the Living and Dead treatments in our experiment (Fig. 2), it appears that the mechanisms of plant growth stimulation were different between the two treatments. Thus, the growth improvement observed in the Dead treatment was unlikely (or at least not primarily) due to improved nutrient (P) uptake. Our experimental system was finely tuned up to allow expression of mycorrhizal growth stimulation by relieving plants from P limitation (Püschel et al. 2016; Püschel et al. 2017; Slavíková et al.

Table 1 Significant association (positive or negative) of prokaryotic taxa detected by amplicon sequencing of root and substrate samples, with the 'Dead' inoculation treatment as compared with the 'Living' inoculation treatment. Analyses were carried out with quantitative data at the different taxonomic levels—phyla (32 operational taxonomic units) or GenBank taxid (roughly corresponding to a prokaryotic species level, 2026 operational taxonomic units), using *t* value biplots

Taxon level	Taxon	Most similar GenBank accession	Association with 'Dead' treatment
Phylum	Nitrospirae	n.a.	Positive
	Firmicutes	n.a.	Positive
	Cand. Peregrinibacteria	n.a.	Positive
	Cand. Saccharibacteria	n.a.	Negative
	Proteobacteria	n.a.	Negative
Species	Unidentified bacterium	HQ290503	Positive
	Verrucomicrobium sp.	KT730050	Positive
	Parviterribacter kavangonensis	KP981370	Positive
	Unidentified Sphingobacteriales	EF636199	Positive
	Streptomyces rishiriensis	KY585942	Positive
	Promicromonospora sp.	KU315840	Positive
	Micromonospora sp.	KU382316	Positive
	Bacillus stamsii	AY189804	Positive
	γ-proteobacterium	AB096215	Positive
	Luteolibacter cuticulihirudinis	JQ429496	Positive
	Streptomyces ossamyceticus	KY585945	Positive
	Paracoccus sp.	KU550051	Positive
	Streptomyces sp.	JX290323	Positive
	Nitrospira sp.	AF155155	Positive
	Knoellia sinensis	KX449277	Negative
	Azospirillum sp.	KX066403	Negative
	Unidentified bacterium	KP174629	Negative
	Amaricoccus sp.	KJ513833	Negative
	Oscillochloris trichoides	DQ139400	Negative
	Sphingomonas sp.	KU925844	Negative
	Unidentified Actinobacteria	HQ663419	Negative
	Brevifollis gellanilyticus	AB552872	Negative
	Unidentified Verrucomicrobia	JF488274	Negative
	Nocardioides sp.	KX028739	Negative
	Phycicoccus ginsenosidimutans	KY012263	Negative

n.a. not applicable for high-level taxa beyond the taxid level

2017). The other nutrients (particularly N, amply supplied with the nutrient solution) were not primarily limiting plant growth. In this regard, it would have been interesting to analyse reasons why the plants in the Living treatment did not grow even larger because they were supplied by the AM fungus with so much more P than plants in the Dead treatment (e.g. space limitation, competition for C between the symbionts), but this goes beyond the scope of this report.

Possible biological mechanisms behind plant growth stimulation by AM fungal necromass

Given the difficulty of explaining the growth stimulation of plants in the Dead treatment with mineral nutrients contained in the necromass (see above), it seems plausible that the growth of plants in the Dead treatment was specifically and directly stimulated by AM fungal metabolites with a signalling function towards the plants. Alternatively, bioactive compounds also could be formed by microbes stimulated upon the addition of AM fungal necromass to the potting substrate—either by degradation/transformation of polymeric or other substances contained in the necromass, or formed de novo (i.e. the necromass would indirectly affect the plants through the microbes). Among the possible AM fungal metabolites, the observed stimulation of plant growth could be due to lipochitooligosaccharides normally involved in the symbiotic dialogue between plants and their AM fungal partners (Genre et al. 2013; Liang et al. 2014). However, because the

Table 2 Significant association (positive or negative) of fungal taxa detected by amplicon sequencing of root and substrate samples, with the 'Dead' inoculation treatment as compared with the 'Living' inoculation treatment. Analyses were carried out with quantitative data at the different taxonomic levels—classes (20 operational taxonomic units) or GenBank taxid (roughly corresponding to a fungal species level, 403 operational taxonomic units), using *t* value biplots

Taxon level	Taxon	Most similar GenBank accession	Association with 'Dead' treatment
Class	Malasseziomycetes	n.a.	Negative
	Tremellomycetes	n.a.	Negative
Species	*Alternaria alternata*	KX987252	Positive
	Fusarium sp.1	KJ920731	Positive
	Fusarium sp.2	LN886532	Positive
	Fusarium merismoides	KU214553	Positive
	Dendryphion nanum	KU500568	Positive
	Pyrenochaeta inflorescentiae	GU361962	Positive
	Arthrinium sp.	KX858879	Positive
	Boeremia exigua	KX272600	Negative
	Boeremia sp.	KX784237	Negative
	Bionectria sp.	KP090208	Negative
	Malassezia restricta	KC152885	Negative
	Exophiala sp.	KY590615	Negative
	Vishniacozyma carnescens	LC203732	Negative
	Unidentified endophyte	KR016097	Negative

n.a. not applicable for high-level taxa beyond the taxid level

necromass was added to the pots together with ungerminated seeds, and *Andropogon* germination may take up to 2 weeks, it is rather unlikely (although not impossible) that signalling compounds from the dead AM biomass would survive in unsterile soil for so long and still be active. Signalling compounds involved in localized or temporarily delimited events in general should have much faster turnover times than days or weeks (Müller and Schier 2011). Or could an early recognition of proximity of AM fungal symbiont, albeit not followed by subsequent events normally involved in establishment of fully functional AM symbiosis (Genre et al. 2008) cause such a strong and persistent effect on plants? We are not aware of any such report in the literature.

Despite the low probability of persistence of signalling compounds in unsterile potting substrate over several weeks, it is possible that some, e.g. lipophilic compounds with longer degradation times than easily water-soluble compounds, could have persisted in the substrate long enough to affect plants. Along these lines, it is possible (although far from demonstrated) that the presence of PIMs detected in the AM fungal biomass here could have contributed to observed plant growth stimulation by the AM fungal necromass. PIMs are common in some prokaryotes, e.g. in the Actinobacteria (Minnikin et al. 1977; Guerin et al. 2009; Sandoval-Calderón et al. 2017), yet they are relatively rare in eukaryotes. However, it should be noted that in the recently published *Rhizophagus irregularis* genome (GenBank: AUPC00000000.2, median total length 125.354 Mb), a gene was identified coding for an enzyme with potential function as 'gpi mannosyltransferase 2' (GenBank: GBC46257.1). In *Saccharomyces cerevisiae*, this enzyme is responsible for the transfer of the second mannose to the glycosylphosphatidylinositol (Fabre et al. 2005). Although different functions have previously been assigned to PIMs in different inter-organismal interactions (Torrelles et al. 2006; Guerin et al. 2007), it remains to be determined whether and to what extent different PIMs exhibit biological activity in the AM symbiosis.

Alternatively, signalling compounds might be released only gradually from the AM fungal necromass either through self-degradation of the compounds contained in it or facilitated through exogenous microbial activity. Chitin (which was demonstrated to be part of the AM fungal cell wall in our study) and chitooligosaccharides (products of chitin hydrolysis by the action of plant or microbial chitinases) belong among the most common elicitors recognized by plant pattern recognition receptor proteins located on plant cell surfaces (Klarzynski et al. 2000; Zhang and Zhou 2010). However, it remains unclear which metabolic pathways could have been upregulated in plants through presence of AM fungal cell wall-derived oligosaccharides to cause the observed plant growth stimulation.

Still another option would be that certain microbial taxa with a strong stimulatory effect on plants were specifically promoted by the addition of the AM fungal necromass. It appears an exciting scenario that such induced changes in

Table 3 The composition of lyso-, monoacyl- and diacyl-phosphatidyl-myo-inositol mannosides (PIMs) from *Rhizophagus irregularis* biomass identified by mass spectrometry of methanol extracts. Experimentally measured mass-to-charge ratios (experimental m/z) of molecular ions of the different compounds are given

Experimental *m/z*	Relative intensity	Name[a]
1155.6085	4.40	PIM_2-L/Po
1381.5530	0.22	Lyso-PIM_5-P/–
1453.6985	0.12	PIM_4-M/L + Po/Po
1455.9478	91.49	Ac_1PIM_2-S, S/S
1559.9223	0.45	Ac_1PIM_3-O, P/P
1645.7983	0.67	PIM_5-P/O + S/Po
1667.9282	0.12	Ac_1PIM_4-M/P/P
1671.8140	0.15	PIM_5-O/O + L/O
1684.0992	0.13	Ac_2PIM_2-Po/Po/L/O
1693.9439	0.45	Ac_1PIM_4-P/P/Po
1744.1051	0.15	Ac_2PIM_3-M/M/P/S
1745.9752	0.14	Ac_1PIM_4-Po/O/O
1904.2303	0.13	Ac_2PIM_3-L/O/O/A
2066.1951	0.16	Ac_2PIM_5-M/M/P/O
2096.0965	0.13	Ac_1PIM_6-L/O/O
2204.3359	0.30	Ac_2PIM_5-O/O/S/S
2250.2322	0.49	Ac_2PIM_6-Po/Po/Po/Po
2306.2948	0.16	Ac_2PIM_6-Po/Po/O/O
2332.3105	0.14	Ac_2PIM_6-Po/L/O/O

[a] *M* myristic acid, *Po* palmitoleic acid, *P* palmitic acid, *L* linoleic acid, *O* oleic acid, *S* stearic acid, *A* arachidonic acid; for further details, please see supplementary information

underground microbiome would be responsible for the observed growth stimulation of our plants. Our detailed microbiome analyses indeed revealed that AM fungal necromass specifically promoted some prokaryotes such as Nitrospirae and Firmicutes, in contrast to other prokaryotic groups (e.g. Cand. Saccharibacteria and Proteobacteria) and certain fungal taxa that also flourished in the treatment amended with the living AM fungus (Tables 1 and 2). There are numerous possible substances that could have caused plants to grow larger and/or faster and which have been shown to be produced by various plant-growth promoting microorganisms (Richardson et al. 2009; Hayat et al. 2010). Thus, our microbiome analyses offer a promising avenue for further investigations aiming at the identification of specific molecular compounds produced by microbes closely associated with AM fungal necromass, and possibly implicated in *Andropogon* growth stimulation.

AM fungal necromass possibly conditioning physicochemical properties of the substrate

Another alternative explanation for the observed stimulatory effects of AM fungal necromass on plant growth could involve its effects on substrate physicochemical properties such as aggregate stability, aeration and/or water repellence, among others. This could happen through significant inputs of substances such as glomalin-related soil proteins through the AM fungal necromass addition (Driver et al. 2005; Purin and Rillig 2007; Peng et al. 2013; Powell and Rillig 2018). Yet, we do not know whether the realized necromass inputs would be enough to cause sufficiently strong physical changes to the substrate to explain what happened to the plants in our experiment. This remains a testable hypothesis, however, with straightforward methodical approaches, e.g. by adding glass wool to pots to mimic hyphal enmeshment of soil particles, or purified proteins from the AM fungal cell walls to model glomalin-related soil protein inputs.

Conclusions

This research demonstrated unexpected and strong growth stimulation of *Andropogon gerardii* by AM fungal necromass and opened several questions for further research. We have speculated on a range of possible mechanisms responsible for the observed effect. Yet, it could as well be a combined effect based on more than one molecular pathway—and unequivocal identification of the cause and effect will require further detailed experimental work. Additionally, validity of the observed phenomenon should be tested across more plant and AM fungal taxa than those we investigated. Detailed biochemical analyses of AM fungal necromass should be completed, particularly with respect to the peptides as well as poly- and oligo-saccharide compounds that may be involved in molecular dialogue between an AM fungus and its host plant (Muller and Harrison 2019). The molecules also should be traced in the soil/substrate environment. Insights into complex soil food webs, as affected by AM fungal necromass, should be sought by a combination of stable isotopic approaches coupled with high throughput sequencing (Lueders et al. 2016). More work in this direction has a great potential to improve our understanding of severely understudied AM fungal biomass recycling in soils (Finlay and Clemmensen 2017) and also our ability to design relevant nonmycorrhizal controls for future experiments.

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Authors' contribution MG and JJ designed and established the experiment. MR, HH and MG prepared the mycorrhizal inoculum (either living or dead). HH and PB helped with harvesting, sample processing and conducting the molecular analyses. JB analysed elemental composition

of plant biomass. STF analysed the composition of fungal cell walls by infrared spectroscopy. MR contributed data on elemental composition of the AM fungal biomass. MG conducted the bioinformatics on the sequencing data. PŠ ran the multivariate statistics. JJ prepared samples of AM fungal biomass for biochemical analyses. TŘ performed the identification of fungal metabolites (PIMs) by mass spectrometry and contributed to writing. KS conducted the chitinases digestion assay including subsequent analyses of chitin oligomers and contributed to writing. JJ wrote the first draft of the manuscript. All authors approved the final version of the manuscript before submission.

Compliance with ethical standards

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

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