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Article

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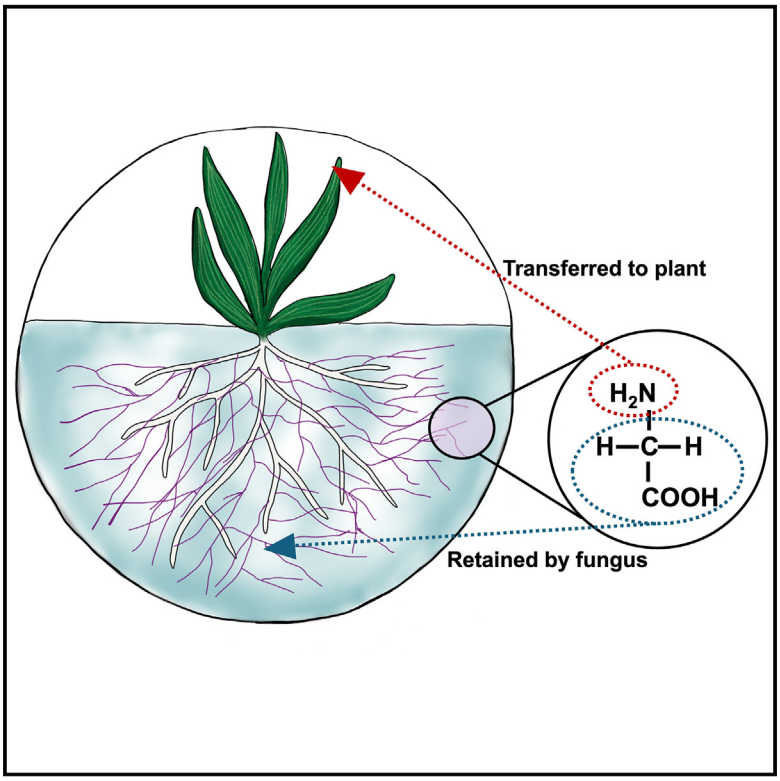
Preferential nitrogen and carbon exchange dynamics in Mucoromycotina "fine root endophyte"-plant symbiosis

Graphical abstract



Highlights

- MFRE preferentially transfer ^{15}N from glycine and ammonium to plants
- MFRE supplied with glycine and ammonium received most plant carbon
- MFRE fungi use organic compounds, retaining C and transferring N to plants
- Plants benefits from MFRE symbiosis depend on substrate nutrient availability

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In brief

Mucoromycotina fine root endophytes (MFRE) are symbiotic fungi that transfer soil nutrients to plants, gaining carbon (C). Howard et al. show that MFRE prefer nitrogen (N) from glycine and ammonium over nitrate and urea, transferring N to the plant and retaining C; N availability influences this, highlighting the importance of MFRE in plant nutrition.

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Preferential nitrogen and carbon exchange dynamics in Mucoromycotina "fine root endophyte"-plant symbiosis

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SUMMARY

Mucoromycotina "fine root endophyte" (MFRE) fungi are an understudied group of plant symbionts that regularly co-occur with arbuscular mycorrhizal fungi. The functional significance of MFRE in plant nutrition remains underexplored, particularly their role in plant nitrogen (N) assimilation from the variety of sources typically found in soils. Using four ^{15}N-labeled N sources to track N transfer between MFRE and *Plantago lanceolata*, applied singly and in tandem, we investigated N source discrimination, preference, and transfer to host plants by MFRE. We traced movement of ^{14}C from plants to MFRE to determine the impact of N source type on plant carbon (C) allocation to MFRE. We found that MFRE preferentially transferred N derived from glycine and ammonium to plant hosts over that derived from nitrate and urea, regardless of other N sources present. MFRE mycelium supplied with glycine and ammonium contained more plant-derived carbon than those supplied with other N sources. We show that the MFRE directly assimilates and metabolizes organic compounds, retaining C to meet its own metabolic requirements and transferring N to plant hosts. Our findings highlight diversity in the function of endomycorrhizal associations, with potentially profound implications for our understanding of the physiology and ecology of plant-fungal symbioses.

INTRODUCTION

Soils are dynamic environments where moisture, temperature, pH, and nutrient balance vary over spatial and temporal gradients. Nitrogen (N), a major plant nutrient, is usually present in soil in many forms simultaneously, at variable concentrations.[1] The majority of soil N is bound within complex, organic molecules derived from the decay of plant, animal, and microbial matter,[2–4] whereas inorganic N, including plant-accessible ammonium and nitrate salts,[1,5] accounts for a much smaller pool with high turnover rates.[6] The form and abundance of N in soils can be affected by both natural and anthropogenic factors, including atmospheric deposition,[7,8] direct application of agricultural fertilizers,[9] and through natural processes such as death and decay.[4,10] The resultant heterogeneity has repercussions for many biotic processes, from the production of microbial N-degrading enzymes[11] to the symbioses formed between plants and mycorrhizal fungi.

Mycorrhizas, intimate symbioses formed between plants and certain groups of soil fungi, occur across nearly all habitats on Earth.[12] These interkingdom partnerships usually enhance plant host acquisition of soil N and phosphorus (P), while the fungal partners benefit through provision of carbon (C)-rich derivatives of photosynthesis, including sugars and lipids.[13,14] The most widespread type of mycorrhizal symbioses are those formed between ~72% of plant species and arbuscular mycorrhizal (AM) fungi belonging to the subphylum Glomeromycotina.[15] AM fungi play an important role in supplying plant hosts with N, particularly in environments where N may be limiting.[16] Soil N concentration affects the frequency of root colonization and efficiency of nutrient transfer in mycorrhizal relationships,[17–19] with N limitation resulting in increased AM-mediated plant N assimilation from soil.[20] As such, soil fertility, including N availability, is a key environmental control on AM benefits derived by host plants by influencing plant C for nutrient (N and P) exchange and host plant photosynthetic capacity.[21,22]

Although research on endomycorrhizas, mycorrhizal fungi that enter living plant cells, has been dominated by AM fungi for decades, another less-well-studied group of soil fungi are emerging as physiologically, ecologically, and evolutionarily important root endophytes.[23–25] Recent advances in molecular detection methods[26] have revealed that fungi of the subphylum Mucoromycotina often colonize plant roots and other tissues (e.g., nonvascular plant thalli) of various plant species, often in

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co-colonization with AM fungi.[27,28] Mucoromycotina is a large subphylum, sister to Glomeromycotina,[29] and consists mainly of saprotrophic and pathogenic species.[30] Mycorrhizal fungi within this subphylum appear to be restricted to the order Endogonales[31–33] and are referred to as Mucoromycotina "fine root endophytes" (MFRE). Like AM fungi, MFRE form associations with a wide range of host plants[34,35] across diverse habitats, from western Europe[36] to Australasia,[37] each with varying edaphic factors, including moisture,[38] organic matter,[39] and nutrient status.[40] While recent investigations have described some key aspects of MFRE function and biology[31,32,35,41–43] the contributions of MFRE to host plant nutrition and responsiveness to environmental factors remain relatively poorly understood, rendering insights into the significance of MFRE in plant communities, soil ecology, and nutrient cycling unclear. Establishing how the availability of nutrients impacts MFRE-plant symbioses and whether they function similarly to AM symbioses are important foundational steps in determining the significance of MFRE in both natural and agricultural ecosystems.

Co-colonization of plants by both MFRE and AM fungi occurs frequently in natural habitats.[44,45] As such, there is strong potential for functional complementarity between the two fungal groups. In the liverworts *Allisonia* and *Neohodgsonia*, dual MFRE-AM fungal associations appear to be functionally complementary in terms of supplying host plants with N and P.[28] Vascular plants also gain nutritional benefits directly from associations with MFRE in terms of fungal transfer of ammonium-N and P to host plants in return for plant-fixed C resources,[31–33] even in the absence of other microbes within monoxenic microcosms.[31] Experimental evidence for MFRE involvement in vascular plant N acquisition is currently limited to a single source of inorganic N, ammonium chloride,[31] and it is unknown how N availability within the environment influences MFRE function. Ammonium (NH_4^+)-N is preferentially transferred to host plants by AM fungi over other N-containing compounds[46,47] and is, therefore, often used in experiments (e.g., Ames et al.[48] and Yang et al.[49]). Considering the putative saprotrophic capabilities of MFRE[31,36,42] and the recent indication that these fungi provide N derived from complex organic matter to liverwort hosts,[42] it is probable that MFRE access, assimilate, and transfer N from a variety of sources in the soil. Given what is known from AM symbioses, it seems likely that the availability of N within the substrate has an impact on the quantity of N transfer by MFRE to host plants, with greater plant-available N concentrations driving lower rates of transfer of N from any source to the host plant.

Inorganic sources of N, such as NH_4^+ and nitrate (NO_3^-), are simple molecules and offer a relatively low energetic cost of assimilation by symbiotic fungi compared with more complex organic compounds containing N. In AM fungi, NH_4^+ is preferentially assimilated over NO_3^-, likely because of the higher energetic cost associated with NO_3^- reduction.[46,47,50] The energetic cost of N assimilation by AM fungi is met through supply of hexoses and lipids by the host plant.[13,14] Given that MFRE are facultative saprotrophs,[31,36] it is possible that at least some of the energetic cost of assimilation of N from the soil may be ameliorated through saprotrophic C acquisition. This would provide MFRE with a physiological niche distinct from AM fungi, offsetting MFRE demand on host plant C resources by assimilation of soil C, while providing plants with access to nutrients from a wider pool of sources in the soil. Despite this, it is possible that MFRE preferentially assimilate inorganic N due to the extra C cost of metabolizing N of organic origin, as is the case for AM fungi.[46,47,50] Unlike AM fungi, MFRE are not obligately biotrophic,[36] and so it may be that organic compounds represent an important source of necessary C for MFRE.

Using *Plantago lanceolata*—a common forb with a wide geographic distribution[51,52] across many habitats[53] and a host for both AM[54,55] and MFRE[56] fungi—colonized by the MFRE isolate *Lyc-1*[31,32] in controlled monoxenic microcosms, we investigated the ability of MFRE to access, assimilate, and transfer ^{15}N from a selection of inorganic and organic compounds (ammonium chloride, sodium nitrate, glycine, and urea) commonly found in soils.[57,58] In a series of experiments where *P. lanceolata* and MFRE isolate *Lyc-1* were grown in monoxenic culture, we simultaneously quantified the allocation of host plant photosynthates passed to MFRE mycelium and fungal-acquired N transferred to the host across multiple N sources when presented in isolation ("N source" experiment; STAR Methods) and in tandem ("fungal choice" experiment; STAR Methods) availabilities, and consider possible underpinning mechanisms of C and N assimilation and transfer between symbionts. In addition, we investigated the effect of substrate N concentration on MFRE-plant nutrient exchange and the fate of organic C bound within glycine using ^{13}C labeling. We used three different nutrient media treatments in this "N concentration experiment" (Figure 1C; STAR Methods). Each treatment was based on 1/2GB5 with different quantities of N. Treatments comprised: "high N" (187.4 $\mu g.g^{-1}N$), "medium N" (93.7 $\mu g.g^{-1}N$), and "low N" (25 $\mu g.g^{-1}N$; full nutrient composition in Table S3). The high-N treatment comprised media containing the same N concentration as the 1/2GB5 used in the previous two experiments. Medium N refers to media containing half the N concentration as the high-N treatment, and the low-N treatment is based on the N concentration of soil of a typical temperate acidic grassland.[59]

RESULTS

N source experiment

Plant growth

There was no effect of MFRE colonization or ^{15}N source type on root biomass (Figure S1A; Table S1); however, shoot biomass (Figure S1B) was driven by MFRE inoculation (ANOVA: $F_{1, 110} = 7.05$, $p < 0.01$) (Figure 1A). Despite this, no differences were observed in biomass between inoculated and uninoculated plants of the same ^{15}N treatment (Tukey's honestly significant difference test, HSD: $p > 0.05$; Figure S1B). The type of ^{15}N source supplied had some effect on shoot biomass (ANOVA: $F_{3, 110} = 3.29$, $p < 0.05$). There was no interaction between the factors (Table S1).

MFRE-mediated plant ^{15}N concentration

The type of ^{15}N source available to MFRE influenced ^{15}N concentration in plant shoots (ANOVA: $F_{3, 74} = 3.7022$, $p < 0.05$; Figure 2A), with plants in microcosms where MFRE was supplied with ^{15}N-glycine accumulating more ^{15}N in the shoots than microcosms treated with either ^{15}N-sodium nitrate or $^{15}N_2$-urea

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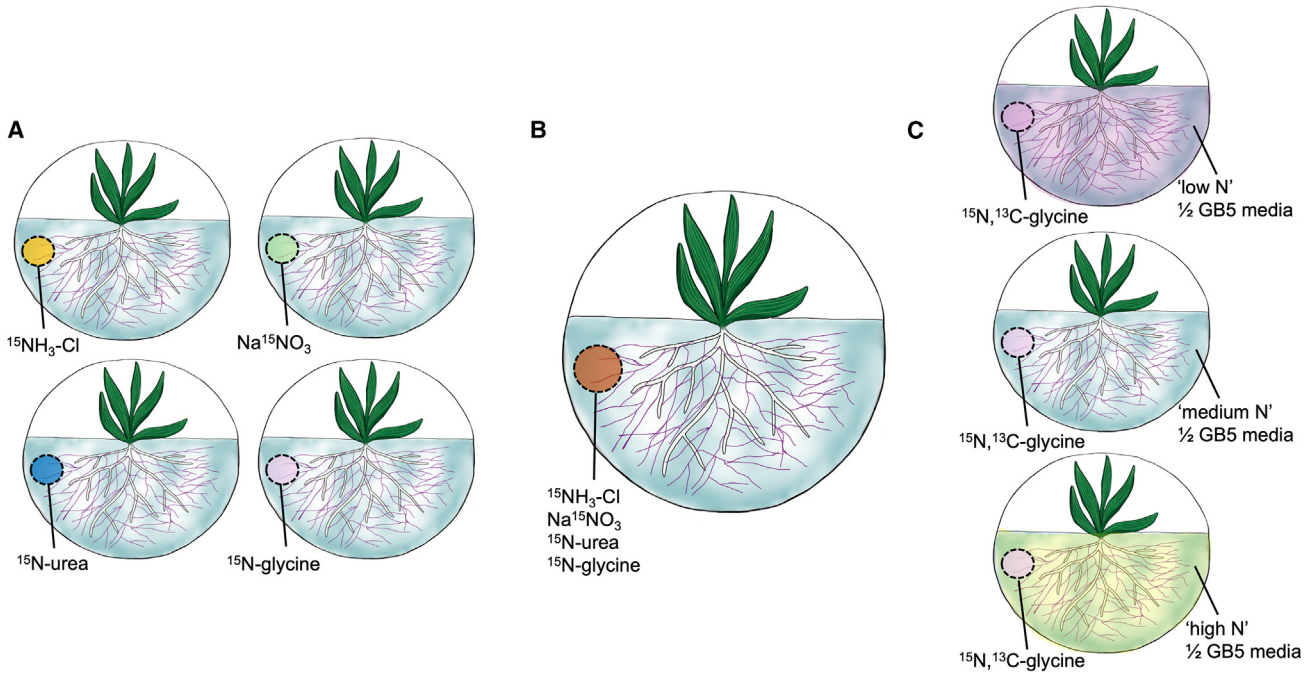

Figure 1. Monoxenic microcosm experiments for tracing movement of C and N between the Mucoromycotina fine root endophyte fungal isolate *Lyc-1* and *Plantago lanceolata*

(A) N source experiments where well contains one of $^{15}NH_3Cl$, $Na^{15}NO_3$, ^{15}N-urea, or ^{15}N-glycine.

(B) Fungal choice experiments where well contains an equal mixture of all sources in (A).

(C) N concentration experiments where all microcosms are supplied with ^{15}N/^{13}C-glycine, with media treatments comprising "high" (187.4 μg g^{-1}), "medium" (93.7 μg g^{-1}), and "low" (25 μg g^{-1}) N content of 1/2GB5 agar (full composition in Table S3). Headspace of microcosms in (A) and (C) were additionally supplied with a 0.25-MBq pulse of $^{14}CO_2$ to track movement of plant photosynthates to MFRE mycelium. See also Figure S1.

(Tukey's HSD: $p < 0.05$), but not those supplied with ^{15}N-ammonium chloride.

Plant-to-fungus C transfer

Quantification of fungal-acquired C in the N source experiment was determined by calculating the mean ^{14}C concentration of media in non-fungal microcosms, representative of diffusion and root exudation, and subtracting that value from the ^{14}C concentration in microcosms with MFRE colonization. ^{12}C was calculated as a function of ^{14}C fixation and the ideal gas law (STAR Methods) and was added to ^{14}C to provide "total C." The trends we observed in plant-to-MFRE C transfer (Figure 2B) mirror—and are stronger than—those observed for MFRE-mediated ^{15}N transfer to plants (Figure 2A). ^{15}N source is a significant driver of the amount of plant C transferred to the MFRE (Kruskal-Wallis: d.f. = 3, $X^2 = 20.256$, $p < 0.001$), with microcosms treated with ^{15}N-glycine or $^{15}NH_4Cl$ transferring significantly more C to MFRE than microcosms treated with either $^{15}N_2$-urea or $Na^{15}NO_3$.

Fungal choice experiment

MFRE-mediated plant ^{15}N transfer and assimilation

When a mixture of N sources was supplied to MFRE, ^{15}N concentration in host plant shoots was strongly influenced by which compound contained ^{15}N (ANOVA: $F_{3, 35} = 4.3933$, $p < 0.01$; Figure 3) (Figure 1B). Plants in microcosms where the glycine or ammonium chloride components of the N source mixture supplied to MFRE were labeled with ^{15}N accumulated more ^{15}N in shoots than microcosms where the labeled ^{15}N source was $Na^{15}NO_3$ (Tukey's HSD: $p < 0.05$). Plants within microcosms where urea contained ^{15}N had intermediate shoot accumulation [^{15}N] (Figure 3) and were not significantly different to other sources.

N concentration experiment

Colonization by MFRE and extraradical MFRE hyphal growth

To study the impact of substrate-N-concentration on MFRE-plant symbioses in terms of root colonization and function, we quantified the root colonization by MFRE and C and N exchanges between symbionts in each microcosm after 7 weeks of growth in monoaxenic systems with varying availabilities of ammonium and nitrate (Figure 1C; Table S3). In all treatments, roots inoculated with MFRE were colonized by abundant fine (<1.5 μm diameter) hyphae, which displayed typical MFRE morphology. We observed irregular branching (Figures 4A–4F), small (1–2 μm diameter) swellings (Figures 4B–4D), larger vesicle-like swellings (~5 μm diameter; Figure 4B), and smaller swellings at hyphae termini (~6.54 μm diameter; Figure 4F) within and around root cells, as previously observed in monoxenically grown plants.[31,32] % Root colonization by MFREs in the low-N treatment was significantly lower than in the medium-N or high-N treatments (Figure 5A, ANOVA: $F_{2, 39} = 6.5694$, $p < 0.01$; Tukey's HSD: $p < 0.05$). The presence of vesicle-like hyphal swellings was also lower in the low-N treatment than in the medium-N or high-N treatments (Figure 5B, ANOVA: $F_{2, 39} = 10.561$, $p < 0.001$). The surface area of

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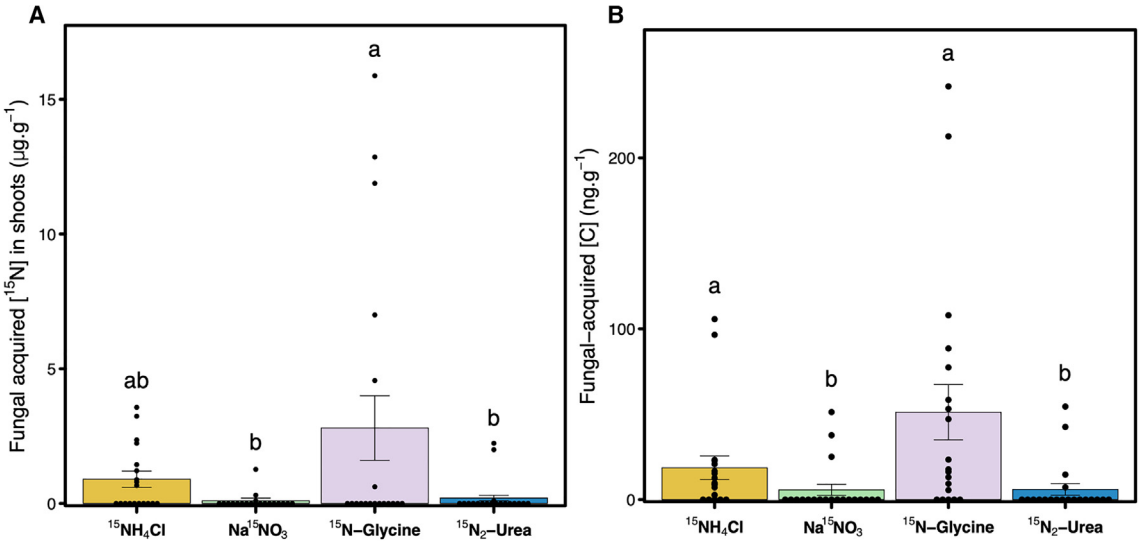

Figure 2. Nutrient-tracing data from N source experiment

(A) Mean fungal-acquired ^{15}N concentration of shoots. Different letters denote significant difference between means (Tukey's HSD: $p < 0.05$). $n = 20$ ($Na^{15}NO_3$, $^{15}N_2$-urea), $n = 19$ ($^{15}NH_4Cl$, ^{15}N-glycine). Error bars indicate ±SE.

(B) Plant-derived C concentration in MFRE hyphae. $n = 20$ ($Na^{15}NO_3$, $^{15}N_2$-urea), $n = 19$ ($^{15}NH_4Cl$, ^{15}N-glycine). Different letters denote significant difference between means (Dunn's post hoc: $p < 0.05$). Error bars indicate ±SE.

extraradical MFRE mycelium extending beyond the root (Figure 5C) was greater at all time points measured in low-N and medium-N treatments compared with high-N ones (ANOVA: $F_{3.81,\ 74.37} = 14.856$, $p < 0.001$).

MFRE-to-plant ^{15}N and plant-to-MFRE C transfer

Given the preference for glycine observed in previous experiments, we used ^{15}N- and ^{13}C-labeled glycine to trace the movement of both N and C from fungus to plant and $^{14}CO_2$ to trace plant-fixed C transfer to the MFRE mycelium. Plants growing in low-N microcosms (full composition in Table S3) contained significantly greater MFRE-acquired [^{15}N] in shoots than those grown in medium- or high-N treatments (Kruskal-Wallis: $X^2 = 9.9668$, d.f. = 2, $p < 0.01$; Dunn test: $p < 0.05$; Figure 6A). There were no significant differences in plant-fixed [C] (ng.g^{-1}) transferred to MFRE mycelium, regardless of N concentration in the media (Figure 6B; Table S1). Plant shoot ^{13}C concentration (Figure 6C) was greater in asymbiotic plants in medium- and low-N treatments compared with MFRE-colonized plants within the same treatment (ANOVA: $F_{1,\ 50} = 20.6347$, $p < 0.001$). Although asymbiotic plants were not affected by the N content of the media, MFRE-colonized plants accumulated more ^{13}C in shoot tissues in the high-N media compared with the low-N media, whereas an intermediate amount of ^{13}C accumulated in shoots of plants grown in medium-N media (ANOVA: $F_{2,\ 50} = 5.0497$, $p < 0.01$; Figure 6C).

DISCUSSION

Our experiments show direct C-for-N exchange between the MFRE (Lyc-1 isolate) and *P. lanceolata*[52,54,55] *in vitro*. Although not all microcosms in our experiments demonstrated C-for-N transfer between MFRE and host plants (likely due to the relatively short time frame of our isotope-tracing period compared with other tracer studies, e.g., Hawkins et al.,[57] Govindarajulu et al.,[60] and Thirkell et al.[61]), there was a preferential transfer and assimilation of ^{15}N derived from glycine and ammonium compared with that derived from nitrate or urea (Figures 2A and 3A) into host plant tissues via MFRE symbionts. This generally corresponded to enhanced photosynthate allocation to the fungus (Figure 2B).

To date, demonstration of resource exchange between MFRE and host plants in the absence of other soil microbes has been limited to a handful of plant species. The only other study of an angiosperm was that of Hoysted et al.,[31] where MFRE transferred N to the legume *Trifolium repens* in return for plant-fixed C resources in the absence of other soil microbes. However, the only source of ^{15}N tracer in this case was $^{15}NH_4Cl$. Further, as a legume, it is likely that *T. repens* is less reliant on fungal symbionts for N acquisition than non-leguminous plants, as N is typically supplied to the plant by N-fixing bacterial symbionts. Our findings expand the known range of sources from which MFRE can access and transfer N to a non-leguminous plant host. Given the range and preferences demonstrated by MFRE in our experiments, the possibility of functional complementarity with AM fungi in dual colonizations, whereby AM fungi play a primary role in supplying host plants with P[46,47] while MFRE supply N from a variety of sources,[31,42] remains open.

The greater MFRE-mediated assimilation and transfer of glycine- and ammonium-^{15}N to *P. lanceolata* compared with the other ^{15}N sources supplied occurred regardless of whether the ^{15}N-ammonium or ^{15}N-glycine tracer was the only ^{15}N source provided to the extraradical MFRE mycelium or whether these sources were part of a mixture (Figures 2A and 3). There was variation in fungal ^{15}N assimilation between microcosms of all treatments and between different experiments. It is possible that there is some effect of fungal biomass and the overall area of fungal hyphal mycelium; however, as these factors were not

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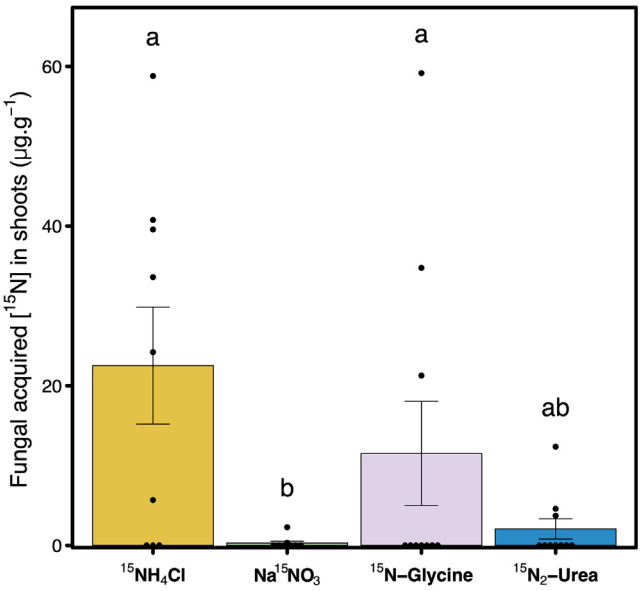

Figure 3. Fungal-acquired ^{15}N content of shoots in fungal choice experiment

Data presented as concentration. Different letters denote significant differences between means (Tukey's HSD: $p < 0.05$). $n = 10$ per treatment, apart from $^{15}NH_4$, in which $n = 9$. Error bars indicate ±SE. See also Figures S2 and S3 and Table S1.

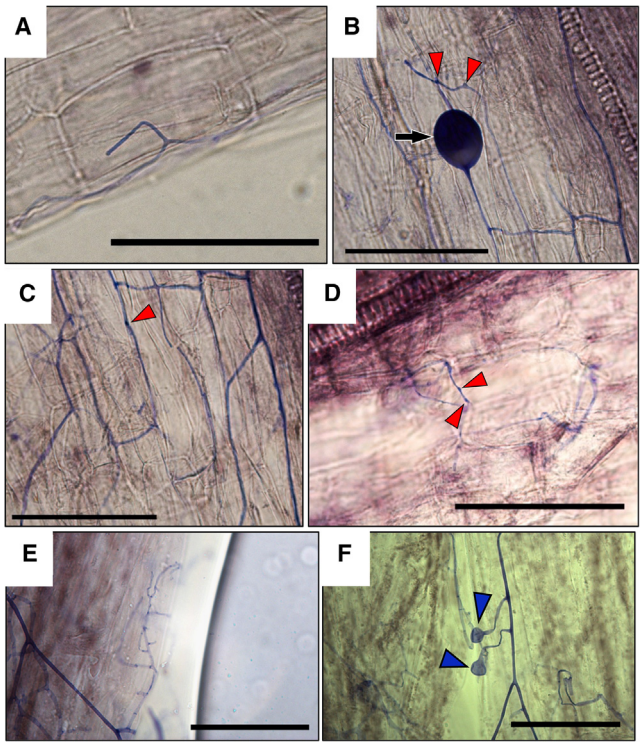

Figure 4. Micrographs of MFRE in *P. lanceolata* roots

Ink-stained roots of *P. lanceolata* colonized by MFRE (in purple) showing fine (<1.5 µm diameter) branching hyphae within cells (A–E), including those with small swellings (B–D; red wedges) as well as larger vesicles (B; black arrow) and terminal swellings (F; blue wedges). All images are from samples taken from monoxenic microcosms 7 weeks post inoculation with MFRE. Scale bars, 50 µm. See also Tables S1 and S2.

quantified in the N source and fungal choice experiments, we are unable to draw any firm conclusions as to the root cause.

The preference for glycine-^{15}N by MFRE in our experimental systems contrasts with the tendency of many AM fungal species to assimilate N from inorganic sources for transfer to host plants.[46,47] When presented with ^{15}N-glycine in non-sterile microcosms, four species of AM fungi showed no direct transfer of the tracer to host plants.[62] As obligate biotrophs, AM fungi lack the molecular toolkit required to degrade organic molecules, such as the extracellular proteases that are commonly found in saprotrophic ericoid and ectomycorrhizal fungi.[63] In contrast, because MFRE can be isolated from host plants and cultured in axenic conditions,[31,36] MFRE should possess at least some degradative capabilities to maintain mycelial growth in the absence of a host plant. Our key findings—that MFREspreferentially assimilate and transfer glycine-derived N to the host plant in return for proportionately similar photosynthetic C and that MFRE-associated plants are inhibited in their acquisition of ^{13}C tracer from glycine while transfer of ^{15}N from glycine is maintained—suggest that MFRE retain and metabolize glycine-derived C skeletons, liberating ammonium-N for transfer to host plants. This provides strong evidence of independent exogenous C acquisition capabilities of MFRE, even when associated with a living host plant. The acquisition of N from some small organic compounds by AM fungi has been demonstrated[64] but to date there is no convincing evidence of AM fungi utilizing C from organic sources.[16]

There was little-to-no transfer of ^{15}N derived from nitrate to host plants by MFRE in any of our experiments, regardless of whether nitrate was included as part of a mixture of N sources or was the only source of N added to the microcosm (Figures 5A and 5B). This contrasts with AM fungi, which frequently assimilate nitrate-N and transfer it to host plants[47,65] and possess nitrate reductases within their genomes.[66] Compared with the other N sources in this study, the relative bioavailability of nitrate-N to both plants[67] and AM fungi[68,69] within the rhizosphere may explain the lack of MFRE-mediated nitrate-N transfer to host plants, as assimilation of nitrate-N would represent a highly competitive niche for MFRE to exploit. A plausible hypothesis would be that MFRE do not possess the requisite molecular or metabolic capacity to exploit soil nitrate pools, having instead evolved capabilities to acquire and metabolize both N and C from organic sources. Ammonium-N, in contrast with nitrate-N, which is also a small inorganic compound, was transferred to plants in amounts comparable with those of glycine-N. This may be evidence that the MFRE assimilates N from soils as ammonium, using similar transporters to AM fungi[50]; however, more research is necessary to confirm this hypothesis.

A number of microcosms in all treatments across all experiments appeared to have very limited or no transfer of ^{15}N from MFRE to host plants. This may be caused by the inherent variability present within these systems[31,33]; however, this could also reflect the response of MFRE symbioses to the relatively high N content of the growth media in these experiments. In the N concentration experiment fewer microcosms contain no ^{15}N transfer, as the media N concentration decreases (Figure 6A).

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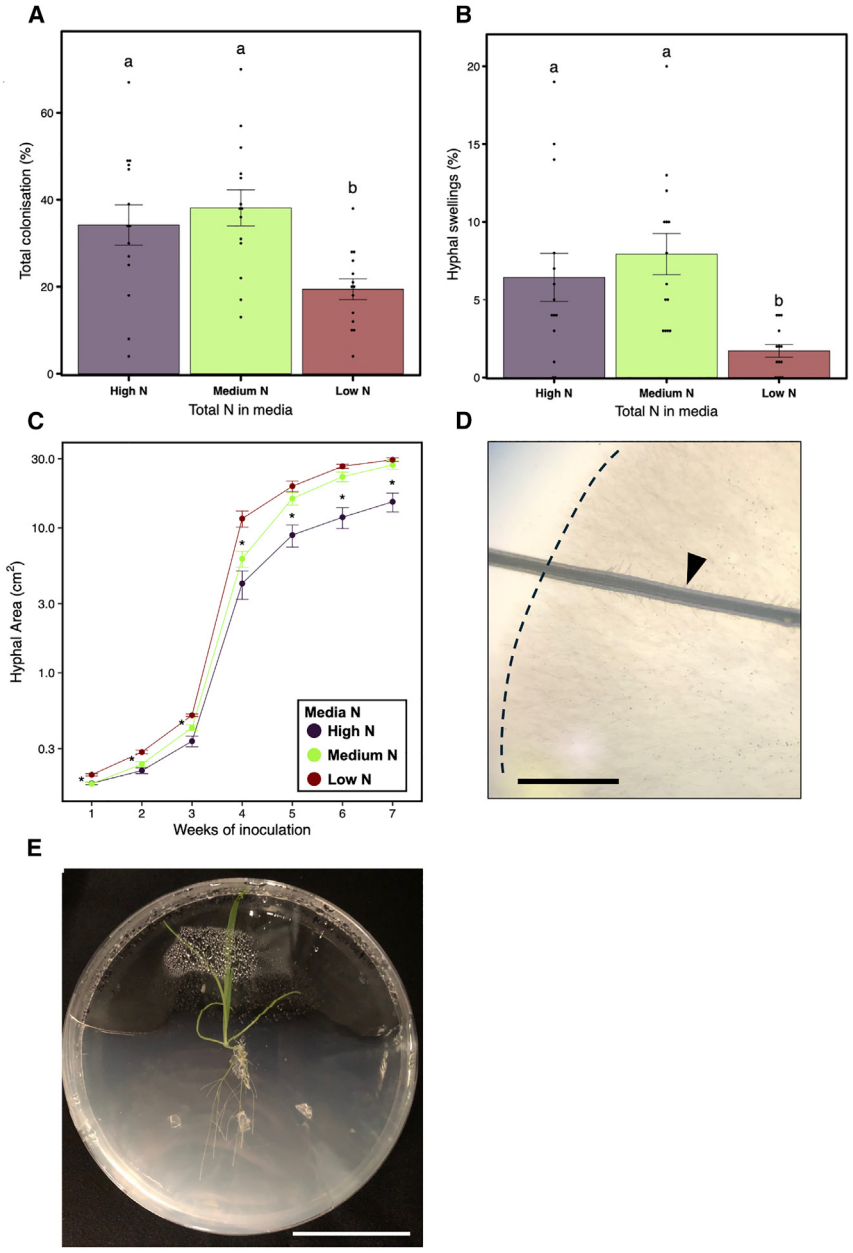

Figure 5. Colonization and mycelial network growth in "N concentration" experiment

(A–E) Colonization of *P. lanceolata* roots by MFRE structures (A and B), hyphal area outside roots (C), close view of MFRE mycelium around plant root (D), and image of whole microcosm showing extent of MFRE mycelium across roots (E).

(A) Total colonization by fungal structures. $n = 14$ per N treatment.

(B) Colonization by spherical "vesicular" structures. $n = 14$ per N treatment.

(C) Growth of extraradical MFRE mycelium in the N concentration experiment. $n = 14$ per N treatment per time point.

(D) Image of *P. lanceolata* root on 1/2GB5 medium (black wedge) and dense area of MFRE hyphae with defined growing edge (dashed line). Scale bar, 500 μm.

(E) Photograph of an example microcosm showing the extent of hyphal mycelium across the host plant root system and growth media. Scale bar, 5 cm. See also Figure S3 and Table S1.

Although MFRE transferred ^{15}N from glycine to host plants, the amount of ^{15}N delivered to host plants from urea, the other organic N source available to the fungi in our experiments, was much lower. This is probably due to the chemical nature of urea; urea is a stable molecule, with a half-life of over 3 years in solution,[70] only after which time it degrades to produce ammonium ions. Urease enzymes that catalyze this reaction are produced by a range of soil microbes,[71] including AM fungi.[63] It may be that MFRE do not produce these enzymes to utilize this substrate, instead scavenging ammonium-N—which our results suggest is readily utilized by MFRE (Figure 5)—from decomposition of urea by other soil-borne microorganisms. The addition of urease-producing soil bacteria to MFRE-only experimental systems, or the development of soil-based systems replete with a rhizosphere microbiome to investigate MFRE-plant symbioses, is now needed to determine whether this is the case.

In AM symbioses, plant reliance on fungal-acquired nutrients is highly context dependent.[72] In particular, the availability of nutrients within the environment can play a large role in determining the stoichiometry of P and N transfers from AM fungi to host plants and shifts in fungal community composition.[72–75] To test whether the nutritional role of MFRE is similarly plastic, we investigated N transfer from MFRE-to-plant across a range of N concentrations supplied to the growth media. Given the preference for assimilation and transfer of N from glycine observed in our other experiments, we supplied the MFRE mycelium with $^{15}N/^{13}C$-labeled glycine in modified 1/2GB5 media (Table S3). We found that host plants relied on MFRE for a greater proportion of their ^{15}N assimilation when grown on reduced N availability media (Figure 6A). The low-N media stimulated growth of a larger extraradical MFRE mycelial network (Figure 5C) and reduced the presence of structures typical of colonization within host roots (Figures 5A and 5B) more than the other media treatments, suggestive of an explorative, foraging growth strategy being deployed by the fungus. In contrast, there was no increase in C allocation from plant hosts to MFRE under the same limited N growth conditions (Figure 6B). However, there was a corresponding decrease in plant acquisition of ^{13}C from glycine in low-N conditions (Figure 6C), indicating assimilation and sequestration of ^{13}C (and ^{12}C) from glycine by MFRE. C acquisition by MFRE from an exogenous source could offset "costs" associated with increased transfer of N by MFRE to hosts[72] in low-N environments. The increased area of MFRE hyphae beyond plant roots in low-N media may explain the greater transfer of ^{15}N observed under the same conditions, as extension of

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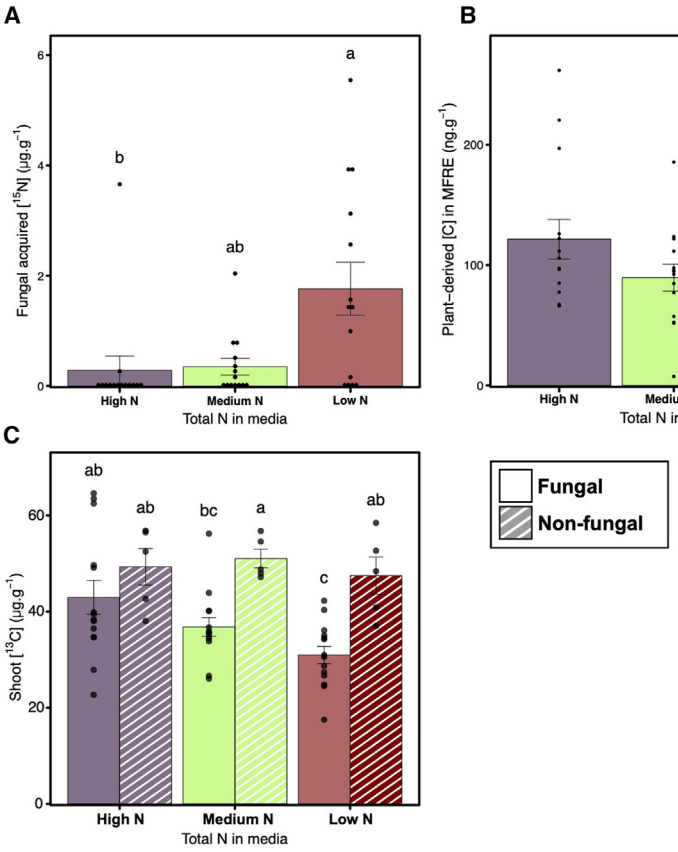

Figure 6. Nutrient-tracing data for the N concentration experiment

(A) Concentration of fungal-derived ^{15}N from labeled glycine in plant shoots. Different letters denote significant differences between means (Dunn's post hoc: $p < 0.05$). $n = 14$ per treatment.

(B) Difference in plant-derived C allocation to agar compared between fungal and non-fungal microcosms. Different letters denote significantly different means (Tukey's HSD: $p < 0.05$). $n = 14$ per treatment.

(C) Plant-glycine-derived ^{13}C concentration compared between fungal (solid bars) and non-fungal (hatched bars) microcosms. Different letters denote significant differences between means (Tukey's HSD: $p < 0.05$). $n = 14$ per treatment. Error bars indicate ±SE. See also Figure S3 and Table S1.

foraging fungal hyphae would increase the likelihood of their encountering the isotope tracer, increasing the amount of tracer transferred to hosts despite no greater root colonization being recorded.

Our finding that MFRE fungi preferentially transfer N from glycine to host plants (Figures 2A and 3) contrasts with our hypothesis that inorganic N would be preferred owing to its relatively simple structure, resulting in more energy-efficient assimilation and metabolism compared with more complex organic compounds. However, our finding that MFRE reduce the assimilation of glycine-derived ^{13}C by plants (Figure 6C), while simultaneously enhancing assimilation of glycine-N (Figure 6A) and receiving plant-derived C (Figure 6B), is consistent with the capacity of MFRE to supplement their plant-fixed C nutrition with C uptake from environmental sources. Access to nutrients bound up in organic compounds potentially provides MFRE with a competitive edge in a crowded symbiotic marketplace, providing the fungi with an advantage when there is not a ready supply of plant-derived C. As such, MFRE may preferentially assimilate organic compounds in the soil as these provide not only N, an exchangeable commodity in plant-fungal symbioses, but also C, which can be used by the fungus to supplement its supply of plant-fixed C. If this is the case, then assimilation of organic compounds could ultimately provide more benefit to MFRE than an inorganic N source. Our experiments support this hypothesis, showing that regardless of N availability in the growth media, MFRE obtained similar amounts of plant-fixed C (Figure 6B) despite greater N being transferred to host plants from glycine. Such physiological plasticity is likely to facilitate persistence of MFRE fungi alongside, and in competition with, other plant endophytic fungi for space and resources within host plant roots.

It should be noted that our experimental systems do not represent the full complexity of soil ecosystems. In nature, plant-MFRE symbioses appear to occur in most scenarios that have been investigated,[35] encompassing many other abiotic factors, including variable light conditions, nutrient availability, and access to water. As such, our findings should not be generalized to all plant-MFRE symbioses in all environmental scenarios; crucially, the data presented exclude the potential for MFRE-microbial interactions such as those between AM fungi and soil bacteria.[76] Such interactions may facilitate the assimilation of a broader range of N sources by MFRE. Nevertheless, our work here represents an important starting point for the exploration of the broader ecological and physiological significance of MFRE fungi to develop a more holistic understanding of plant-fungal symbioses.

In summary, we show that MFRE preferentially transfer N derived from glycine to host plants rather than the simpler inorganic N compounds offered in our experiments. This is likely because organic compounds provide both N and C, which the fungi use to supplement their nutrition. The ability to break down and assimilate complex organic nutrients may give MFRE fungi a competitive advantage in symbiotic environments, and further work investigating the nutritional discrimination between these symbiotic fungal clades is now urgently required.

RESOURCE AVAILABILITY

Lead contact

More information or resource requests should be forwarded to the lead contact, Katie Field (k.j.field@sheffield.ac.uk).

Materials availability

This study did not generate new unique reagents

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

N.O.A.H. and K.J.F. conceived and designed the experiments. N.O.A.H., E.D., A.W., and K.J.F. performed the experiments and laboratory analyses. N.O.A.H. analyzed and interpreted the data. N.O.A.H. wrote the manuscript, with contributions from all other authors. All authors revised and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Lactic acid 90%	Acros	189870010
^{14}C-sodium bicarbonate	Perkin Elmer	NEC086H001MC
Ethanol	Sigma	32221
Pelikan Brilliant Black ink	N/A	N/A
Poly(vinyl alcohol)	Sigma	363146
Glycerol	Acros	158920025
CarbonCount	Meridian Biotechnologies	CT/10
CarbonTrap	Meridian Biotechnologies	CC/10
^{15}N-Glycine (≥ 98% atom % ^{15}N)	Sigma	299294
^{15}N-Ammonium chloride (≥ 98% atom % ^{15}N)	Sigma	299251
Sodium ^{15}N-nitrate (≥ 98% atom % ^{15}N)	Sigma	364606
$^{15}N_2$-Urea (≥ 98% atom % ^{15}N)	Sigma	316830
Experimental models: Organisms/strains		
Mucoromycotina 'fine root endophyte'	Field et al.[36]	N/A
Plantago lanceolata	Yellow Flag wildflowers	N/A
Software and algorithms		
R (v4.3.0)	R	http://R-project.org
R Studio (v2023.3.0.386)	RStudio	https://rstudio.com
ImageJ (v1.53a)	Schneider et al.[77]	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Plant growth conditions

In all experiments, monoxenic microcosms (Figure 1) were established using *Plantago lanceolata* seedlings (Yellow Flag Wildflowers, Gloucester, UK) with MFRE (*Lyc-1*) mycelium introduced from axenically-grown stocks (see above). *P. lanceolata* is a non-leguminous, mycorrhizal forb, common across a range of diverse habitats[53] and with a wide distribution.[51] It is a commonly used plant model for AM studies[54] with the relatively small size, propensity for mycorrhization (identified as hosting MFRE in wild-collected plants[56]) and rapid development making it a very tractable and ecologically relevant plant species for our experiments. 140 mm sterile triple vented Petri-dishes were filled with ~60 mL of ½GB5, or one of the three treatments in N concentration experiments, poured on a gradient that allowed for plant development in an upright position. *P. lanceolata* seedlings sterilised in a 4.5% sodium hypochlorite solution were germinated on flat 'nursery' plates of ½GB5 under 16:8hr (day:night) at room temperature. Seven days after sterilisation, individual germinated seedlings were transferred to experimental microcosms under sterile conditions.

Fungal inoculum and microcosm establishment

MFRE isolate *Lyc-1*, initially isolated from *Lycopodiella inundata*,[31,32] was maintained on Gamborg B5 basal medium at 50% concentration (1.6g.L^{-1}; Sigma-Aldrich; 187.4 μg.g^{-1}N; Table S3) buffered with 0.5g.L^{-1} MES (Sigma-Aldrich) solidified with 1% agar (referred to as ½GB5). Cultures were kept in the dark and incubated at 25°C. Immediately following transplant of *P. lanceolata* seedlings to individual microcosms, three small (approximately 1.25 cm^3) sections of ½GB5 agar containing abundant MFRE hyphae and spores were placed adjacent to emerging roots. Experimental microcosms were sealed with Parafilm and the 'belowground' agar portion of each plate was wrapped in aluminium foil to reduce light penetration into agar media. These microcosms were maintained in 16:8hr day:night conditions at a constant temperature of 25°C.

Experimental microcosms

Using monoxenic *P. lanceolata*-MFRE microcosms (below), we established three experiments to test our hypotheses:

(i) **To investigate the capability of MFRE to assimilate and transfer N to the host plant from diverse sources** we conducted an '*N source experiment*' whereby different ^{15}N-labelled isotopes of compounds abundant in soils and transferred to plants by AM fungi[57,58] (ammonium, nitrate, urea, glycine; 1 mg.ml^{-1}) were supplied to MFRE mycelium and subsequently measured in plant tissues while plant-derived C was traced into MFRE mycelium (Figure 1A).

(ii) **To assess MFRE ^{15}N source preference**, we conducted a '*fungal choice experiment*' whereby microcosms were simultaneously labelled with all four sources of N as the 'N source experiment', providing a choice of N source to MFRE mycelium (1 mg.ml^{-1} per source; Figure 1B).

(iii) **To determine the effect of substrate N concentration variability** on MFRE-plant nutrient exchange and the fate of organic C bound within complex organic N sources, three different nutrient media treatments were employed in an '*N concentration experiment*' (Figure 1C). Each treatment was based on ½GB5 but with inclusion of differing quantities of N, concentrations being relevant to previous experimental systems (total N in media of experiments i and ii is equivalent to the 'High N' treatment of experiment iii) as well as a limestone grassland in the Peak district ([59]; 'Low N'). Treatments comprised: 'High N' (187.4 μg.g^{-1}N), 'Medium N' (93.7 μg.g^{-1}N), and 'Low N' (25 μg.g^{-1}N) (Full nutrient composition in Table S3).

METHOD DETAILS

Colonisation of P. lanceolata roots by MFRE and mycelial growth

After growing plants and fungi together in microcosms for seven weeks, '*N concentration*' experiment plants were harvested and approximately 25% of the root system from each microcosm were stained using methods modified from Vierheilig et al.[78] Quantification of colonisation by MFRE was not possible for the '*N source*' and '*fungal choice*' experiments due to the limited root biomass. Briefly, roots were placed into a 10% solution of KOH for 1hr at 70ºC, rinsed in tap water, placed in ink-vinegar stain (5% Pelikan Brilliant Black, 5% acetic acid, 90% d.H_2O) for 1hr, then rinsed again and placed in 1% acetic acid overnight to de-stain. The stained root material was mounted on slides in PVLG (Polyvinyl-Lacto-Glycerol) and colonisation counted under 40 x objective magnification (Ceti Max II; Medline Scientific, Chalgrove, UK). Representative images (Figure 4) were obtained under 100 x objective magnification (Leica DM6; Leica Microsystems, Wetzlar, Germany).

We measured the two-dimensional area of MFRE extraradical mycelium in each microcosm of the 'N concentration' experiment weekly from the point of inoculation until the systems were harvested seven weeks later. The outline of the furthest extent of MFRE mycelial networks (Figure 5D) were digitized and mycelial area determined using ImageJ (v1.53a[77]).

^{15}N, ^{13}C, and ^{14}C isotope tracing

i) '*N source*' *experiment*: Seven weeks after seedlings were placed in individual microcosms, a ~2.5ml well was dug into the agar near to the margins of the MFRE mycelium, away from plant roots, filled with 100 μL of a 1 mg mL^{-1} solution of a single ^{15}N-labelled compound (total 0.1 mg ^{15}N labelled compound per plate; one of ammonium chloride ($^{15}NH_4Cl$, ≥ 98% atom % ^{15}N, 27.53 μg ^{15}N; Sigma-Aldrich), sodium nitrate ($Na^{15}NO_3$, ≥ 98% atom % ^{15}N, 17.44 μg ^{15}N; Sigma-Aldrich), glycine ($C_2H_5{}^{15}NO_2$, ≥ 98% atom % ^{15}N,19.72 μg ^{15}N; Sigma-Aldrich) and Urea ($CH_4{}^{15}N_2O$, ≥ 98% atom % ^{15}N, 48.36 μg ^{15}N; Sigma-Aldrich) and backfilled with ½GB5 media. To control for diffusion of the ^{15}N solution into the agar and subsequent direct plant assimilation, non-fungal control microcosms were also established. (n = 10 control microcosms for each ^{15}N treatment apart from ^{15}N-ammonium chloride which n = 9 controls due to microbial contamination). In total n = 20 ($Na^{15}NO_3$, $^{15}N_2$-urea), n = 19 ($^{15}NH_4Cl$, ^{15}N-Glycine).

ii) '*Fungal choice*' *experiment*: Seven weeks after inoculation with MFRE, wells were filled with 25 μL of a 4 mg.mL^{-1} solution of each N source used previously (i.e. ammonium chloride, sodium nitrate, glycine, and urea). These were applied in four treatments, with only one of the sources in each containing the ^{15}N label. As such, each treatment comprised three unlabelled N sources and one ^{15}N-labelled N source (0.1 mg compound per source, 0.4 mg compound in total per microcosm). Each well was backfilled with ½GB5 as previously described. The fungal 'choice' experiment comprised 10 fungal experimental microcosms. To control for diffusion of isotope through the agar medium, uninoculated control microcosms were established (n = 10 uninoculated microcosms per treatment). In total there were 20 microcosms established per treatment

iii) N concentration experiment: Building on the observations from experiments i) and ii), seven weeks post inoculation, all microcosms of each N concentration treatment ('High N': 187.4 μg.g^{-1}N, 'Medium N': 93.7 μg.g^{-1}N, and 'Low N': 25 μg.g^{-1}N) were labelled with a solution of 100 μl 1 mg.ml^{-1} ^{15}N-glycine tracer added to wells cut into the agar portion of the microcosms and backfilled with ½GB5 media, as described above (n = 5 per treatment). To determine the fate of glycine bound-C, we labelled using stable ^{15}N- (19.72 μg ^{15}N) and to determine the fate of glycine bound-C in the presence of MFRE, we used ^{13}C-labelled glycine (17.09 μg ^{13}C per plate). Shoot ^{15}N/^{13}C concentrations were determined using IRMS. To control for isotope diffusion and non-MFRE mediated N/C distribution, non-fungal control microcosms were established (n = 5 uninoculated microcosms per treatment).

In 'N source' and 'N concentration' experiments, immediately after ^{15}N/^{13}C addition into wells, the surface of the agar portion of the microcosm was covered with a clear PVC sheet and sealed with anhydrous lanolin. A 0.25 MBq $^{14}CO_2$ pulse was liberated into the headspace of sealed plates from 6.75 μl ^{14}C-labelled sodium bicarbonate (2.14 GBq/mmol) by the addition of 2 ml 90% lactic acid. Microcosms were incubated for 24 hrs to allow for $^{14}CO_2$ fixation and movement of ^{15}N (and ^{13}C) and ^{14}C between plants and MFRE. At the end of the labelling period, 2 ml 2M KOH was introduced into small containers within the microcosms to absorb any remaining $^{14}CO_2$. After 1 hr, all plant materials were removed carefully from the agar, separating plant shoots from roots, and removing as much

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excess agar from root material as possible prior to freeze-drying. To assess ^{14}C transfer to MFRE the agar (containing MFRE fungal mycelium in all microcosms apart from uncolonized controls) was also freeze-dried and homogenised. 10-30 mg freeze-dried agar was weighed into CombustoCones (Perkin Elmer, Beaconsfield, UK) prior to sample oxidation (Sample Oxidiser 307, Perkin Elmer, Beaconsfield, UK) and ^{14}C quantification via liquid scintillation counting (Packard Tri-Carb 4910TR, Perkin Elmer, Beaconsfield, UK). Total carbon (^{12}C + ^{14}C) fixed by the plant and transferred to MFRE within the agar was calculated as a function of the total volume and CO_2 content of the labelling chamber and the proportion of the supplied $^{14}CO_2$ label fixed by the plants. The difference in carbon between fungal and non-fungal plants is equivalent to the total C transferred from plant to MFRE within the fungal microcosms, assuming no alteration in plant root C exudation under fungal colonisation.

In all experiments, all plant shoots were harvested and freeze-dried 24 hr after isotope addition. Between 0.1 and 5mg freeze-dried shoot tissue were measured into tin capsules (Sercon, Crewe, UK) and the abundance of ^{15}N in samples determined by IRMS (Isotope Ratio Mass Spectrometry) using an ANCA GSL 20–20 Mass Spectrometer (Sercon PDZ Europa 2020 Isotope Ratio Mass Spectrometer coupled to a PDZ ANCA GSL preparation unit). Data were collected as atom $\%^{15}N$ and as %N using un-labelled control plants for background detection.

Equations

The following equations were used to determine plant and fungal ^{15}N and ^{14}C content.

Total C content

Total carbon assimilated by the plant was calculated using the following equations modified from Hoysted et al.[31]:

$$T_{pf} = \left(\frac{A}{A_{sp}} m_a\right) + (P_r \times m_c)$$

where T_{pf} = Transfer of carbon from plant to fungus, A = radioactivity of the agar tissue sample (Bq); A_{sp} = specific activity of the source (Bq Mol^{-1}), m_a = atomic mass of ^{14}C, P_r = proportion of the total ^{14}C label supplied present in the agar tissue; m_c = mass of C in the CO_2 present in the labelling chamber (g) (from the ideal gas law):

$$m_{cd} = M_{cd}\left(\frac{PV_{cd}}{RT}\right) \therefore m_c = m_{cd} \times 0.27292$$

where m_{cd} is mass of CO_2 (g), M_{cd} is molecular mass of CO_2 (44.01 $g.mol^{-1}$), P is total pressure (kPa); V_{cd} is the volume of CO_2 in the chamber (0.000049m^3); R is the universal gas constant ($J.K^{-1}.mol^{-1}$); T, absolute temperature (K); m_c, mass of C in the CO_2 present in the labelling chamber (g), where 0.27292 is the proportion of C in CO_2 on a mass fraction basis. To determine the amount of C transfer to agar that was mediated by MFRE alone, the average concentration of ^{14}C in non-fungal controls was subtracted from the ^{14}C concentration in individual experimental microcosms.

^{15}N content

Plant tissue concentration of ^{15}N was calculated using the following equations from Hoysted et al.[31]:

$$M_{Ex} = \left(\frac{At_{lab} - At_{cont}}{100}\right)\left(M\left[\frac{\%E}{100}\right]\right)$$

where M_{Ex} is mass (excess) of ^{15}N in samples (g), At_{lab} is atom percentage of ^{15}N in the experimental microcosms, At_{cont} is the atom percentage of ^{15}N in unlabelled control plant material, this was generated by growing *P. lanceolata* seedlings in microcosms as described above but with no isotope labels added into the systems. M is the sample biomass (g) and $\%E$ is the total percentage of N. This was then converted to μg to obtain concentration per mg of plant tissue and then further expressed per g of plant biomass ([^{15}N]). The average [^{15}N] of non-fungal control microcosms for each ^{15}N treatment was then subtracted from the [^{15}N] for each experimental microcosm within that treatment.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were conducted using R[60] and R studio (v2023.3.0.386[79]), using packages 'dplyr' (v1.1.2[80]), 'car' (v3.1-2[81]), 'rosetta' (v0.3.12[82]), 'stats' (v4.3.0[79]), 'agricolae' (v1.3-5[83]) Isotope tracing data were analysed using analysis of variance (ANOVA) with post hoc Tukey testing (as indicated). Data were checked for normality and homogeneity of variance. Where assumptions were not met, either a square root or logarithmic transformation was performed (Table S1), or a non-parametric test Kruskal-Wallis with Dunn's post hoc test (as indicated) was conducted. Plant biomass was compared between Fungal and non-Fungal plants using either a student's T-test, or Wilcoxon signed-rank test where assumptions of normality and homogeneity of variance were not met. Hypha area growth data were analysed using a two-way repeated measures ANOVA with a bonferroni correction. Figures were created in R (v2023.3.0.386[79]) using the 'ggplot2' package (v3.4.2[84]).