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RESEARCH ARTICLE

Fungal symbiont diversity drives growth of *Holcus lanatus* depending on soil nutrient availability

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Abstract

1. Arbuscular mycorrhizal (AM) fungi frequently colonise plant roots and can affect plant morphology and physiology through their contribution to plant nutrition. However, the functional role of AM fungi in the presence of other microbial symbionts, including widespread Mucoromycotina 'fine root endophytes' (MFRE) fungi, remains largely unknown.
2. While both AM fungi and MFRE transfer nutrients, including nitrogen, from inorganic and organic sources to host plants, their combined effects on co-colonised plants have only been investigated in liverworts. Here, we compare the morphology and physiology of the grass *Holcus lanatus* grown with an AM fungal community versus a more diverse symbiotic fungal community containing both AM fungi and MFRE.
3. *Holcus lanatus* plants were grown in the presence of either a diverse MFRE+AM fungi soil inoculum or a multi-species AM fungal inoculum. Plant traits associated with growth were quantified, along with fungal transfer of ^{15}N tracer to plants from a variety of sources (ammonium chloride, alanine, glycine and algal necromass).
4. *Holcus lanatus* grown with the AM fungal community had greater root and shoot growth during early development and prior to the addition of ^{15}N-labelled sources, compared with plants grown with the more diverse symbiotic fungal community. When nitrogen sources were made available to the fungal symbionts in the pot microcosms, plants growing with the MFRE+AM fungi soil inoculum had a faster growth rate than plants growing with the AM fungal community. At harvest, *H. lanatus* grown with the AM fungal community had a larger biomass, and there were no differences in ^{15}N tracer assimilation in plants across the two fungal community treatments.
5. Our results demonstrate that the diversity of fungal inocula in conjunction with soil nutrient availability determine the benefits derived by plants from diverse fungal symbionts. Our research contributes to understanding host plant outcomes in diverse multi-symbiont scenarios.

KEYWORDS
^{15}N isotope tracing, arbuscular mycorrhizal fungi, Endogonales, *Holcus lanatus*, Mucoromycotina 'fine root endophytes', mycorrhizas, plant–soil interactions, symbiosis

1 | INTRODUCTION

In nature, the colonisation of plant roots by diverse symbiotic soil fungi known as mycorrhizal fungi is the prevailing condition for the majority of plants (Brundrett & Tedersoo, 2018; Orchard, Standish, Dickie, et al., 2017; Teste et al., 2020). Mycorrhizal symbioses involve the bi-directional exchange of fungal acquired nutrients, including nitrogen (N) and phosphorus (P), for plant-fixed carbon (C) (Smith & Read, 2008) and are thought to have facilitated plant terrestrialisation ca. 500 Mya (Field, Rimington, et al., 2015; Morris et al., 2018). Today, most plants associate with soil fungi as a means of increasing their plasticity and fitness; however, these associations are complex and potential benefits to the fungi or plants can depend on environmental conditions, host/symbiont genotypes and additional biotic interactions (Johnson et al., 1997; Kuyper & Jansa, 2023).

One of the most widely studied groups of mycorrhizal fungi are the Glomeromycotina arbuscular mycorrhizal (AM) fungi, which form intracellular structures in the roots of >70% of vascular plants (Brundrett & Tedersoo, 2018; Smith & Read, 2008). The AM fungi can be important in host plant P and N nutrition, providing plants with up to 100% of their nutritional needs for these critical macronutrients (Hawkins et al., 2000; Rozmoš et al., 2022; Smith et al., 2011; Smith & Read, 2008; Thirkell et al., 2016, 2019, 2020; van der Heijden et al., 2015; Xie et al., 2022). The functional significance of mycorrhizal symbioses is probably best understood as a 'mutualism–parasitism continuum' whereby the functionality of individual associations between host plants and fungal symbiont(s) is context-dependent (Johnson et al., 1997). Several abiotic and biotic factors affect the function of AM fungal–plant symbioses including soil nutrient availability, moisture and the presence of other microbes (Deepika & Kothamasi, 2015; Kuyper & Jansa, 2023). Phosphorus transfer from AM fungi to plants, for example, is generally inversely correlated with P availability in the substrate, that is, the more bioavailable P present in the rhizosphere, the less plants may rely on AM fungal-acquired P (Olsson et al., 2010; Smith et al., 2011, 2015). In contrast, the effects of soil N availability on AM fungal N transfer are less well-understood and require further scrutiny (Thirkell et al., 2019; Xie et al., 2022).

Mucoromycotina 'fine root endophytes' (MFRE) often co-colonise plant roots alongside AM fungi (Orchard, Standish, Dickie, et al., 2017). MFRE are an understudied group of Endogonales soil fungi that are common across a wide variety of habitats (Albornoz et al., 2021; Kowal et al., 2022; Orchard, Standish, Dickie, et al., 2017). Although MFRE are molecularly and morphologically distinct from Glomeromycotina AM fungi (Orchard, Hilton, Bending, et al., 2017), they share some functional characteristics with the latter, such as obtaining C from plants in exchange for N and P (Field et al., 2016, 2019; Field, Rimington, et al., 2015; Hoysted et al., 2019, 2023). However, unlike strictly biotrophic AM fungi, MFRE can be isolated and grown in vitro in the absence of a plant host (Field, Rimington, et al., 2015; Hoysted et al., 2023). This suggests that MFRE may persist in the soil in a free-living state, probably due to being equipped with transporters and enzymatic pathways necessary to obtain nutrients from complex organic nutrient sources (Howard et al., 2022). Other Mucoromycotina fungi closely related to symbiotic Endogonales are saprotrophic, indicating that MFRE may well share at least some of the molecular machinery required for a facultative saprotrophic lifestyle (Spatafora et al., 2016). The putative saprotrophic capabilities of MFRE (Field, Rimington, et al., 2015) and their assumed ability to access and transfer N from organic sources to plants—albeit only tested so far with non-vascular plants (e.g., liverworts; Field et al., 2019)—support the hypothesis that MFRE have a complementary role in plant nutrition alongside AM fungi, with potentially important ecological implications for plant community structure and nutrient cycling, especially with regard to N (Field et al., 2019; Howard et al., 2022; Hoysted et al., 2019, 2023).

In plants, N is a key component of the most abundant proteins and pigments, such as RuBisCO and chlorophyll; in fungi, it is needed for hyphal proliferation and the production of nutrient transporters in membranes (Howard et al., 2022). Nitrogen is present in soils in concentrations that can range from 0.39 to 32 g kg^{-1} depending on edaphic factors (Bronson, 2008). The two main inorganic forms of N are ammonium (NH_4^+) and nitrate (NO_3^-), which usually account for ~10% of total soil N (Bronson, 2008; Leghari et al., 2016) and are thus prone to becoming depleted and consequently limiting plant growth. Organic sources of N (e.g., amino acids, peptides and proteins) on the other hand are abundant, comprising as much as >95% of the total soil N in some ecosystems (Näsholm et al., 2009; Stams et al., 1990; Stein & Klotz, 2016). Plants are able to take up NH_4^+, NO_3^- and some amino acids directly into their roots (Ganeteg et al., 2017; Näsholm et al., 2001), but tend to rely on microbial processing of complex organic compounds and materials prior to their assimilation (Stein & Klotz, 2016).

Associations with MFRE that have saprotrophic capabilities could offer host plants enhanced access to organic soil N sources beyond that afforded by associations with AM fungi alone (Field et al., 2019; Howard et al., 2022). Given recent reports that these two groups of fungi proliferate under different edaphic conditions (Albornoz et al., 2021, 2022; Mansfield et al., 2023), it is important that we learn more about their respective and combined roles in plant N acquisition as this will allow us to better predict how land management may affect plant community reliance on fungal acquired N in both managed and natural habitats. Agricultural run-off mitigation (Juncal et al., 2023) and crushed silicate rock applications to land for improved carbon sequestration (e.g., Kantzas et al., 2022) are two land management strategies that likely have environmental effects beyond those intended. These effects may include altering the edaphic conditions of nearby habitats, and in turn, potentially affecting fungal community composition. While we may be able to quantify changes in the presence and abundance of different fungal

groups in these non-target habitats, coupling these data with a solid understanding of the functionality of different plant–fungal associations provides more insight into how plant communities may be impacted. Understanding the roles of AM fungi and MFRE in more detail is also necessary for critical interpretation of mycorrhizal research experiments that do not consider the potential presence of MFRE in unpasteurised soil (e.g., Hartman et al., 2023; reviewed in Orchard, Hilton, Bending, et al., 2017; Sinanaj et al., 2021). A better understanding of MFRE functionality from an ecological perspective (i.e., in the presence of other rhizosphere biota) will promote a more holistic understanding of the outcomes of plant–fungal interactions.

While functional complementarity between AM fungal species has been reported in *Medicago truncatula* and leek colonised by up to three AM fungal species: *Glomus mosseae (=Funneliformis mosseae)*, *G. claroideum (=Entrophospora claroidea)* and *G. intraradices (=Rhizophagus irregularis)* (Jansa et al., 2008), plant host benefits from co-colonisation with mycorrhizal fungi of two distinct fungal clades, for example, AM fungi and MFRE, remains an important knowledge gap. Using the grass *Holcus lanatus* (Yorkshire Fog) which hosts AM fungi and MFRE and is common across European grasslands (Ayling et al., 2021; Hoysted et al., 2019; McGonigle & Fitter, 1990), we tested the effect of a diverse symbiotic fungal community on plant growth and assimilation of fungal acquired N from a range of inorganic and organic sources in soil-based systems, and compared this effect with that elicited by a community with AM fungi only. We hypothesised that (i) the presence of MFRE in the fungal community benefits *H. lanatus* by increasing its assimilation of fungal acquired N, which in turn positively impacts plant growth; and (ii) *H. lanatus* receives more fungal acquired N from organic sources when colonised by a diverse symbiotic fungal community containing MFRE, than with an AM fungal community.

2 | MATERIALS AND METHODS

2.1 | Replication statement

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Plant	Plant	6 for each combination of fungal inoculation treatment × ^{15}N-labelled nutrient source
Plant	Plant	48 for plant morphological and physiological traits per fungal inoculation treatment

2.2 | Symbiotic fungal inocula

Soil was sourced in March 2020 from a long-term (c. 20 years) pasture at the University of Leeds Farm, UK (53°52′29″ N and 1°19′23″ W) where MFRE fungi have been previously molecularly and morphologically detected (Sinanaj et al., 2021). The soil was dry sieved through a 2-mm sieve to remove rocks and root fragments, before being wet sieved through sieves of pore sizes 106 and 25 μm using a sieve shaker (AS 200 control, Retsch, Germany) as per Sinanaj et al. (2021). The 106–25 μm soil fraction, which excludes most, but not all, AM fungal spore diameters (Albornoz et al., 2020), was collected and dried in an oven at 40°C to produce a soil inoculum enriched in MFRE fungal propagules (Sinanaj et al., 2021), hereafter referred to as the MFRE+AM fungi soil inoculum. The sieving step ensured MFRE were well-represented in our MFRE+AM fungi treatment.

Commercial AM fungal inoculum (PlantWorks, UK) containing a mix of AM fungal species (*Rhizophagus intraradices*, *Glomus microaggregatum*, *Diversispora* spp., *Funneliformis mosseae*, *Funneliformis geosporum* and *Entrophospora claroidea*) was used to inoculate the AM fungal community pots. This inoculum was selected to provide germinating seedings with the option of associating with a range of AM fungal symbionts that occur across UK and European grasslands (Zubek et al., 2022), while reducing the risk of contaminant MFRE propagules, which can be high in AM fungal inoculum derived from wild soil. Differences in the nutrient levels and bacterial communities in the carrier matrix/soil of the two inocula were controlled for across pots (Figure 1a). This was done by supplementing the AM fungal community pots with autoclaved MFRE+AM fungi soil inoculum, and 4 mL of a microbial wash obtained from 50 g of fresh MFRE+AM fungi soil inoculum suspended in 1X phosphate buffered saline (PBS) and passed through a 11 μm pore size filter paper (Whatman, UK); similarly, the MFRE+AM fungi pots were supplemented with autoclaved AM fungal inoculum, along with 4 mL of a microbial wash obtained from 50 g of fresh AM fungal inoculum using PBS and filtration (see Appendix S1).

Our aims in this experiment were to compare the ecologically relevant functional significance of single-clade and multi-clade fungal communities. *Holcus lanatus* without mycorrhizal symbionts is atypical (Ayling et al., 2021; McGonigle & Fitter, 1990); therefore, we did not include a non-mycorrhizal treatment in our experiment. We also did not include an MFRE-only inoculation treatment, as MFRE rarely colonise plants in the absence of AM fungi (Orchard, Standish, Dickie, et al., 2017), with only a few notable exceptions (e.g., the liverwort *Treubia lacunosa* and the lycophyte *Lycopodiella inundata*) (Bidartondo et al., 2011; Hoysted et al., 2019).

2.3 | Assembly of pot microcosms

A total of 48 plastic pots (80 × 80 × 85 mm), disinfected with a 1% solution of RELY+ON™ VIRKON™ (UK), were set up for each of the two fungal inoculation treatments (Figure 1c). For the MFRE+AM fungi treatment, MFRE+AM fungi soil inoculum was mixed with autoclaved sand at a ratio of 1:81, as per Albornoz et al. (2020), before being supplemented with autoclaved AM fungal inoculum and microbial wash to produce the final pot substrate (Figure 1a). For the AM fungal community treatment, 10 g of commercial AM fungal

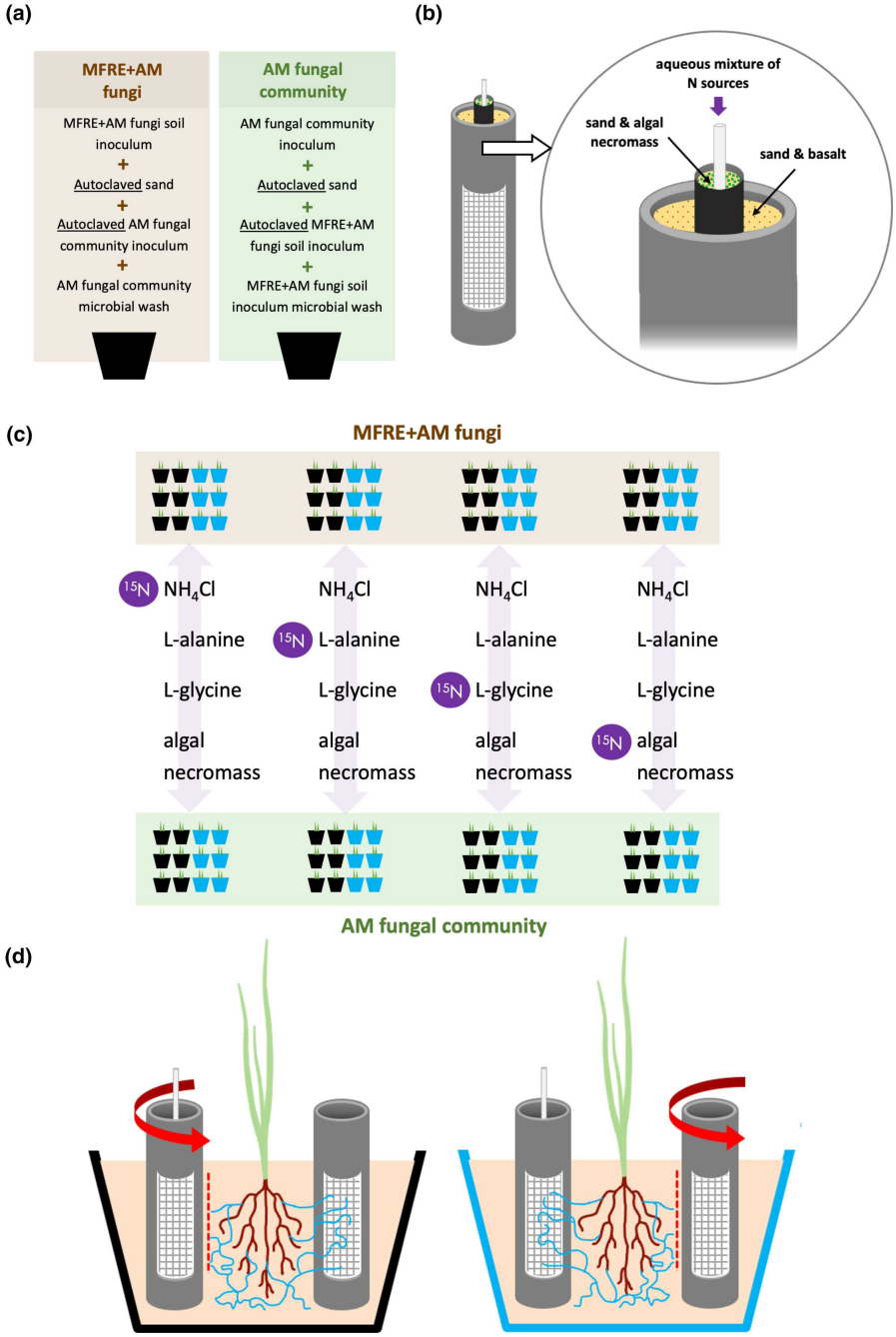

FIGURE 1 Experiment set-up detailing the substrates and nitrogen (N) sources made available in the pot microcosms of each fungal treatment. (a) Substrate composition of each pot treatment. (b) Assembly of the custom-made plastic core containing organic and inorganic N sources. The purple arrow indicates the opening of the capillary tube where the aqueous N-containing compounds are pipetted into once the straw (black cylindrical barrier) is removed to expose the algae-coated sand within the core. (c) Schematic detailing the number of pots per fungal inoculation treatment and per ^{15}N-labelled nutrient source made available to the fungal communities. Fungi were able to access the N sources in half of the pots (light blue). The other half of the pots (black) served as controls where fungal access to the sources was prevented. (d) Pots with static (right) and rotated (left) cores containing isotope tracers. Fungal hyphae are shown in light blue; red arrows indicate the cores that are rotated 90°.

inoculum was diluted in 1 kg autoclaved sand, as recommended by the supplier, then combined with autoclaved MFRE+AM fungi soil inoculum and the corresponding microbial wash to produce the final substrate (Figure 1a).

Two custom-made cylindrical plastic cores covered with 10 μm nylon mesh, fine enough to prevent root ingrowth but coarse enough to allow penetration by mycorrhizal fungal hyphae, were inserted at equidistant positions within each pot (Figure 1b). Through

these cores, ^{15}N-labelled and unlabelled inorganic and organic nutrient sources were made available to the fungi after 8 weeks, subsequently allowing the quantification of the flux of fungal acquired ^{15}N to plants (Field et al., 2019; Figure 1d). One of the two cores contained a perforated capillary tube inserted centrally within a plastic straw, which was filled with 1.6 g of sand coated in freeze-dried *Chlorella vulgaris* (strain CCAP 211/21A), that is, an algal necromass organic nutrient source. The algal necromass was prepared by culturing *C. vulgaris* in either ^{15}N-labelled or unlabelled media, followed by freeze-drying and mixing with autoclaved sand. The ^{15}N-labelled algae-coated sand was diluted with unlabelled algae-coated sand to 1.79%, before being used as a labelled organic N source (Appendix S2). The tubing in the core was for the delivery of the aqueous N sources (ammonium chloride, L-glycine and L-alanine), while the straw served to minimise the exposure of the algal necromass to degradative microorganisms within the pot substrate, until the moment the N sources were made available in the microcosms. Cores were filled with a mixture of autoclaved sand and 1% fine ground tertiary basalt (Oregon, USA) to encourage fungal ingrowth (Quirk et al., 2012).

Holcus lanatus seeds (Emorsgate Seeds, UK) were surface sterilised in 5% sodium hypochlorite for 2 min and washed three times in sterile dH_2O. The seeds were sown generously into the pots and, upon germination, were thinned to no more than five seedlings per pot.

2.4 | Plant growth conditions and morphological measurements

Plants were grown in a controlled environment chamber (MicroClima 1000, Snijders Labs, The Netherlands) with a 16-h photoperiod, 20°C day temperature, 15°C night temperature, 70% relative humidity and 350 μmol^{-1} irradiance. Pots were watered every three days from above with 30 mL reverse osmosis (RO) water and repositioned regularly within the chamber to control for block effects. The number of leaves and tillers of individual plants within each pot were recorded weekly, as well as the maximum canopy height for each pot. The mean tiller number and leaf number in each pot was calculated and used in downstream statistical analyses. Chlorophyll content was measured just above the midpoint of the longest leaf in each pot once leaves were large enough to be examined using a SPAD meter (Minolta, Japan).

2.5 | Addition of nitrogen sources

After 8 weeks, inorganic (ammonium chloride) and organic (L-glycine, L-alanine and algal necromass) N sources were made available to the fungi in the pots. Ammonium chloride was chosen over other inorganic sources because ammonium ions (NH_4^+) are less prone to leaching (Stein & Klotz, 2016). The organic N sources were chosen to represent the complexity of organic N in soil (Näsholm et al., 2009). Both L-glycine and L-alanine have been implicated in AM fungi symbioses as compounds that are directly transferred from fungus to plant (Cappellazzo et al., 2008; Hawkins et al., 2000) while algal necromass was previously used in studies on AM fungi and MFRE symbioses as a tractable representative of complex organic matter in soil (Field et al., 2019; Thirkell et al., 2016).

Before the N sources were made available, the presence of AM fungi and MFRE was confirmed in the roots of *H. lanatus* grown alongside the experimental plants under the same conditions, using acidified ink staining (Vierheilig et al., 1998) (Figure S1). Each experimental pot received all four N sources; however, only one source was ^{15}N-labelled, while the other three were non-labelled to allow fungal preference for inorganic and organic N to be explored (Figure 1c). The ^{15}N-labelled and non-labelled algal necromass, which was added during microcosm assembly, was exposed to microbes and fungi within the core by careful removal of the plastic straw barrier in the cores (as in Field et al., 2019). 100 μL of a 1 mg mL^{-1} aqueous mixture containing the other three N sources (combined to result in equal numbers of N atoms from each compound) was subsequently pipetted into the capillary tube of these cores, totalling 0.0206 mg N per pot (see Appendix S2). Concurrently, 10 mL of 0.2X strength Hoagland solution, totalling 0.409 mg N, was added to the centre of each pot as minimal fertiliser (Appendix S3).

There were 12 pots for each of the eight combinations of ^{15}N-labelled source × fungal inoculation treatment (Figure 1c). In half of these pots, the core containing the ^{15}N isotope tracer was rotated 90° every three days to sever hyphal connections between its contents and *H. lanatus* (Figure 1d). The rotated cores served as a control for distinguishing between ^{15}N acquired by plants through fungi and ^{15}N uptake by plants as a result of diffusion of the tracer out of the core and/or alternative microbial N cycling processes (Johnson et al., 2001; Leifheit et al., 2014).

2.6 | Harvest and quantification of fungal acquired ^{15}N in plant tissues

Pots were harvested 3 weeks after the ^{15}N and N sources were made available (as per Field et al., 2019). Plant shoots, plant roots, bulk soil and core soils were separated, weighed, sampled and freeze-dried. Sub-samples (~2 g) of fresh soil were used to carry out soil hyphal extractions, followed by quantification of hyphal length densities using Trypan blue staining and light microscopy at 400× magnification (Brundrett et al., 1994). Fresh samples of primary and lateral roots, collected from across the root systems in each pot, were stored in 70% ethanol at 4°C (Orchard, Standish, Nicol, et al., 2017) prior to staining with acidified ink and quantification of the % total root length (%TRL) colonised by fungi using light microscopy. Values for %TRL colonisation were obtained by inspecting ~100 Fields of View (FOVs) of stained roots per pot.

The amount of ^{15}N in freeze-dried plant shoot and root material was determined by weighing out 1.5–4 mg homogenised

plant material into 6 × 4 mm^2 tin capsules (Sercon Ltd. Irvine, UK) for analysis with continuous-flow mass spectrometry (PDZ Europa 2020 IRMS, Sercon Ltd. Irvine, UK). A published equation using ^{15}N atom % values from the mass spectrometry output (Field et al., 2016; see Appendix S4) was used to calculate fungal acquired ^{15}N in the *H. lanatus* samples. The average amount of ^{15}N in the plants without fungal access to the ^{15}N tracer (i.e., pots with the tracer in the rotated core) was first calculated. This average was then subtracted from the amount of ^{15}N in each plant with intact fungal connections to the core containing the ^{15}N tracer, resulting in fungal acquired ^{15}N and accounting for non-symbiotic fungal mediated movement of ^{15}N.

2.7 | Statistical analyses

Statistical analyses were performed using the software package SPSS Version 26 (IBM Analytics, New York, USA). Data were checked for homogeneity and normality. Depending on these properties, an independent samples Student's *t*-test or a Mann–Whitney *U* test was used to analyse the effect of fungal treatment on *H. lanatus* morphological features, chlorophyll content, biomass and total N content in shoots and roots. The same tests were used to analyse the fungal composition of the substrate and plant roots, as well as the relative growth rate (RGR) of *H. lanatus*, which was calculated using the maximum canopy height data (Appendix S4). Data collected from all pots ($n = 96$) were used to analyse the above variables, except for fungal composition of substrate and plant roots, which were analysed using data obtained from half of the pots ($n = 48$).

To investigate the effect of symbiotic fungal community on the development of *H. lanatus* after the N sources were made available in the pots, the percentage change in dependent variables that were measured 8 weeks of post-sowing and 11 weeks of post-sowing was calculated and analysed using an independent samples Student's t-test for parametric data, and a Mann–Whitney *U* test for nonparametric data. Data from pots where fungi had access to the N sources were used ($n = 48$). The fungal acquired ^{15}N tracer data were analysed using a one-way analysis of variance (ANOVA) or, where assumptions for this test were not met, Kruskal–Wallis tests with post hoc tests. Each combination of ^{15}N-labelled source x fungal inoculation treatment was represented by six data points.

3 | RESULTS

3.1 | Fungal community composition within pots at harvest

At harvest, the MFRE+AM fungi pots that had access to the core containing the ^{15}N and N sources ($n = 24$) had substrate with extraradical fungal hyphae, of which 73.4% ± 2.3 (mean ± SE) were MFRE and 26.6% ± 2.3 were AM fungi. The fungal hyphal densities (m hyphae g^{-1} dry substrate) in these pots reflected the % composition data, as fine MFRE hyphae were more abundant than coarser AM fungal hyphae ($U = 79$, $p < 0.001$; Figure S2A). However, in roots of *H. lanatus* sampled from these pots, the % total root length (%TRL) colonised by MFRE versus AM fungi was not significantly different ($U = 225.5$, $p = 0.192$) (Figure S2C). Only coarse AM fungal hyphae were observed in the roots and substrate of the AM fungal community pots ($n = 24$; Figure S2B,D). While the fungal community composition within the pots was consistent with the inoculation treatment applied, the roots sampled generally exhibited relatively low fungal colonisation, resulting in %TRL colonisation values that ranged between 0 and 19.4% in the MFRE+AM fungi pots, and between 0 and 8.3% in the AM fungal community pots (Figure S2D).

3.2 | *Holcus lanatus* above-ground traits are affected by the composition of fungal symbionts within the rhizosphere

There were striking morphological differences between *H. lanatus* grown in the two fungal inoculation treatments before N sources were made available within the pots (Figure 2a). Plants in the MFRE+AM fungi pots had fewer tillers ($U = 378.5$, $p < 0.001$), fewer leaves ($U = 347$, $p < 0.001$), and a reduced maximum canopy height per pot ($U = 220$, $p < 0.001$), compared to plants in the AM fungal community pots. The chlorophyll content and relative growth rate (RGR) of plants grown in the MFRE+AM fungi soil inoculum were also lower ($t = 3.522$, df = 94, $p = 0.001$; and $t = 3.958$, df = 94, $p < 0.001$; respectively).

Three weeks after the N sources were made available in the pots, some of the fungal inoculation-linked differences had disappeared (Figure 2a). There were no significant differences in leaf number per plant ($U = 231.5$, $p = 0.242$) or chlorophyll content ($t = -1.036$, df = 46, $p = 0.306$). Three weeks following the addition of the N sources, the % change in leaf number, maximum canopy height, chlorophyll content and RGR was significantly greater in the MFRE+AM fungi pots (Figure 2b–e). The % change in tiller number was similar across treatments (Figure S3). Despite the rapid development of *H. lanatus* in the MFRE+AM fungi pots after introduction of the N sources, the plants in the AM fungal community pots had a greater shoot and root biomass at harvest, and also a greater root-to-shoot ratio (Figure 3).

3.3 | Fungal transfer of inorganic and organic ^{15}N tracer to *H. lanatus* by symbiotic fungi

Total shoot nitrogen (N)—the sum of the N taken up directly by *H. lanatus* roots and fungal acquired N—was significantly greater in the AM fungal community pots in terms of absolute values (Table 1; Figure 4a), while total shoot N concentration was similar between treatments (Table 1; Figure 4b). Plant roots in the AM

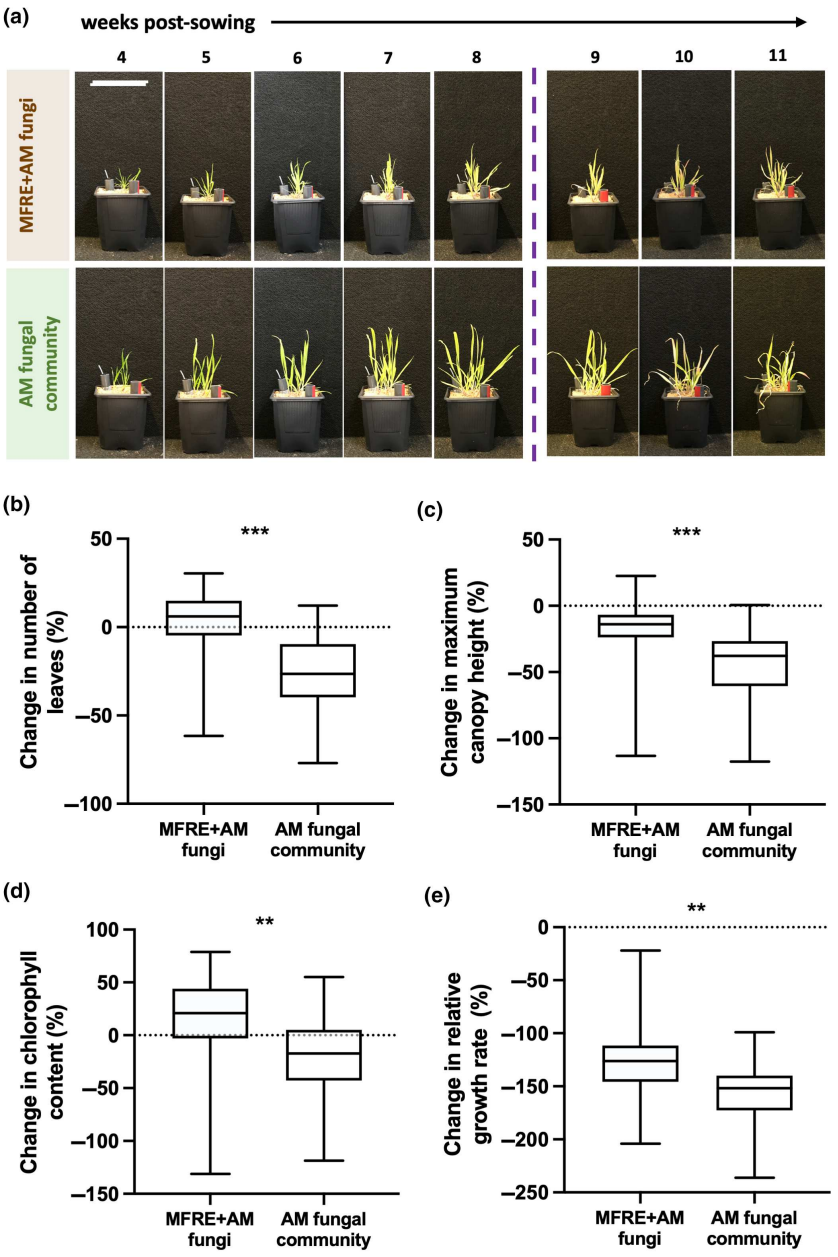

FIGURE 2 Development of *Holcus lanatus* grown in two fungal treatments. (a) Representative MFRE+AM fungi and AM fungal community pot microcosms after 4–11 weeks of plant growth. The dashed line indicates the point when N sources were made available to the fungi in half of the pots. (b) Percentage change in the number of leaves per plant, (c) pot maximum canopy height, (d) chlorophyll content of the longest leaf in each pot, and (e) relative growth rate per pot ($n = 48$), over the 3 weeks after the introduction of the N sources. ** and *** indicate a significant difference (p-value <0.01 and p-value <0.001, respectively).

fungal community pots had more total N in terms of absolute values; however, roots in the MFRE+AM fungi pots had a higher N concentration (Table 1; Figure S4A,B).

Fungal transfer of ^{15}N tracer to *H. lanatus* occurred in both treatments across pots with inorganic and organic ^{15}N-labelled sources (Table 1; Figure 4c,d). Fungal acquired ^{15}N across the different pots was comparable in terms of absolute quantities in plant shoots and roots, as well as when normalised to shoot biomass and root biomass (Table 1; Figure 4c,d; Figure S4C,D).

4 | DISCUSSION

Unravelling the extent to which diverse symbiotic fungal clades affect plant growth is crucial for a holistic understanding of the significance of mycorrhizal symbioses in the structure and function of plant communities across ecosystems. We aimed to identify potential differences in host plant assimilation of N from various nutrient sources and the ensuing effects on plant growth when the host plant was grown in the presence of two contrasting fungal communities. Under our experimental conditions, fungal acquired ^{15}N tracer in leaf tissues at harvest was similar across all plants, irrespective of the available N source, suggesting that the presence of MFRE alongside AM fungi did not enhance fungal transfer of N to plants. We were not able to discern whether MFRE or AM fungi were responsible for supplying plants with ^{15}N in the MFRE+AM fungi pots; however, given the similar %TRL colonisation by each fungal symbiont, and recent research confirming direct N transfer from MFRE to a vascular plant in vitro (Hoysted et al., 2023), it is likely that both fungal symbionts contributed to the N nutrition of *H.*

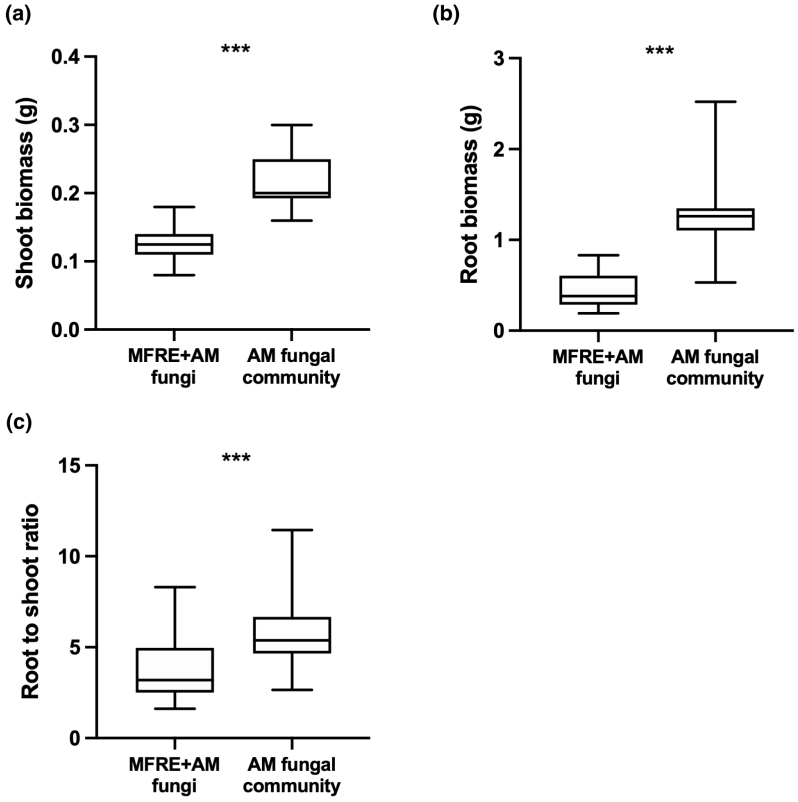

FIGURE 3 Harvested biomass (dry) and root-to-shoot ratio of *Holcus lanatus* in pots where the fungal community had direct access to the N sources. (a) Shoot biomass, (b) root biomass and (c) root-to-shoot ratio in the pots of each fungal treatment ($n = 24$). *** indicates a significant difference ($p < 0.001$).

lanatus in our experiment. We observed striking differences in plant morphological and physiological characteristics between the two fungal inoculation treatments. Plants from the MFRE+AM fungi pots had a lower biomass at harvest and developed fewer above-ground structures during early growth, compared with plants from the AM fungal community pots. This suggests that there were imbalances in nutrient exchange between *H. lanatus* and its fungal symbionts over the course of the experiment. Our data do not support our hypothesis that the presence of MFRE in the fungal community benefits *H. lanatus* by increasing its assimilation of fungal acquired N, but instead paint a more complex picture.

While functional complementarity between AM and MFRE symbioses in terms of host plant nutrition (Field et al., 2019) may exist in grasses, the broader impact of AM fungi and MFRE co-colonisation on plant performance and development is important to consider. At harvest, N content and biomass were greater in plants grown with the AM fungal community. The large root biomass and root-to-shoot ratio of these plants indicate that over the course of the experiment they invested more resources in the expansion of their root system, potentially to increase nutrient foraging and uptake via both the root and mycorrhizal pathways (Smith & Read, 2008). Per cent TRL colonisation by AM fungi was relatively low in these pots; therefore, it is possible that direct nutrient uptake into roots was the primary driver for above-ground plant growth. It follows that the larger amount of shoot N in *H. lanatus* from the AM fungal community pots compared with MFRE+AM fungi pots is linked to the larger plant biomass at harvest. The N concentration of shoots in both treatments was not statistically significant, suggesting that N was not limiting at harvest. Nevertheless, the smaller size of plants from the MFRE+AM fungi pots brings into question whether they experienced stress during their development and whether they were more N limited than their AM fungal community counterparts during early growth.

Rate of host plant growth throughout the experiment was related to the fungal inoculation treatment. Plants associating with both MFRE and AM fungi initially appeared stunted when compared to plants associating with only AM fungi. The smaller number of leaves and tillers, and the lower chlorophyll content of the plants in the MFRE+AM fungi pots prior to the addition of N sources suggest that the presence of MFRE in soil limits early plant growth and development in low nutrient substrates like the sand-based one of this experiment. Although the presence of MFRE did not appear to have a positive influence on the relative growth rate of *H. lanatus* in the early stages of the experiment, this changed once the N content in the pots was increased through the addition of the diverse N sources and Hoagland's solution. Following nutrient addition, leaf number, maximum canopy height and chlorophyll content all increased more for the plants in the MFRE+AM fungi pots compared to those in the AM fungal community pots during the 3 weeks prior to harvest. Given that these variables are indicative of plant N usage, this implies that the presence of MFRE improved N nutrition of *H. lanatus* during this specific period. This may have been either through MFRE contributing directly to N transfer to plants (Hoysted et al., 2023) after their own fungal N demand was met, MFRE releasing N stored in their fungal structures into plant roots, or MFRE indirectly

TABLE 1 Summary of statistical tests carried out on data for total and fungal acquired nitrogen in *Holcus lanatus* at harvest.

Variable tested	Test type	*p*-value	Result
Total shoot nitrogen (absolute)	Student's *t*-test; $t=6.721$, df = 46	<0.001	Significantly greater in AM fungal community pots
Total shoot nitrogen (concentration)	Mann–Whitney *U* test; $U=231.5$	0.244	Similar between fungal treatments
Total root nitrogen (absolute)	Mann–Whitney *U* test; $U=169$	0.014	Significantly greater in AM fungal community pots
Total root nitrogen (concentration)	Student's *t*-test; $t=-4.985$, df = 46	<0.001	Significantly greater in MFRE+AM fungi pots
Fungal acquired ^{15}N in shoots grown in MFRE+AM fungi pots (absolute)	ANOVA; $F=0.646$, df = 3	0.595	Similar amount regardless of type of nitrogen tracer
Fungal acquired ^{15}N in shoots grown in AM fungal community pots (absolute)	Kruskal–Wallis; $H=7.217$, df = 3	0.065	Similar amount regardless of type of nitrogen tracer
Fungal acquired ^{15}N in roots grown in MFRE+AM fungi pots (absolute)	Kruskal–Wallis; $H=1.041$, df = 3	0.791	Similar amount regardless of type of nitrogen tracer
Fungal acquired ^{15}N in roots grown in AM fungal community pots (absolute)	Kruskal–Wallis; $H=4.594$, df = 3	0.204	Similar amount regardless of type of nitrogen tracer
Fungal acquired ^{15}N in shoots grown in MFRE+AM fungi pots (concentration)	Kruskal–Wallis; $H=3.902$, df = 3	0.272	Similar concentration regardless of type of nitrogen tracer
Fungal acquired ^{15}N in shoots grown in AM fungal community pots (concentration)	Kruskal–Wallis; $H=8.447$, df = 3	0.038; non-significant Dunn–Bonferroni post hoc test	Similar concentration regardless of type of nitrogen tracer
Fungal acquired ^{15}N in roots grown in MFRE+AM fungi pots (concentration)	Kruskal–Wallis; $H=2.937$, df = 3	0.401	Similar concentration regardless of type of nitrogen tracer
Fungal acquired ^{15}N in roots grown in AM fungal community pots (concentration)	Kruskal–Wallis; $H=4.332$, df = 3	0.228	Similar concentration regardless of type of nitrogen tracer

Note: Significant *p*-values are highlighted in grey.

enhancing N transfer through synergistic interactions with AM fungi and other microbes (e.g. recruitment of N-mineralising rhizospheric bacteria) (Faghihinia et al., 2023).

Several mechanisms may have resulted in the differences in plant growth-related traits observed between the fungal treatments over the course of the experiment. Competition between the soil fungal communities and *H. lanatus* for the limited pool of macronutrients is likely to have been fierce within our pots (van der Heijden et al., 2008), especially during the first 8 weeks of the experiment when no fertiliser was applied to the substrates. Isotope tracing experiments with a similar experimental design and also using monocotyledonous species (e.g., different cultivars of wheat) colonised by AM fungi, feature weekly applications of a nutrient solution to pots to facilitate plant growth (Elliott et al., 2021; Thirkell et al., 2020). Here, fertiliser was purposely not applied in the early stages of the experiment to encourage colonisation and host plant reliance on fungal symbionts for nutrient acquisition once plant-accessible nutrients were depleted. Despite this, the MFRE may have relied more on a saprotrophic mode of nutrition (Field, Rimington, et al., 2015) than on nutritional mutualism with *H. lanatus*, consequently, reducing the available pool of nutrients to AM fungi and plants, resulting in less above-ground biomass production for the latter.

The saprotrophic capabilities of MFRE may thus represent a double-edged sword to plants in the context of plant-MFRE symbioses; MFRE are likely to liberate nutrients from a range of plant-inaccessible sources, but they may only share these nutrients with their host plants under select environmental conditions. It is likely that MFRE are more symbiotic in high N environments (Albornoz et al., 2022) where their fungal N demand does not become a limitation to plant assimilation of N. This has ecological implications for plant community structure, as plant species forming prolific associations with MFRE may be out-competed by other plant species associating with different symbionts in N-limited environments

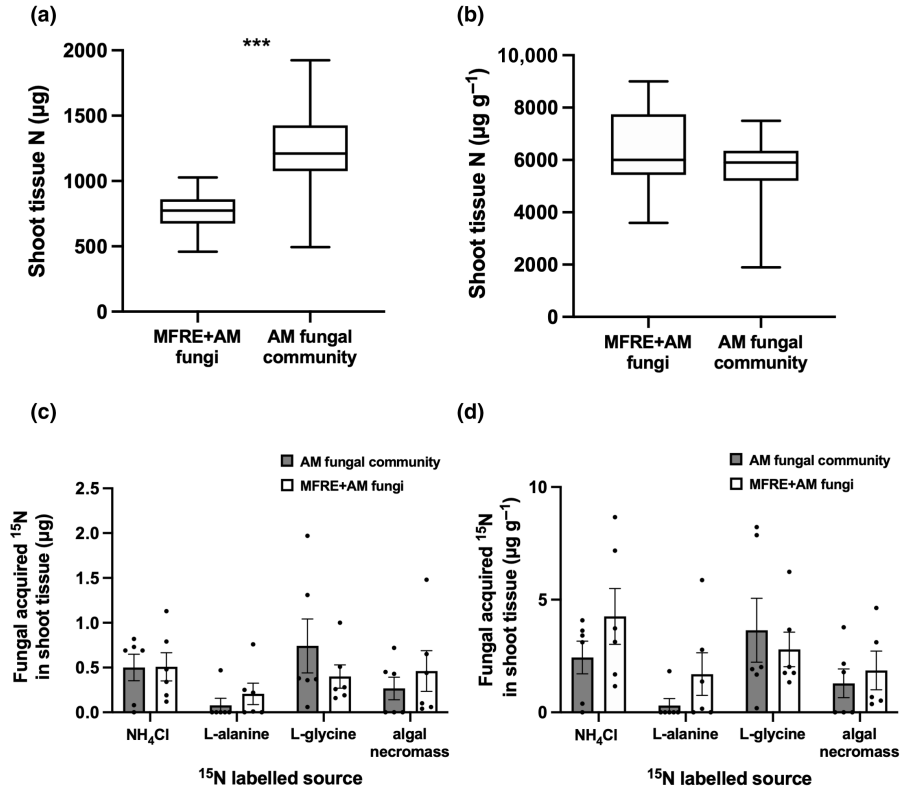

FIGURE 4 Nitrogen (N) content within *Holcus lanatus* shoots at harvest. (a) Mass and (b) concentration of total N for the pots of each treatment where the fungal community had direct access to the added N sources ($n = 24$). *** indicates a significant difference (p-value <0.001). (c) Mass and (d) concentration of fungal acquired ^{15}N from pots of each treatment supplied with either an inorganic or organic ^{15}N-labelled source ($n = 6$).

where MFRE may switch to their saprotrophic mode of nutrition. A mechanism to explain several cases of growth depression reported in plants colonised by mycorrhizal fungi involves plant suppression of direct P uptake in anticipation of fungal acquired P that is ultimately not received, especially due to low root colonisation (Smith et al., 2009). This mechanism, which is likely to extend to the uptake of other nutrients including N, may have been at play in *H. lanatus* grown in the MFRE+AM fungi pots. The MFRE may have colonised enough root to elicit strong downregulation of genes coding for plant transporters involved in direct nutrient uptake, for example, *AMT1;1* (Mayer & Ludewig, 2006), but failed to supply the plants with enough nutrients, leading to stunted growth. This is an example of the risk faced by plants when making significant developmental changes and resource investments to facilitate outsourcing of nutrient acquisition to symbionts. On the other hand, if MFRE are indeed more beneficial to plants under high N environments, then dual plant associations with MFRE and AM fungi may offer some plants increased plasticity to shifts in abiotic conditions.

Another factor that may have contributed to the inoculation-linked plant growth differences in our experiment is the role of MFRE in relation to plant phenology. Hoysted et al. (2021) found that the nutritional function of MFRE symbioses in *Lycopodiella inundata* depends upon plant developmental stage, noting that juvenile *L. inundata* sporophytes exchange no nutrients with MFRE, while adult sporophytes transfer C to MFRE in exchange for P and N. A similar phenological interaction may have occurred between MFRE and *H. lanatus* in the MFRE+AM fungi pots. *Holcus lanatus* in its early growth stages may have received no nutritional benefits from MFRE—or inadequate ones in terms of the C it may have supplied the fungi with—and may have experienced growth depression due to competition for nutrients with the partially saprotrophic MFRE. Older *H. lanatus* plants, capable of photosynthesising more, may have benefited from MFRE colonisation due to their potentially greater abundance and subsequent allocation of photosynthates to the fungi in exchange for nutrients. However, this scenario is speculative, given that the addition of N sources and fertiliser is likely to have been the key driver of inoculation-linked plant growth differences at the later growth stages of *H. lanatus*. Nevertheless, the possibility that the developmental stage of *H. lanatus* determines whether it benefits from nutrient exchanges with MFRE brings into question whether this could be the case in other MFRE host plants; after all, changes in mycorrhizal functioning across plant lifecycles are known, for example, the symbiosis between AM fungi and the fern *Ophioglossum vulgatum* (Field, Leake, et al., 2015). The link between plant phenology and the symbiotic function of MFRE and AM fungi has implications at the ecosystem level, as it is likely to influence seedling

establishment in competitive environments. Together with the ability of mycorrhizal fungi to divert nutrients to plant species that provide the most benefits (Müller, 2021), the activity of symbiotic fungal communities is critical in shaping plant communities.

In both fungal inoculation treatments, the type of labelled N source made available did not result in significant differences in the amount of fungal acquired ^{15}N tracer detected in shoots across plants at harvest. As such, our second hypothesis, that *H. lanatus* receives more fungal acquired N from organic sources when colonised by a diverse symbiotic fungal community containing MFRE, is not supported. Although no fungal preference for nutrient sources was observed, this may have been a result of the length of time that the fungal communities were exposed to the N sources. Had access to the N sources been extended beyond 3 weeks, a preference may have emerged and more ^{15}N tracer may also have been detected in plant shoots. A final aspect to consider is the potential differences in the AM fungal taxa between the fungal inoculation treatments. The commercial AM fungal inoculum that was used contained species, such as *Funneliformis mosseae* (formerly *Glomus mosseae*), which are present in grasslands and are known to colonise and provide benefits to *H. lanatus* (Gonzalez-Chavez et al., 2002; Zubek et al., 2022). However, we are not certain whether the abundance of these species was similar in the MFRE+AM fungi soil inoculum. While the focus of our experiment was at the subphylum level (Mucoromycotina vs. Glomeromycotina), it is important to note that interactions between different AM fungal species can impact symbioses with plant hosts (Cope et al., 2022; Jansa et al., 2008). Future research exploring the functionality of fungal symbioses with MFRE and AM fungi at a finer species scale using fungal cultures as inoculum in controlled systems (Hoysted et al., 2023) and using soil fungal communities that have been genetically, metabolically and physiologically characterised, will be necessary to build on the results of our experiment and further the mycorrhizal field.

5 | CONCLUSIONS

Our findings demonstrate a differential response of the common European grass species *H. lanatus* to contrasting multi-clade and single-clade fungal communities. We show that the function and impact of these multi-symbiont interactions are dynamic and may depend on soil nutrient availability, competition and potentially plant phenology. The combination of MFRE with AM fungi may be detrimental to the growth of *H. lanatus* under low nutrient conditions or during early growth; however, this appears to be ameliorated by increasing availability of soil N. Further research is needed to define the nutrient thresholds and plant developmental stages under which MFRE provide net nutrient and growth benefits to host plants—particularly in multi-symbiont scenarios. This will be key to fully understanding the impact of ecologically-relevant, complex symbiotic fungal communities in shaping plant community structure and function.

AUTHOR CONTRIBUTIONS

BS, KJF, SP and MIB conceived and designed the investigation. BS undertook the experiments, analysed the results and led the writing; all authors discussed results and commented on the manuscript. BS and KJF agree to serve as the authors responsible for contact.

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CONFLICT OF INTEREST STATEMENT

Katie Field is a senior editor of *Functional Ecology*, but took no part in the peer review and decision-making processes for this paper. The authors declare no other conflict of interest.

DATA AVAILABILITY STATEMENT

Data deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.pc866t1ww (Sinanaj et al., 2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1: Fungal morphologies in arbuscular mycorrhizal fungal community pots and MFRE+AM fungi pots.

Figure S2: Fungal symbiont data for the pot substrates and *Holcus lanatus* roots at harvest.

Figure S3: Percentage change in the number of tillers per plant after the addition of the N sources.

Figure S4: Nitrogen (N) content within *Holcus lanatus* roots at harvest.

Appendix S1: Preparation of the bacterial filtrates added to the pot substrates.

Appendix S2: Preparation of the algal necromass and aqueous N sources added to cores.

Appendix S3: Recipe for Hoagland's solution added to pots after 8 weeks of plant growth.

Appendix S4: Equations for calculating fungal acquired labelled N in plant tissues, and relative growth rate (RGR).

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