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ARTICLE

Organic nitrogen utilisation by an arbuscular mycorrhizal fungus is mediated by specific soil bacteria and a protist

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Arbuscular mycorrhizal (AM) fungi lack efficient exoenzymes to access organic nutrients directly. Nevertheless, the fungi often obtain and further channel to their host plants a significant share of nitrogen (N) and/or phosphorus from such resources, presumably via cooperation with other soil microorganisms. Because it is challenging to disentangle individual microbial players and processes in complex soil, we took a synthetic approach here to study ^{15}N-labelled chitin (an organic N source) recycling via microbial loop in AM fungal hyphosphere. To this end, we employed a compartmented in vitro cultivation system and monoxenic culture of *Rhizophagus irregularis* associated with *Cichorium intybus* roots, various soil bacteria, and the protist *Polysphondylium pallidum*. We showed that upon presence of *Paenibacillus* sp. in its hyphosphere, the AM fungus (and associated plant roots) obtained several-fold larger quantities of N from the chitin than it did with any other bacteria, whether chitinolytic or not. Moreover, we demonstrated that adding *P. pallidum* to the hyphosphere with *Paenibacillus* sp. further increased by at least 65% the gain of N from the chitin by the AM fungus compared to the hyphosphere without protists. We thus directly demonstrate microbial interplay possibly involved in efficient organic N utilisation by AM fungal hyphae.

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INTRODUCTION

Arbuscular mycorrhizal (AM) fungi have accompanied terrestrial plants for approximately a half billion years [1], playing a particularly important role in their host plants' acquisition of phosphorus (P) from soil in exchange for reduced carbon (C) provided by the plants [2–4]. The P is taken up by the AM fungal hyphae from the soil solution chiefly as orthophosphate [5, 6]. Efficient acquisition of P from organic sources such as phytic acid by the AM fungal hyphae has also been documented upon close cooperation between hyphae and such other soil microorganisms as the bacterium *Rahnella aquatilis* [7–10]. This is because the AM fungi lack genes coding for potent exoenzymes, thus preventing them from efficiently accessing organic nutrient sources in soil on their own [11, 12].

Earlier research has shown that AM fungal hyphae also take up nitrogen (N) from the soil as both ammonium and nitrate ions [13, 14]. Further, it has been demonstrated that the hyphae could obtain significant amounts of N also from such organic sources as plant litter or chitin, sometimes transferring part of this N to their host plants, even as they compete with those plants for limited soil N under other circumstances [15–20]. It long has been assumed that other soil microbes play important roles in mineralisation and/or further processing of organic N before it can be taken up by AM fungal hyphae, similarly as in the case of organic P [21–24]. Furthermore, bacterial grazers have been posited to facilitate release of N to the soil solution as free ammonium ions from the microbes they ingest [18, 25]. Direct experimental evidence of such inter-kingdom associations in utilisation of organic N by AM fungal hyphae and their associated mycorrhizal plants has nevertheless not been reported [26].

The aim of this research was to establish an experimental in vitro system to study interactions between AM fungal hyphae and other microbes in their hyphosphere. Using such experimental system and different chemical forms of N, we quantified rates of N transfer from a root-free zone into the roots while controlling for passive N diffusion. In so doing, we addressed three hypotheses:

(1) The AM fungal hyphae alone cannot take up significant amounts of N from an organic N source such as chitin, in contrast to N administered as ammonium ions, even if they are capable of producing chitinases and deaminases [27].
(2) Chitinolytic bacteria inoculated in the root-free zone will enhance hyphal access to N supplied as chitin to the same compartment.
(3) Including protists into the root-free zone would further increase the rates of N release from the chitin and thus the amounts of N taken up by the AM fungal hyphae from that zone and eventually transferred to the roots.

We used an experimental system where Ri T-DNA transformed chicory (*Cichorium intybus* L.) roots were used instead of a full plant, because a green plant would require light and inevitably necessitate active cooling of the experimental system. We wanted to avoid this for logistical reasons. Moreover, some autotrophic in vitro culture systems established previously [28, 29] had required expansion of plant leaves in free air, thus imposing

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additional technical challenges to be overcome to prevent microbial contamination of the rhizosphere and hyphosphere zones. Such experimental systems as we employed here, which can easily be established in copious numbers due to efficient clonal propagation of roots, previously proved themselves extremely valuable for addressing such specific questions in mycorrhizal physiology and ecology as relate to trading resources between AM fungus and its plant host(s) or microbial interactions in AM fungal hyphosphere [10, 30, 31].

MATERIALS AND METHODS

Biological materials

For the experiments described here, we used monoxenic culture of *Rhizophagus irregularis* Walker & Schüßler genotype SYM5 (also known as LPA9 or BEG236) originally obtained from *Asphodelus* sp. rhizosphere in Greece during 1980. The culture has been maintained in vitro in association with Ri T-DNA transformed chicory roots for several years prior to the research described here [32]. To establish a non-mycorrhizal (NM) control, we used the same root culture as above without the AM fungus (i.e. NM roots). The prokaryotes used here were of various provenances (Table 1), maintained on solid lysogeny broth (1.5% agar) for at least five generations, and stored in 30% glycerol at −80 °C prior to use. Four of them exhibited chitinolytic activity when crab-shell chitin was provided as the only C and N source (Table 1 and supplementary Figs. S1–S3). Identity of the different bacterial strains was revealed by sequencing amplicons of their rRNA genes as described previously [33]. The culture of the protist *Polysphondylium pallidum* (Amoebozoa) was originally obtained from spruce bark compost [34] and subcultured for at least five generations using *Escherichia coli* co-culture on lysogeny broth agar (1.5%). Thereafter, it was inoculated separately to each of the prokaryotes (Table 1) using spores carefully recovered from sporangiophores above the surface of the agar plates in order to avoid contaminating the follow-up cultures with *E. coli*.

^{15}N-labelled chitin

Isotopically (^{15}N) labelled chitin was prepared from *Zygorhynchus* sp. cell walls as previously [18]. Its elemental and isotopic composition as well as biochemical structure and purity were then analysed as described elsewhere [33]. Briefly, the chitin batch for Experiment 1 contained 5.8% N and 42.3% C by weight and its ^{15}N share was 29.6 atom%. The chitin batch for Experiment 2 contained 5.3% N and 45.8% C by weight, with the N being fully isotopically labelled (>98 atom% represented by ^{15}N).

Experimental system (microcosms)

The experimental system used here was constructed as three-compartment in vitro cultivation vessel consisting of a large, sterile Petri dish (diameter 15 cm, 2 cm height, made from polystyrene) and two smaller compartments (Fig. 1 and supplementary Fig. S4). One of the smaller compartments (the root compartment or rhizobox) was made from a lid of a small (6 cm diameter) polystyrene Petri dish with a hole for root transfer drilled into its top, and the bottom opening sealed with 42 µm nylon mesh (Silk & Progress, Brněnec, Czech Republic). The rim of the rhizobox was dipped in chloroform before pressing it against the mesh, which firmly glued the rhizobox walls to the mesh. After preparing the rhizoboxes, they were sterilised by γ-rays (>25 kGy, Bioster, Veverská Bítýška, Czech Republic). The second smaller compartment (termed here the labelling compartment) was made from the bottom of a small (6 cm diameter) Petri dish and was inserted at least 5 mm from the rhizobox. The large Petri dish was filled with 100 ml of standard modified Strullu and Romand (MSR) medium, pH 5.5 [35], containing 93 µg P and 379 µmol N, supplemented with 1% (w:v) sucrose, and solidified with 0.3% (w:v) gelling agent (Phytagel, Merck, Darmstadt, Germany). The P and N concentrations in Phytagel powder were 560 µg g^{-1} and 46 µmol g^{-1}, respectively, which (together with the MSR medium) ensured luxurious P supply and rather limited N supply to the roots and AM fungi (details not shown). The sterile labelling compartment was inserted into the liquid medium freshly poured into the large Petri dish before it solidified and was held down by a sterile metal plug. The rim of the labelling compartment protruded at least 1 mm above the medium (see supplementary Fig. S4 for details). After the medium solidified, the rhizobox was placed on top of the MSR medium and the metal plug was removed from the labelling compartment. Mycorrhizal or NM roots were added to the rhizobox through the hole (supplementary Fig. S4) and incubated at 24 °C in darkness for 61 or 75 days in Experiment 1 or 2, respectively, before the roots filled the rhizobox and AM fungal hyphae (if applicable) colonised the entire volume of the MSR medium (Fig. 2). Thereafter, the labelling compartment was filled with N-free MSR medium with or without an added ^{15}N-labelled N source and bacteria and/or protists were further added or not added as specified below. In Experiment 1, sucrose was added to both the large Petri dish volume and the labelling compartment and the N concentration in the labelling compartments was increased fourfold as compared to the rest of the microcosm, thereby establishing an N-rich patch similarly as in a previous unsterile pot experiment [18]. In Experiment 2, sucrose was omitted from the labelling compartment because we had noted possible interference between sucrose and growth of some of the bacteria. Besides, in Experiment 2, the N concentration in the labelling compartment was adjusted to equal that in the full-strength MSR medium.

Experiment 1

This experiment was carried out to test whether the AM fungal hyphae were able to take up and transport a significant quantity of N supplied as chitin towards the roots compared to N supplied as ammonium. Further, we tested whether any of the bacteria added to the hyphosphere would improve AM fungal access to the N bound in chitin. Because the standard cultivation media (MSR) for monoxenic AM fungal cultures contains sucrose, and also because we wanted to supply chitin as the only N source (and not the only N and C source) in the labelling compartment, we also supplied sucrose to the labelling compartment in this experiment. Altogether, 287 experimental microcosms without any visible bacterial contaminations and with AM fungal hyphae (in the mycorrhizal treatment) profusely colonising the root-free MSR medium were included into this experiment. Each labelling compartment was filled with 15 ml of N-free MSR medium (where KNO_3 and $Ca(NO_3)_2$ were replaced by KCl and $CaCl_2$, respectively) supplemented with 1% sucrose and solidified with 0.3% Phytagel. To this medium was added either 60 mg ^{15}N-labelled chitin per compartment or an equal amount of N provided as NH_4Cl (30% ^{15}N atom %) or left without N amendment. Amendments with ^{15}N-labelled compounds effectively elevated the N concentration in the labelling compartment fourfold as compared to the medium filling the large plate volumes. Thereafter, the plates were incubated for another 26 days to allow colonisation of the labelling compartments by AM fungal hyphae in the mycorrhizal treatment. At that time point, fresh (4 days old) bacterial liquid cultures (lysogeny broth) produced from glycerol stocks upon reciprocal shaking (110 rpm, 2 cm) at 24 °C were added or not added to the labelling compartment as 3 × 20 µl of the respective bacterial suspension or of sterile lysogeny broth. The plates were then incubated for an additional 20 days at 24 °C in darkness before harvest.

The full factorial design with 6–8 replicate plates per treatment combination included three factors: (1) AM fungus (two levels, present or absent), (2) ^{15}N amendment of the labelling compartment (three levels, chitin, ammonium chloride, or none), and (3) bacterial inoculation of the labelling compartment (six levels). The latter included either none or one of the following bacterial strains ID 1, 5, 9, 10, or 15 (Table 1). In addition, 4–6 mycorrhizal and NM plates with chitin as the ^{15}N-labelled N source in the labelling compartment were established per selection of other bacterial treatments (i.e. strains ID 2, 8, 16, or 17; see Table 1 for additional details).

Experiment 2

This experiment was mainly directed to testing whether including a protist into the experimental system containing plant (roots), AM fungus, and bacteria would further improve the efficiency of utilising organic N from AM fungal hyphosphere. Because we previously had noted some interferences between sucrose amendment of cultivation media and growth of several bacteria included in the research described here (details in supplementary Figs. S1 and S2), we omitted sucrose from the labelling compartment in this experiment. This effectively made the chitin the only N and the main C source for the saprotrophic microbes in the labelling compartment, considering that C inputs were comparably low from the hyphal exudates and AM fungal necromass to the labelling compartment.

This experiment included 182 experimental microcosms. All were free of bacterial contamination upon filling the labelling compartments, and all mycorrhizal plates produced extensive hyphal networks in the root-free compartment. Each labelling compartment was filled with 15 ml of N-free MSR medium devoid of sucrose and solidified with 0.3% Phytagel. Each of the labelling compartments contained 15 mg of ^{15}N-labelled chitin,

Table 1. Identities and provenances of bacterial strains used in this study.

Isolate ID	Bacterial culture details	Provenance	Chitinolytic activity detected[b]	Sporulation of *Polysphondylium* observed in association with the bacteria[c]	Taxonomic affiliation by 16S rRNA gene fragment sequencing (sequence similarity of closest match in GenBank)
1	Isolate D22	Isolated from grassland soil, Czech Republic	Yes	n.a.	*Janthinobacterium* sp. (100%), Oxalobacteraceae, β-proteobacteria
2	Isolate D23	Isolated from grassland soil, Czech Republic	Yes	Yes, abundant	*Janthinobacterium* sp. (100%), Oxalobacteraceae, β-proteobacteria
5	*Paenibacillus chitinolyticus* CCM 4527	Public culture collection, originally isolated from forest soil, Japan	Yes	Yes, abundant	*Paenibacillus* sp. (100%), Paenibacillaceae, Firmicutes
8	Isolate M-N	Isolated from a mycorrhizal inoculum pot[a]	No	No	*Gordonia* sp. (100%), Nocardiaceae, Actinobacteria
9	Isolate M-R	Isolated from a mycorrhizal inoculum pot[a]	No	n.a.	*Rhodococcus* sp. (100%), Nocardiaceae, Actinobacteria
10	Isolate NM-A	Isolated from a non-mycorrhizal inoculum pot[a]	No	n.a.	*Arthrobacter* sp. (99%), Micrococcaceae, Actinobacteria
12	Isolate M-F	Isolated from a mycorrhizal inoculum pot[a]	No	Yes	*Bacillus* sp. (94%), Bacillaceae 1, Firmicutes
13	Isolate M-U	Isolated from a mycorrhizal inoculum pot[a]	No	No	*Mycobacterium* sp. (99%), Mycobacteriaceae, Actinobacteria
15	Isolate NM-H	Isolated from a non-mycorrhizal inoculum pot[a]	No	Yes	*Rhodococcus* sp. (100%), Nocardiaceae, Actinobacteria
16	Isolate NM-O	Isolated from a non-mycorrhizal inoculum pot[a]	No	n.a.	*Nocardia* sp. (100%), Nocardiaceae, Actinobacteria
17	*Paenibacillus ehimensis* CCM 4526	Public culture collection, originally isolated from garden soil, Japan	Yes	Yes, abundant	*Paenibacillus* sp. (100%), Paenibacillaceae, Firmicutes

n.a. not analysed.
[a]Isolated using yeast extract agar supplemented with colloidal chitin.
[b]See supplementary Figs. S1, S2 and S3 for details.
[c]See supplementary Fig. S6 for photos.

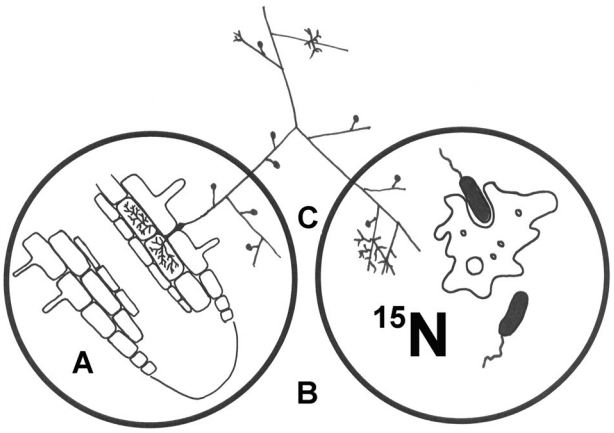

Fig. 1 Experimental system (a microcosm) employed in this study. The system was prepared in a large Petri dish (not shown here). Inside that dish, a small rhizobox (**A**), made of a small Petri dish and delimited from the rest of the system with a 42 µm nylon mesh, contained the Ri T-DNA transformed *Cichorium intybus* roots, either non-mycorrhizal or mycorrhizal. Mycorrhizal fungal hyphae growing out of the mycorrhizal roots through the mesh colonised the MSR medium [35] filling the large dish volume (**B**) and eventually reached the labelling compartment (**C**). The labelling compartment was made of another small Petri dish and was filled with a modified (nitrogen-free) MSR medium, with (Experiment 1) or without (Experiment 2) sucrose and with or without an added ^{15}N-labelled nitrogen source (either mineral or organic) and various bacteria combined or not combined with a protist grazer (more details in supplementary Fig. S4).

corresponding to the N concentration in the medium filling the large plates. The plates were then incubated for 26 days to allow for efficient colonisation of the labelling compartments by the AM fungal hyphae in the mycorrhizal treatment. Subsequently, fresh (4 days old) bacterial liquid cultures produced as above were added or not added to the labelling compartment as 1 × 60 µl of the bacterial suspension or of sterile lysogeny broth. The plates were incubated for a further 13 days at 24 °C in darkness. Thereafter, the spots inoculated with bacteria in the labelling compartment were either inoculated or not inoculated with spores of the protist *P. pallidum* produced on cultures of the respective bacteria, with the exceptions of bacteria ID 8 and 13, which had not supported protist sporulation in previous co-cultures. Protist spores recovered from aerial sporangiophores formed while co-culturing the protist with bacterium ID 2 were used instead. The plates were then incubated for an additional 9 days at 24 °C in darkness before harvest.

The full factorial design included 4–5 replicate NM plates and 6–7 replicate mycorrhizal plates per each microbial inoculation combination. Three experimental factors were considered: (1) AM fungus (two levels, present or absent); (2) bacterial inoculation of the labelling compartment (eight levels, inoculated with one of the bacterial strains ID 2, 5, 8, 12, 13, 15, 17, or left uninoculated), and protist inoculation (two levels, inoculated or uninoculated).

Harvest and analyses

Microcosms with roots escaping from the rhizobox were removed from both experiments unless the roots grew no closer than within 1 cm of the labelling compartment. Further, plates with visible bacterial contamination were excluded from the experiments unless this was strictly delimited spatially to individual and easily removable colonies in the large plate volume. Such contaminating colonies were removed prior to extracting roots and AM fungal hyphae from the plates.

Upon harvest, the plates were opened, roots from the rhizobox removed, dried at 65 °C for 3 days, weighed and then pulverised in a ball mill (MM 200, Retsch, Haan, Germany). Their C and N concentrations and isotopic composition of N were analysed using a Flash 2000 elemental analyser coupled with a Delta V Advantage isotope-ratio mass spectrometer (Thermo Fischer Scientific, Bremen, Germany). The hyphae from the root-free zone (not including the labelling compartment) were collected by filtering through Omnipore membrane filters (5 µm pore size, 47 mm diameter, Merck Millipore, Burlington, MA, USA) after the Phytagel was dissolved in 10 mM potassium citrate buffer (pH 6.0). Dry weights of the hyphae were recorded after drying at 65 °C for 3 days and their C and N concentrations and N isotopic compositions were analysed as above. Phosphorus concentrations were analysed in randomly selected root and hyphal samples from Experiment 1 by Malachite green colorimetry [36] after dry incineration of the samples (550 °C, 12 h) and extraction of the ashes with concentrated nitric acid [37]. In Experiment 2, we further quantified the development of AM fungal hyphae, bacteria, and the protist using quantitative real-time PCR (details and results in the Supplementary Information).

Calculations and statistical analyses

The N, C, and P contents of the roots and AM fungal hyphae per microcosm were calculated from the respective element concentrations and dry biomass of the samples. Excess ^{15}N values (i.e. the amounts of N originating from the isotopically enriched inputs) in the root and the hyphal samples were calculated by considering molar N concentrations in the samples, their ^{15}N abundance given as atom%, and using ^{15}N abundance of samples without ^{15}N-labeled compounds added to the labelling compartment as an isotopic baseline. Percentage of ^{15}N transfer from the labelling compartment to the AM fungal hyphae and/or roots was then calculated using a two-source mixing model employing a mass balance equations framework as detailed elsewhere [38]. Knowing the levels of isotopic enrichment of the labelled compounds and their inputs per experimental system (i.e. microcosm), we calculated the fraction of ^{15}N supplied per microcosm and recovered either in the roots or the AM fungal hyphae.

Data were analysed using one-, two-, or three-way analysis of variance (ANOVA), followed by post hoc Duncan's multiple range test separating treatment means if ANOVA proved significant ($p < 0.05$). These analyses were carried out in Statgraphics Plus for Windows v. 3.1 (Statgraphics Technologies, Inc., The Plains, VA, USA). In case of significant heteroscedasticity of data (checked by Bartlett's test), log (x + 0.1) transformation was employed prior to statistical analyses.

RESULTS

Colonisation of labelling compartment by AM fungal hyphae

Rapid colonisation of the labelling compartment by finely branched AM fungal hyphae was observed when N was supplied as NH_4Cl in Experiment 1. That was in contrast to a labelling compartment completely devoid of N (Fig. 2). When the labelling compartment was supplied with chitin in Experiment 1, the speed of AM fungal hyphae ingrowth was comparable to that with NH_4Cl, but the hyphal morphology differed. The chitin-fed hyphae were long, spread further, and were only sparsely branched (Fig. 2). In Experiment 2, the development of AM fungus in the labelling compartment was either not affected by the different bacterial isolates or was stimulated by isolates ID 2, 5 and 12 (supplementary Fig. S5). In the same experiment, AM fungus development in the labelling compartment was not significantly affected by presence of the protist *Polysphondylium pallidum* (supplementary Table S1).

^{15}N transfer to AM fungal hyphae and roots—effect of N source and microbes

In Experiment 1, transfer of isotopically labelled N from the labelling compartment to the NM roots or mycorrhizal roots with the AM fungal hyphae connected to them was affected by all three experimental factors (i.e. presence of AM fungus, identity of bacterial isolate, and form of N supplied) and their interactions (supplementary Table S2). When N was supplied as NH_4Cl, transfer of N was ~10% of the added N appearing in the mycorrhizal roots and their associated AM fungal hyphae, whereas values below 1% were recorded in the NM roots (Fig. 3). Further, transfer of N added as NH_4Cl from the labelling compartment to the NM roots or mycorrhizal roots with AM fungal hyphae was not affected by presence or identity of prokaryotes in the labelling compartment (Fig. 3, supplementary Table S3). When N was supplied to the

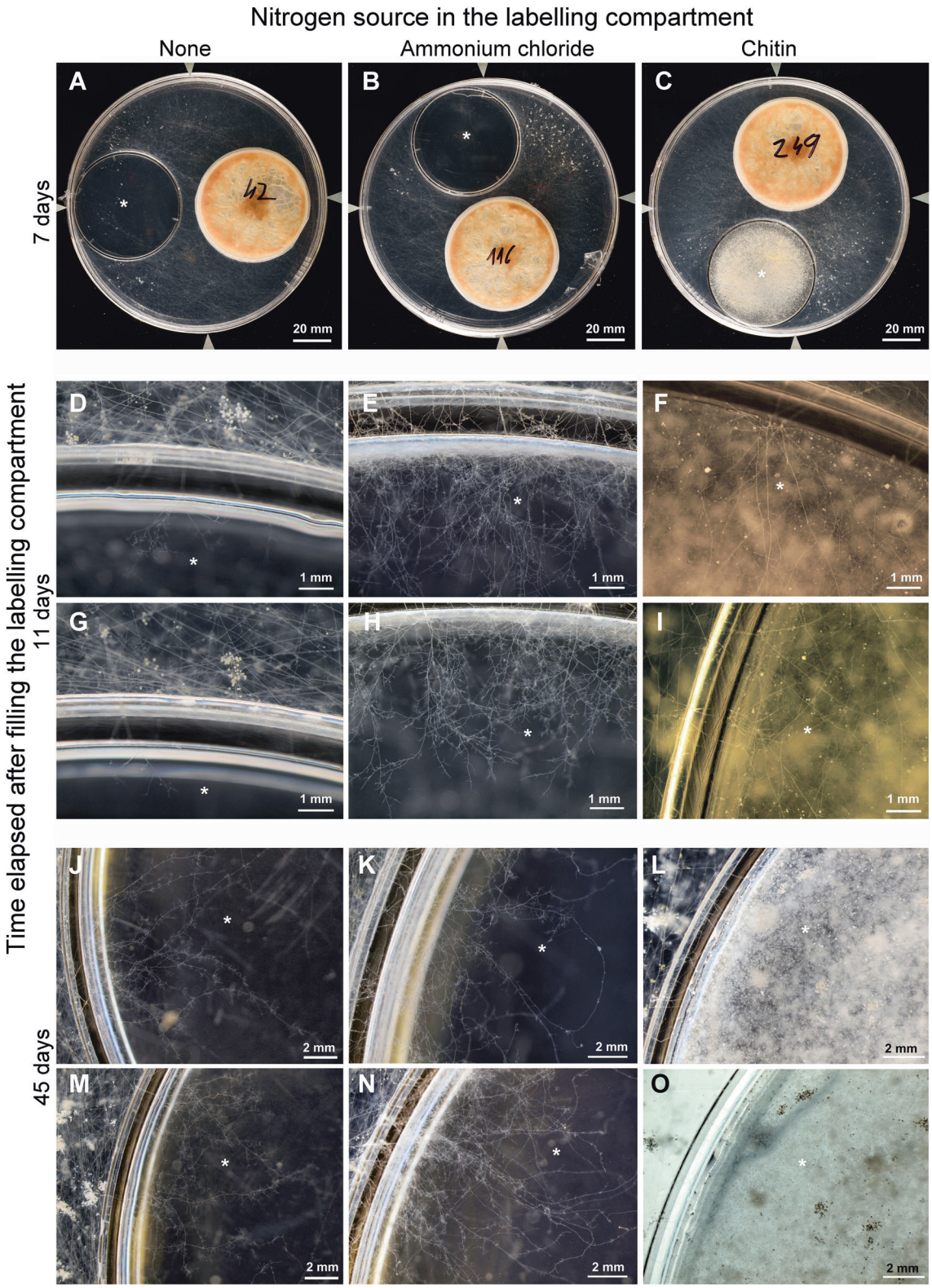

Fig. 2 Development of *Rhizophagus irregularis* hyphae and spores in Experiment 1 as affected by mineral- (ammonium chloride) or organic- (chitin) nitrogen addition to the labelling compartment (marked with asterisk) in comparison to a nitrogen-free control. Growth of the *R. irregularis* was supported by Ri T-DNA transformed *Cichorium intybus* roots in rhizoboxes (small dishes delimited from the main dish volume by a 42 µm nylon mesh), visible in the upper row (**A**–**C**), where they are labelled with handwritten plate numbers. The AM fungal hyphae and spores developed in the main dish volume filled with the standard MSR medium [35] and supplemented with 1% sucrose after emerging from roots pre-colonised with *Rhizophagus* and inoculated into the rhizoboxes. After 2 months of growth, labelling compartment was filled with MSR medium supplemented with 1% sucrose and devoid of nitrogen (**A**, **D**, **G**, **J**, **M**), or with added ^{15}N-labelled ammonium chloride (**B**, **E**, **H**, **K**, **N**) or ^{15}N-chitin extracted from *Zygorhynchus* sp. cell walls (**C**, **F**, **I**, **L**, **O**). Fungal development was then observed and photographed at the edge of the labelling compartment under an Olympus SZX10 stereomicroscope at different time points (see left edge of panel for details).

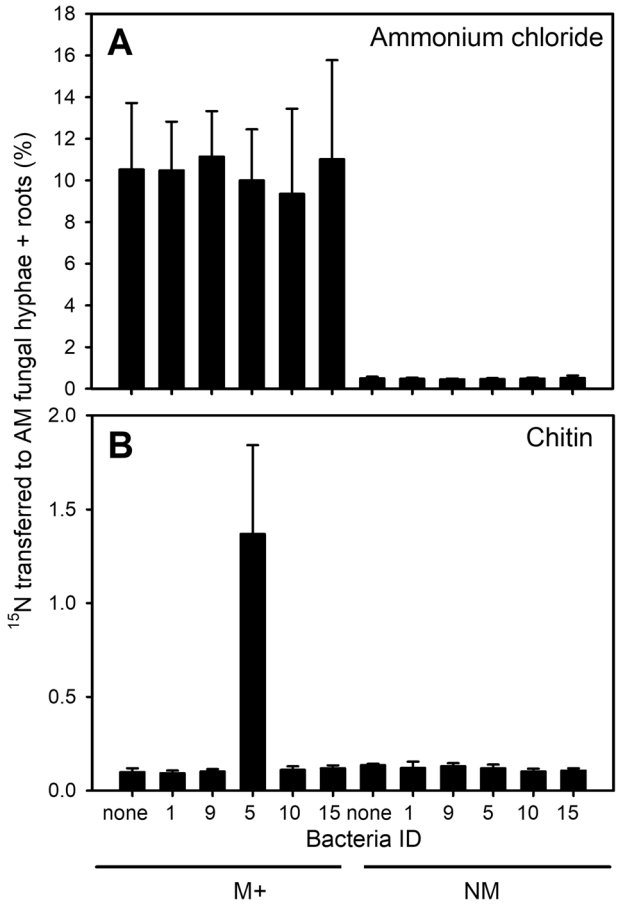

Fig. 3 Transfer rates of ^{15}N applied into the labelling compartment to the roots together with the mycorrhizal hyphae attached to them (if applicable) in Experiment 1 according to presence or absence of mycorrhizal fungus in the experimental system (M +, mycorrhizal; NM, non-mycorrhizal) and identity of selected bacterial isolates added to the labelling compartment. Shown are mean values (and standard deviations) of 5–8 replicates per treatment. Presented values illustrate the fraction of ^{15}N-labelled mineral (**A**) or organic (**B**) nitrogen source applied into the labelling compartment and transferred to the non-mycorrhizal roots or mycorrhizal roots together with their attached mycorrhizal hyphae extracted from the large dish volume at the end of the experiment (%).

labelling compartment as chitin in Experiment 1, its uptake to NM roots was low (i.e. all values below 0.17% of the supplied ^{15}N, except a single outlier reaching 0.31%), regardless of the identity of prokaryotes added. The uptake of N from chitin by mycorrhizal roots and the AM fungal hyphae connected to them was comparable to that of the NM roots (reaching up to 0.15% of the supplied ^{15}N) in all bacterial treatments except the bacterial isolate ID 5 (*Paenibacillus chitinolyticus*). That bacterium significantly increased N acquisition from chitin via AM fungal hyphae to a mean 1.43% of the supplied ^{15}N (Fig. 3). This resulted in high statistical significance of both AM fungal presence and identity of bacteria inoculated in the labelling compartment as well as their interaction in chitin-supplemented microcosms (Fig. 3, supplementary Table S4). Further, we found that another isolate of *Paenibacillus* sp. (ID 17) significantly increased N transfer from chitin to the roots and the AM fungal hyphae outside the labelling compartment (reaching a mean 0.52% of the supplied ^{15}N) as compared to the other bacteria (Fig. 4). All primary data to Experiment 1 are available as Supplementary Data 1 file accompanying this paper.

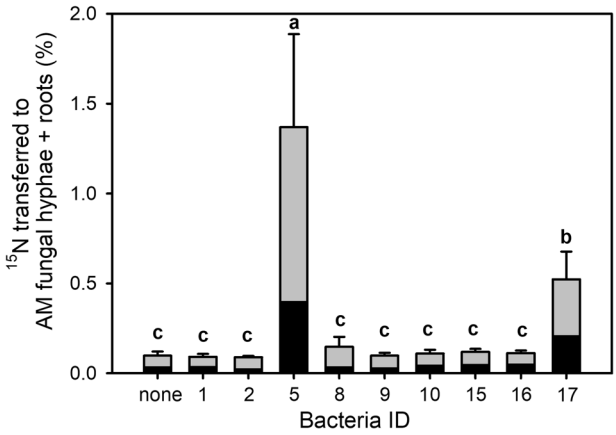

Fig. 4 Transfer rates of ^{15}N-labelled nitrogen applied into the labelling compartment in the form of chitin to the roots (grey bars) or to the mycorrhizal hyphae in the root-free zone of the large Petri dish (black bars) in Experiment 1. Only results from mycorrhizal microcosms are included in this analysis. Shown are mean values (and standard deviations) of 4–6 replicates per treatment of the total ^{15}N-transfer values (i.e. excess ^{15}N contained in the roots and their attached mycorrhizal fungal hyphae). Different letters indicate significant differences between treatment means with respect to the amount of ^{15}N-labelled nitrogen applied as chitin into the labelling compartment and transferred to the roots and their attached mycorrhizal fungal hyphae, expressed as percentage of total ^{15}N supplied in the labelling compartment. Means were separated by Duncan's post-hoc test ($p < 0.05$), following significant ANOVA ($F_{9,50} = 27.3$, $p < 0.001$). Data were log (x + 0.1) transformed prior to ANOVA.

In Experiment 2, we observed generally higher rates of ^{15}N transfer from the labelling compartment to the mycorrhizal roots with their attached AM fungal hyphae as compared to Experiment 1 (Fig. 5). Significantly higher transfer rates of N from chitin were recorded in mycorrhizal microcosms with isolate ID 5 added to the labelling compartment (mean 11.5% of the supplied ^{15}N) as compared to other bacterial isolates or to N transfer from the labelling compartment to NM roots. In the NM microcosms, the values remained below 1.36% of the supplied ^{15}N (Fig. 5, see supplementary Table S5 for ANOVA results). Further, presence of the protist *Polysphondylium pallidum* in the labelling compartment significantly increased the rate of N transfer to the roots and their associated AM fungal hyphae (supplementary Table S5). This effect obviously was driven by highly significant ($p = 0.011$, one-way ANOVA) increase of N transfer due to protist inoculation in mycorrhizal microcosms with added bacterium ID 5. The mean ^{15}N transfer rates in the relevant treatments were 8.5% and 14% of the supplied ^{15}N, which were detected in the roots and the extraradical AM fungal hyphae without and with the protists, respectively (Fig. 5). All primary data to Experiment 2 are available as Supplementary Data 2 file accompanying this paper.

^{15}N allocation to roots and AM fungal hyphae

Only marginal differences (ANOVA $F_{9,50} = 1.96$, $p = 0.07$) were observed between the bacterial treatments in terms of ^{15}N allocation from chitin to the AM fungal hyphae and mycorrhizal roots (i.e. considering only those mycorrhizal microcosms amended with chitin) in Experiment 1 (Fig. 4). Somewhat lower ^{15}N allocation to hyphae was noted upon inoculation with bacterium ID 2 to the labelling compartment (<28% of the total ^{15}N transferred out of the labelling compartment), in contrast to isolates ID 16 and ID 17, which induced more than 45% of the chitin-derived ^{15}N detected outside of the labelling compartment to be allocated to the hyphae. Allocation of ^{15}N from ammonium

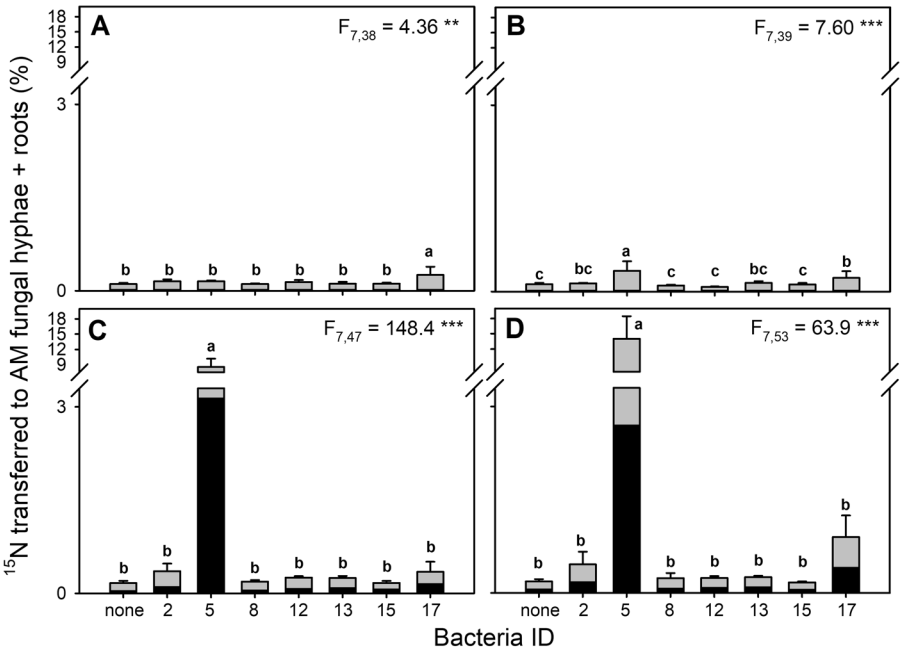

Fig. 5 Transfer rates of ^{15}N-labelled nitrogen applied to the labelling compartment in the form of chitin to the roots (grey bars) or to the mycorrhizal hyphae in the root-free zone of the large Petri dish (black bars) in Experiment 2. Results are shown separately for non-mycorrhizal plates without protists (**A**), non-mycorrhizal plates with protists (**B**), mycorrhizal plates without protists (**C**), and mycorrhizal plates with protists (**D**). Shown are mean values (and standard deviations) of 4–7 replicates per bacterial treatment. Different lower-case letters in each graph indicate significant differences between treatment means within the individual graphs. The numbers presented are percentages of ^{15}N-labelled nitrogen applied as chitin into the labelling compartment, and recovered outside the labelling compartment either in the roots or in the mycorrhizal hyphae. Means were separated by Duncan's post-hoc test ($p < 0.05$), following significant ANOVAs (respective F values and p value ranges are shown for each individual graph). Data were log(x + 0.1) transformed prior to ANOVA. Statistical comparisons were carried out separately for each of the four scenarios (i.e. presence or absence of the mycorrhizal fungus and presence or absence of the protist). *** $p < 0.001$, ** $0.01 > p \geq 0.001$.

chloride to the roots and the AM fungal hyphae was not significantly affected by any of the bacteria ($p > 0.1$). Gross mean ^{15}N allocation to the hyphae across all bacterial treatments was 27 ± 4.5% ($n = 59$) of the ^{15}N originating from the NH_4Cl and detected outside of the labelling compartment in either the root or hyphal biomass.

In Experiment 2, significant differences were detected in chitin-derived ^{15}N allocation between roots and the hyphae in mycorrhizal microcosms when the protists were present. In the absence of protists, only marginally significant differences were observed between bacterial isolates ($F_{7,47} = 2.12$, $p = 0.064$), ranging from <30% of the entire amount of chitin-derived N outside of the labelling compartment being detected in the hyphae (bacteria ID 8 and ID 2) to >43% (bacterium ID 15). When protists were present, allocation of the ^{15}N to roots and the hyphae was significantly affected by bacterial identity ($F_{7,53} = 4.26$, $p = 0.001$), with the bacterium ID 5 showing the smallest allocation to hyphae (21%). Further, bacterium ID 8 also showed low (~33%) ^{15}N allocation to hyphae, very similar to the bacteria-free treatment with 34% of the ^{15}N being detected in the hyphae. All the aforementioned treatments showed significantly lower values than did the treatment with bacterium ID 17 in the labelling compartment together with the protist, in which case 46% of all chitin-derived N detected outside of the labelling compartment was allocated to the hyphae.

DISCUSSION

Limited uptake of N from chitin by AM fungal hyphae alone

Our isotopic analyses revealed that the AM fungal hyphae could not release and take up significant amounts of N bound in chitin on their own, despite that chitinase genes have recently been uncovered in the genomes of several AM fungal species [27]. These results support our first hypothesis. On the other hand, and consistent with previous literature [20, 39, 40], the hyphae of the AM fungus could take up and transport large quantities of ammonium ions from the labelling compartment towards the roots, regardless of the identity of prokaryotes added or not added to that compartment. Passive diffusion of NH_4^+ ions from the labelling compartment towards the roots was obviously very effectively blocked (Fig. 3). We nevertheless noted a low but still measurable ^{15}N transfer to roots from the labelling compartment where either NH_4Cl or chitin was added in both mycorrhizal and NM microcosms. This might have been due to diffusion of NH_3 gas, which has recently been proposed as an alternative and largely neglected pathway of lateral N flow from soil to AM fungal hyphae and/or roots [41]. Whereas spontaneous production of ammonia from ammonium ions in aqueous solution is long known [42], production of ammonia gas from fungal chitin would require either spontaneous degradation of chitin due to residual chitinase and/or deaminase activities or presence of free ammonium ions in the chitin preparation. Both are theoretically possible and would require further analyses to clarify, but neither of these potential issues invalidate the results of our experiments. They only would increase the background ^{15}N.

Some chitinolytic bacteria improve utilisation of chitin-bound N by AM fungal hyphae

In our research, two isolates of chitinolytic *Paenibacillus* sp. increased utilisation of chitin as N source by the AM fungal hyphae, albeit to different extents or with different temporal dynamics. Differences in the latter are suggested due to

differential allocation of chitin-derived ^{15}N to the roots and the AM fungal hyphae outside of the labelling compartment between treatments added with bacteria ID 5 and ID 17 (see above).

Paenibacillus sp. has repeatedly been isolated from spores of AM fungi [43, 44], and it also has been demonstrated to bind to both vital and non-vital AM fungal hyphae under laboratory conditions [45, 46]. A number of significant interactive effects have previously been described between various AM fungi and *Paenibacillus* isolates [47–49]. One genotype of *Paenibacillus validus* has been shown to stimulate asymbiotic growth (i.e. formation of new spores from a germinating spore in the absence of a host plant) of an AM fungus *Rhizophagus* sp. [50]. We do not yet know how frequently members of *Paenibacillus* sp. actually occur in the AM fungal hyphosphere. It seems, though, that they are not restricted solely to biofilms at the AM fungal hyphae surfaces but occur both in the hyphosphere and surrounding soil [24]. This means they could be regarded as opportunistic inhabitants of the AM fungal hyphosphere and possibly involved also in recycling of AM fungal necromass [33].

Interestingly, two other chitinolytic bacteria (*Janthinobacterium* sp.) did not support acquisition of N from chitin by the AM fungus in our experiments. This is surprising inasmuch as we confirmed that those bacteria obviously expressed chitinolytic activity, particularly when offered chitin as the only C and N source (i.e. in absence of sucrose) and without contact to AM fungal hyphae (supplementary Figs. S1 and S2). Moreover, *Janthinobacterium* sp. is efficiently grazed by the protist *Polysphondylium pallidum* (supplementary Figs. S5 and S6). This means that, at least in the presence of protist [25], chitin-derived N should be released from the *Janthinobacterium* cells, allowing for detection of a significant N transfer from chitin to the AM fungal hyphae. This should be the case even if the bacterium was able to evade competition with the AM fungal hyphae for chitin's primary degradation products. Our results thus suggest differential expression of chitin-degrading genes in the *Janthinobacterium* sp. depending upon the presence of AM fungal hyphae and thus indicating limited validity of our second hypothesis.

Compared to *Paenibacillus* sp., there is less experimental evidence for specific interactions between AM fungal hyphae and *Janthinobacterium* sp., although this bacterial genus (or its close relatives) has previously been reported from both AM fungal spores and hyphae [45, 51]. Our results could possibly be explained such that, in the presence of AM fungal hyphae, *Janthinobacterium* sp. (which was actually growing profusely in Experiment 2 in the absence of sucrose; see supplementary Fig. S5 for data) would preferentially utilise complex AM fungal exudates [7, 10, 52] as compared to exogenously provided chitin, and the chitin would possibly be utilised only in absence of the AM fungus.

Different C and N stoichiometry

It is clear that omitting sucrose from the cultivation media in the labelling compartment in Experiment 2 strongly increased chitin mineralisation by the prokaryotes, even though the AM fungal hyphae's capacity for ammonium ions uptake obviously was not strongly hampered by sucrose (Fig. 3). Whereas the transfer of N from the labelling compartment to the AM fungal hyphae and roots in Experiment 1 (with sucrose) reached just up to 2% of the N supplied, only in the absence of sucrose (Experiment 2) did we reach N transfer rates above 10%. Only the latter results were thus comparable to those of our previous experiments conducted with unsterile potting substrates [18, 20]. This seems to be due at least in part to the fact that in Experiment 2 chitin and the living AM fungal hyphae were the only C sources for the prokaryotes in the labelling compartment. Increased need of C from the chitin by the bacteria in Experiment 2 may thus explain the order of magnitude faster mineralisation of chitin as compared to Experiment 1.

Protists speed up AM fungal uptake of N from chitin

We demonstrated that prokaryote-assisted acquisition of N by AM fungal hyphae from chitin accelerated when a eukaryotic grazer (a protist) was present together with the prokaryote in the organic N zone. These results provide strong support to our third hypothesis. However, significant variation in population size of the protists depending upon bacterial identity was noted in Experiment 2 (supplementary Fig. S5). This was not necessarily co-incident with chitin mineralisation rates and the rates of N transfer from chitin to the AM fungal hyphae. Further research is therefore justified as to the mechanisms and pathways of organic N recycling in AM fungal hyphosphere.

Conclusions and future perspectives

Resource stoichiometry and temporal dynamics of inter-kingdom interactions and population dynamics in AM fungal hyphosphere should be addressed in future research, which should also be expanded to other AM fungal species, bacteria, and protists (ideally isolated from a single ecosystem). Knowledge gained from simplified in vitro experiments should then be confirmed under unsterile soil conditions, using a full (green) plant model, stable isotope probing, co-occurrence networks, and (possibly also) by direct observation of microbial interactions and element transports, as well as utilising dense spatiotemporal sampling schemes. Other protists are likely to dominate grasslands or agricultural soils as compared to forests, and thus protists like *Vermamoeba* sp. may be more relevant for future studies as opposed to *Polysphondylium* sp. [53]. Such research will be most relevant to soils and AM fungal symbiosis-dominated ecosystems reliant on organic nutrient recycling, such as tropical rainforests, grasslands, and/or organically managed farms [54–56].

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AUTHOR CONTRIBUTIONS

MR and JJ conceived and developed the study. MR, PB and HH isolated, sequenced and cultured the different microbes included in the research described here. PB and MK prepared and analysed isotopically labelled chitin. MR, PB, HH, MK, MD and KG all

contributed to establishment and harvest of the two main experiments and sample processing for the different (elemental, isotopic and molecular) analyses. MR conducted the bacterial cultivation assays. MR, MK, MD and KG conducted quantitative molecular analyses in Experiment 2 (including development and calibration of microbial taxa-specific qPCR markers). JJ drafted the paper. All authors contributed to its revisions and approved the final version.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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