Functional significance of Mucoromycotina 'fine root endophyte' fungi in the nitrogen nutrition of host plants

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

The University of Sheffield
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Submission Date: 13/11/2024

Acknowledgements

First and foremost, I would like to thank my supervisors Professors Katie Field and Tim Daniell for their support and feedback, without which this thesis would have remained a jumble of notes and figures scattered between in my lab book and computer.

I'd also like to thank Dr Alex Williams and Emily Durant (the MFRE dream team) for persevering with me, and for taking the edge off some fairly gnarly times. You pair mean the world to me, (and who else could I convince to help out harvesting experiments?) I couldn't have asked for better lab partners (I tried but better ones were unavailable, you guys are pretty cool too though!).

I'm grateful for the comradery of the entire Field lab (and Daniell lab hangers on) past and present: Miles, Little Alex, Emily M, James, Pedzi, David, Izzy, Besiana, Grace H, Jacob, and Sara. To Lizzy Parker, I can only hope to one day repay your constant support and mentorship since I was a lowly undergrad looking for lab experience.

Thanks to my parents, Steve and Sasha, my grandparents, Liz and Brian, and Conroy and Leslie, for teaching me to love learning and always allowing me to be my best, you are not obliged to read any more of this thesis, just know it wouldn't exist without you, and I'm eternally indebted to you all.

Finally, I'd like to thank the most understanding and patient woman in the world, Natalie for keeping me grounded and simply making life worthwhile.

Abstract

Mycorrhizal associations between fungi and plant roots have globally significant impacts on terrestrial nutrient cycling. Mucoromycotina 'fine root endophytes' (MFRE) are a distinct group of mycorrhiza-forming fungi that associate with the roots of a range of host plant species. Previous misidentification and assignment as arbuscular mycorrhizal (AM) fungi of the Glomeromycotina – sister subphylum to the Mucoromycotina – has resulted in systematic under investigation of these fungi. Therefore, it is now important to establish how MFRE-plant symbioses function.

In this thesis I develop novel monoxenic microcosms, and non-sterile mesocosms that are used in experiments throughout my project. Monoxenic microcosms are first used to investigate the nutritional function of MFRE in the absence of a soil microbiome. I then use non-sterile mesocosms to investigate the effects of a soil microbiome on the nutrient dynamics between MFRE and *Plantago lanceolata.*

From these experiments I establish that in the absence of other soil microbes, MFRE preferentially assimilates nitrogen from glycine and ammonium chloride for transfer to *Plantago*. This occurs regardless of whether these sources of nitrogen are applied singly or in equal mixtures with other sources of nitrogen. I also determine that the availability of nitrogen to MFRE and host plants affects the amount of nitrogen and carbon exchanged between MFRE and *Plantago*. Under reduced inorganic nitrogen conditions, MFRE transports proportionately more nitrogen to *Plantago* hosts than when inorganic nitrogen is more available. This variation in nitrogen transfer from MFRE occurs concurrently with no alteration in the photosynthetic carbon transfer from *Plantago* to MFRE and a significant reduction in colonisation by MFRE of *Plantago* roots.

My research adds new nuance to our knowledge of MFRE symbiotic functions with plants. I present clear evidence that MFRE are functionally distinct from AM fungi. I show that MFRE have a significant capacity to assimilate organic compounds and utilise their carbon and nitrogen components differently, while altering its relative benefit to host plants.

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Declaration

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means. This work has not been previously presented for an award at this, or any other, university."

The introduction section includes work from – **Howard, N., Pressel, S., Kaye, R. S., Daniell, T. J., & Field, K. J. (2022).** The potential role of Mucoromycotina 'fine root endophytes' in plant nitrogen nutrition. *Physiologia Plantarum*, 174(3), e13715-n/a. https://doi.org/10.1111/ppl.13715

Contributions to the manuscript were as follows: KJF, SP and TJD conceived the concept and framing for the paper. NH and RAK wrote the first draft, SP constructed figures 1 and 2, NH drew figures 3 and 4, all authors commented and developed subsequent versions of the manuscript and approved the final version.

Chapters 3 and 4 of this thesis include work from – **Howard, N. O. A., Williams, A., Durant, E., Pressel, S., Daniell, T. J., Field, K. J. (2024).** Preferential assimilation, metabolism, and transfer of organic nitrogen to host plants by Mucoromycotina 'fine root endophytes'. *Current Biology*. (In press). https://doi.org/10.1101/2024.07.31.606011

Contributions to the manuscript were as follows: N.H. and K.J.F. conceived and designed the experiments; N.H., E.D., A.W., and K.J.F performed the experiments and laboratory analyses; N.H. analysed and interpreted the data; N.H. wrote the manuscript with contributions from all other authors. All authors revised and approved the final manuscript.

Chapter 1: General Introduction

1.1 Plants & Soil macronutrients

Plant growth and productivity is limited by availability of primary resources required for survival including light, water, and carbon (C) in the form of atmospheric CO_2. Plants also require micro- and macronutrients; often derived from the soil after microbial turn-over, and/or direct microbial acquisition via symbiosis. The macronutrients nitrogen (N) and phosphorus (P) are used for a variety of metabolic and structural functions in plants (Kraiser *et al.,* 2011; Plaxton & Lambers, 2015; Pallardy, 2008). For example, phosphate is essential for plant growth, especially the production of DNA, cell plasma membranes and ATP (Plaxton & Lambers, 2015; Li *et al.,* 2015). A large amount of phosphate present in soils is inaccessible to plants owing to the phosphate binding qualities of many soils (Plante, 2007) whereby phosphates are adsorbed to metal ions, such as Fe^{+} and Al^{+}, or clay particles, becoming biologically unavailable to plants (Xu *et al.,* 2019; Xiong *et al.,* 2022). P is also limited in many ecosystems, including both aquatic (van Donk *et al.,* 1989; Conley *et al.,* 2009) and terrestrial (Vitousek *et al.,* 2010).

Plants also require N for primary production of amino acids and secondary metabolites (Gupta, 2020). Both P and N macronutrients are each necessary for the assimilation of the other (Rufty *et al.,* 1990) and are thus inextricably linked. The phosphate starvation response (PSR) upregulates phosphate transporters and phosphatases to increase P assimilation while limiting plant shoor and root growth to reduce P demand (Nagatoshi *et al.,* 2023). This process is downregulated under N starvation (Ueda & Yanagisawa, 2019). Therefore, dual fertilisation with N and P synergistically increases plant productivity over fertilisation with only one element (Krouk & Kiba, 2020)

1.2 Nitrogen as a key macronutrient for plants

N is a key macronutrient in plant nutrition (Evans, 1989) with many inputs into soil systems (several examples in Figure 1.1). As a result of global soil N limitation (Vitousek

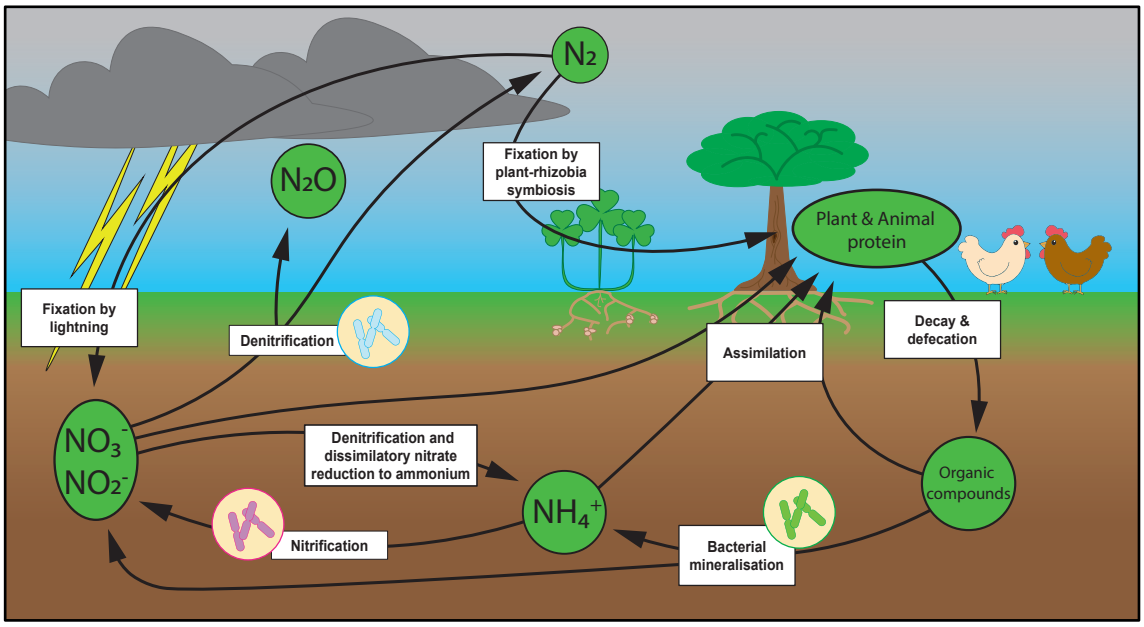

Figure 1.1 Inputs of different N sources into soils. Arrows indicate direction of N transformation. Green circles represent N pools. Constructed using findings of, Rao & Puttanna, 2000; Phelps, 2004; Powlson & Addiscott, 2005; Gioseffi et al., 2012; Andrews et al., 2013. This is not a full list of N inputs.

& Haworth, 1991), it is a key driving force in plant competition and evolution (Pankoke *et al.,* 2015; Kang *et al.,* 2015). N occurs within soils in both inorganic mineral (e.g., ammonium and nitrate salts; Matsumoto *et al.,* 2000) and organic forms (derived from plant, animal and microbial decay; Greenfield, 2001). The majority of inorganic N in soils is in the form of nitrate (NO_3^-; Powlson & Addiscott, 2005; Andrews *et al.,* 2013), most of which is derived from the aerobic metabolism of certain clades of soil bacteria and archaea (Rao & Puttanna, 2000; Powlson & Addiscott, 2005). Accordingly, plants acquire most of their N requirements from direct assimilation of NO_3^- (Andrews *et al.,* 2013), while other, scarcer, N sources such as ammonium and amino acids are directly assimilated to a lesser extent (Gioseffi *et al.,* 2012). Organic N can be as high as 90% of total soil N in some habitats (e.g., moorland soil in the vicinity of *Calluna vulgaris*; Abuarghub & Read, 1988). Major types of organic N-containing compounds found within soils include free amino acids, polypeptides and proteins, purines, pyrimidines and vitamins. There is evidence that organic N is important for many plant N budgets across a

variety of ecosystems as plant-available inorganic N pools are often limiting (Talbot and Treseder, 2010).

1.3 Plant adaptations to environmental N limitation

>500 million years (My) of plant evolution has driven a huge array of plant adaptations and strategies to enhance N access and assimilation in N-limited environments, including in extreme cases carnivory to directly access different N pools (Roberts & Oosting, 1958; Bott *et al.,* 2008). More widespread are interactions with symbiotic associations with soil microbes which provide indirect access to otherwise unavailable soil N pools (Smith & Read, 2008; Phillips *et al.,* 2011). A diverse array of microorganisms occupy the rhizosphere and surrounding soil, and play an important role in plant N nutrition through cycling and degradation of mineral and organic N (Phillips *et al.,* 2011; Truu *et al.,* 2020) and some by forming symbioses with plants. For example, rhizobia bacteria colonize the roots of legumes, inducing nodule formation, which facilitates atmospheric N_2 fixation by the bacteria and transfer of N_2 to the host plant in return for plant-fixed C (Andrews & Andrews, 2017). Atmospheric fixation in leguminous root nodules is a major conduit for N flux into soils and is a starting point for the release of organic and inorganic N into soil systems from plant shoot, root, and nodule decay (Peoples *et al.,* 2009).

A different nutritional symbiosis is formed between the vast majority of plants and certain groups of soil fungi; these partnerships are known as mycorrhizal symbioses (from the greek *mýkēs* “fungus”, *rhiza* “root” and *syn* “together”, *bios* “life”; Brundrett, 2009; Brundrett and Tedersoo, 2018). Mycorrhizal fungi have, until recently, been classified into four main groups based on colonization structures, morphology, and host range (Brundrett, 2009; Brundrett and Tedersoo, 2018; Table 1.1; Figure 1.2), all of which show evidence of N transfer from the fungi to the host plants in one form or another (Fochi *et al*., 2017; Makarov, 2019; Stuart and Plett, 2020). The most commonly occurring groups of mycorrhizal fungi are spread across three fungal phyla: Mucoromycota, Ascomycota, and Basidiomycota (Spatafora *et al*., 2016; Stuart and

Mycorrhizal group / Distinguishing features	Arbuscular Mycorrhizas	Ectomycorrhizas	Ericoid Mycorrhizas	Orchid Mycorrhizas	Mucoromycotina Fine Root Endophytes (MFRE)
Host Range	Angiosperms, gymnosperms, ferns, liverworts, lycopods, hornworts	2 gymnosperm lineages, 28 angiosperm lineages	Ericaceae, diapensiaceae (Ericales)	Orchidaceae	Angiosperms (*Trifolium* spp.), liverworts (*Neohodgsonia mirabilis*), lycopods (*Lycopoiella inundata*)
Lifestyle	Obligate biotroph	Partial saprotroph	Partial saprotroph	Partial saprotroph	Partial Saprotroph
Known forms of N assimilated	NH_4^+, NO_3^-, souble proteins	NH_4^+, NO_3^-	NH_4^+, NO_3^-	NH_4^+, amino acids	NH_4^+
N forms transferred to plant host	NH_4^+, potentially NO_3^-	NH_4^+, potentially amino acids	NH_4^+	Amino acids?	NH_4^+?
Notes on colonisation structures and fungal morphology	Intracellular formation of arbuscules, coils, vesicles, formation of 'coarse' (>3µm diameter) hyphae, large (91 to >300µm diameter) spores	Intercellular hyphal proliferation (Hartig net)	Intracellular hyphal complex within external layer of root epidermal cells	Intracellular formaltion of pelotons	Intracellular formation of arbuscules, coils, vesicles, 'fine' (<2µm diameter) hyphae, 'fan-like' hyphal branching, small (10-12µm diameter) spores

Table 1.1 Summary of key points to compare and contrast the different mycorrhizal types using the extensive literature on non-MFRE mycorrhizal types, and comparatively sparse literature on MFRE. Columns in green indicate the four traditional groupings of mycorrhizal fungi. The blue column shows Mucoromycotina 'fine root endophyte' (MFRE) as a fifth mycorrhizal group to be considered along with the four existing groups (Chalot & Brun, 1998; Brundrett, 2009; Smith & Read, 2010; Fochi et al., 2017; Brundrett & Tedersoo, 2018; Hoysted et al., 2019; Rimington et al., 2020; Sinanaj et al., 2021). From Howard *et al.* (2022)

Plett, 2020). Within Mucoromycota, arbuscular mycorrhizal fungi — the most researched and geographically widespread (Větrovský *et al.,* 2023) group of mycorrhiza-forming fungi — are found within the subphylum Glomeromycotina (syn. Glomeromycota) (Schüßler and Walker, 2010). Arbuscular mycorrhizal (AM) fungi are estimated to form associations with ~72% of vascular plants (Brundrett and Tedersoo, 2018). Recent molecular, cytological, and physiological evidence suggests another group of widely occurring (Orchard *et al.,* 2017b), mycorrhiza-forming (Hoysted *et al.,* 2019) fungi should now be considered alongside these;

'fine root endophytes' of the subphylum Mucoromycotina, within the Mucoromycota (Spatafora *et al*., 2016; Orchard *et al*., 2017a; Walker *et al*., 2018; Table 1.1).

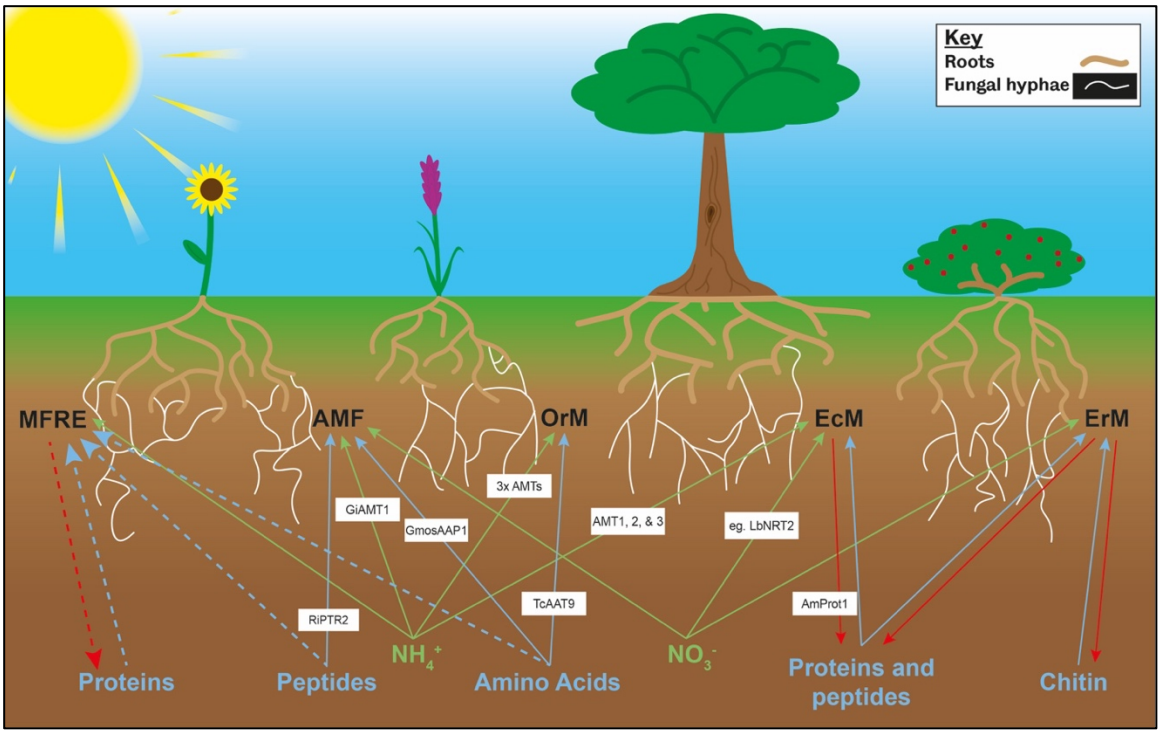

Figure 1.2 Forms of nitrogen present in soil and capabilities of mycorrhiza-forming fungi to access and assimilate them with known key transporters and enzymes involved shown. Solid arrows represent known assimilation whereas dashed arrows represent possible assimilation pathways. Red arrows indicate secretion of enzymes by fungi to degrade organic nutrient sources, green and blue arrows indicate inorganic and organic N sources respectively (Nehls *et al.,* 2001; López-Pedrosa *et al.,* 2006; Cappellazzo *et al*., 2008; Belmondo *et al*., 2014; Fochi *et al*., 2017; Stuart and Plett, 2020)

1.4 Arbuscular Mycorrhizas

The most common mode of endomycorrhizal symbiosis, and the most commonly occurring of all mycorrhizas, are the arbuscular mycorrhizal fungi (Brundrett & Tedersoo, 2018) which form tree-like branching structures (arbuscules; Figure 1.3) within plant root cells with close adherence to the plasma membrane in order to maximise surface area for nutrient exchange (Smith & Read, 2008). These

associations have been suggested to have been a crucial development in the early colonisation of the land by plants >500 million years ago (Morris *et al.,* 2018), facilitating early non-vascular plant assimilation of nutrients from primitive soils (Heckman *et al.,* 2001) as well as providing the molecular basis for other symbiotic interactions within plants (Geurts & Vleeshouwers, 2012). Nutrient exchange between plant root cells and arbuscules occurs via transporter proteins such as SWEET transport proteins (An *et al.,* 2019) which facilitate the exchange of photosynthetically derived hexoses from plant to fungus. These plant-derived sugars are the sole source of C available to AM fungi, they are obligate biotrophs and cannot assimilate C without engaging in symbioses with plants (Smith & Read, 2008). Notable exceptions to this are non-photosynthetic mycoheterotrophic plants (Walder & van der Heijden, 2015). These plants connect to AM fungal networks from which they extract all of their C requirement without photosynthesising. In addition, the C within the parasitised AM fungal network necessarily derives from the photosynthesis of other, interconnected, autotrophic plants engaged in typical mutualism with AM fungal networks (Selosse & Roy, 2009).

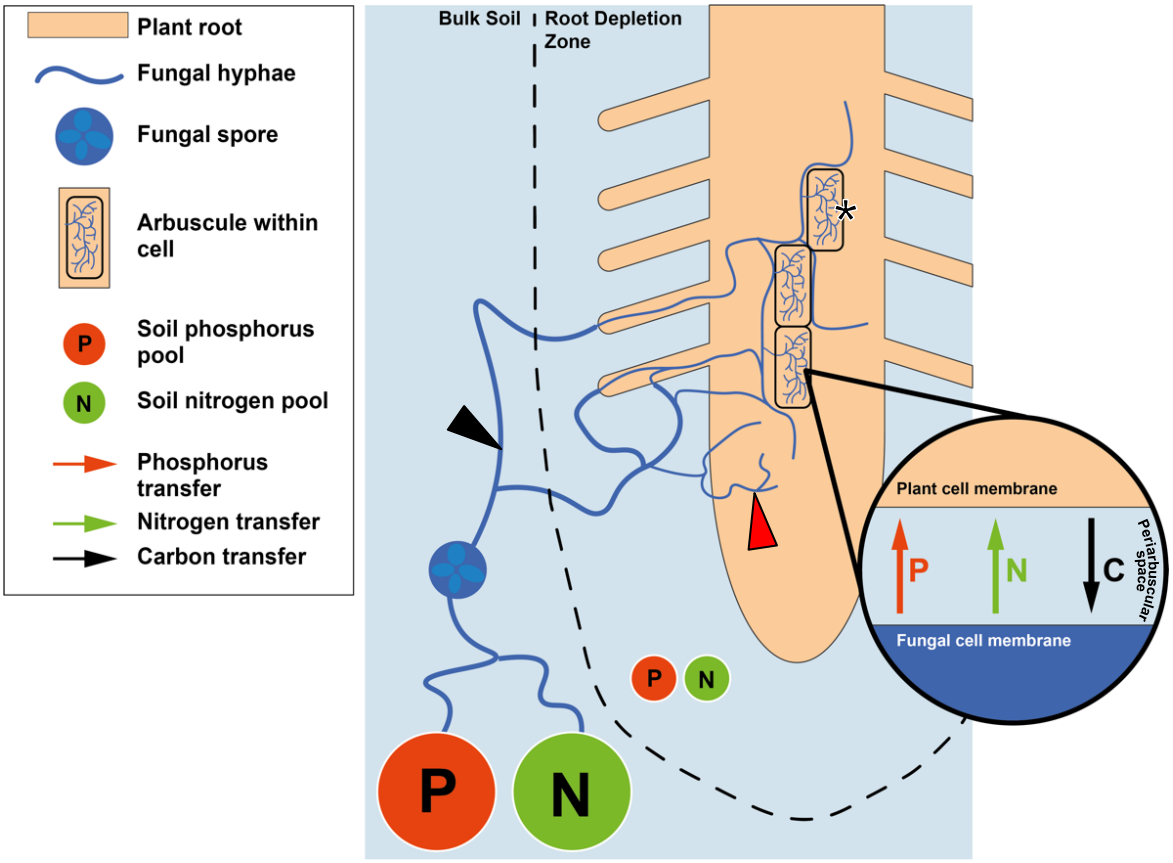

Figure 1.3 Schematic diagram of arbuscular mycorrhizal assimilation of soil N and P beyond root depletion zone. Also depicted are arbuscules within plant cells (*), intercellular hyphae (red wedge), extraradical hyphae (black wedge) and N & P-for-C exchange at periarbuscular interface (enlarged). Watts *et al.*, (2023).

Mycorrhizal fungi play an influential role in soil chemistry; during their normal growth, AM fungi assimilate N and P from their environment to exchange with plant hosts for C. The more AM fungi present in soils, the greater their impact on the N and P cycling within them. In so doing, AM fungi prevent nutrient leaching and gaseous N emissions from soils (Cavagnaro *et al.,* 2015). As such, AM fungi are significant drivers of soil N cycling and their capacity to assimilate different N-containing compounds, limited capacity thereof, govern the available nutritional niches for other, non-AM plant-fungal partners.

1.4.1 Nitrogen preferences of AM fungi

N assimilation by plants is often enhanced when in association with AM fungi (Xie *et al.,* 2022). AM fungi have the capacity to assimilate many different N-containing compounds, from organic forms of N such as amino acids (Hawkins *et al.,* 2000), and organic matter (Leigh *et al.,* 2009; Thirkell *et al.,* 2019), to inorganic compounds often used in agricultural fertilisers (Jach-Smith & Jackson, 2020). While uptake, use, and exchange of various N sources are carried out by many species of AM fungi, the rates at which these processes occur vary depending on the type of N available and fungal species present, with inevitable repercussions on nutritional niches and therefore, feedback onto the species composition of a given environment (Antunes *et al.,* 2012).

Many different N containing compounds are directly utilised by AM fungi including ammonium (NH_4^+), nitrate (NO_3^-), urea, amino acids (AA), and soluble proteins (Jin *et al.,* 2005). There is however some evidence to suggest that inorganic N is favoured over organic N when both are available (Whiteside *et al.,* 2012). It is possible that the relative inability of AM fungi to derive nutrition from organic sources is a primary driver of their obligately biotrophic status (Smith & Read, 2008). A potential preference for NH_4^+ over NO_3^- as an inorganic source of N has even been shown by Toussaint *et al.* (2004). As Tanaka & Yano (2005) show, NO_3^- is taken up by AM fungi *Glomus aggregatum* at similar rates to NH_4^- at similar concentrations, however transfer of NO_3^- to crimson clover (*Trifolium incarnatum* L) plants occurred at much lower rates than that of NH_4^+. This has been hypothesized to be due to the need for energetically costly reduction of NO_3^- to be carried out by the fungus in order for fungal uptake to be achieved (Marzluf, 1997).

The preference for ammonium over nitrate as a main source of N nutrition is not a universal phenomenon among AM fungi however, some studies indicate greater transfer of N to plant hosts from AM fungi when supplied with nitrate (Ngwene *et al.,* 2013). Nitrate is one of the most common and most mobile N sources in soil. AM fungi import NO_3^- by a H^+-mediated symporter GiNT (Bago *et al.,* 1996), expressed in the (extraradical mycelium) ERM (Tian *et al.,* 2010), before transfer to host plants.

Ammonium uptake by AM fungi, on the other hand, is achieved via transport proteins such as the high affinity ammonium transporter GintAMT1 expressed in

the ERM of *Glomus intraradices* in the presence of low concentrations of NH_4^+ (López-Pedrosa *et al.,* 2006). The GintAMT2 transporter is a high affinity ammonium transporter, however it is expressed in the intraradical mycelium (IRM) of the same species to scavenge excess NH_4^+ released during the fungal metabolism of other N sources (Pérez-Tienda *et al.,* 2011). GintAMT3 is a low affinity ammonium transporter that is localised in the intraradical mycelium (IRM) of AM fungi colonising plant roots and is upregulated under reduced inorganic N concentration (Calabrese *et al.,* (2016).

Organic N sources are also present in the soils and are scavenged by AM fungi in a range of different ways, such as the amino acid permease GmosAAP1, found to be expressed by the AM fungus *Glomus mosseae* which is capable of actively importing proline via a H^+ and pH-mediated mechanism (Cappellazzo *et al.,* 2008). This same transporter is expressed in both the IRM and ERM and has the capacity to transport multiple amino acids such as arginine, asparagine, and glutamine (Jin *et al.,* 2005). A dipeptide transporter RiPTR2 has been found in the widespread AM fungus *Rhizophagus irregularis* in both the ERM and IRM, suggesting that AM fungi can metabolise products of partial protein breakdown (Belmondo *et al.,* 2014). One of the larger organic N pools in soils comprises organic polymers such as proteins from partially degraded plant necromass. While it would seem a highly desirable N pool to utilise, many AM fungi species do not appear capable of polymeric N degradation, relying instead on the organic breakdown resulting from bacterial metabolism while, conversely, many ectomycorrhizal and ericoid mycorrhizal fungi seem to be capable of organic N breakdown, indicating that this is potentially an ability that has been lost or had not evolved before the different groups diverged (Talbot & Treseder, 2010).

1.4.2 AM fungi nitrogen transfer to host plants

In Arbuscular mycorrhizas, fungus-to-plant N transfer occurs across the periarbuscular space – the space between fungal and plant cell membranes – (Figure 1.4b) although the mechanism by which fungal hyphae release N into this space is currently unknown. Aquaporins may be involved as suggested by the identification of two fungal aquaporins expressed in the extraradical mycelium and arbuscules of maize roots inoculated with *R. irregularis* (Li *et al*., 2013; Chen *et al*.,

2018a). Recently, two *R. irregularis* genes with homology to *Saccharomyces cerevisiae AMMONIA TRANSPORT OUTWARD PROTEIN 3* (*ATO3*) have been identified; these could also be candidates for export of ammonia into the interfacial apoplast (Chen *et al*., 2018a).

Uptake of N from the interfacial apoplast into the plant occurs via transport proteins such as the ammonium transporter GmAMT4.1 expressed in soybean root cortical cell membranes when in partnership with *Glomus intraradices* (Kobae *et al*., 2010). There are four other AM-induced AMT genes identified in soybean, two in sorghum, two in tomato, and three in *Medicago truncatula* (Jin *et al*., 2012; Chen *et al*., 2018a). In addition to ammonium transporters, nitrate transporters in the NRT2 family have been identified as AM fungi-induced in tomato, *M. truncatula,* and *Lotus japonicus*, although their subcellular localisation and transport activities remain unknown and potential transfer of nitrate in the symbiosis is poorly understood (Hogekamp *et al*., 2011). Some organic forms of N may also potentially be transferred; AM fungi-upregulated amino acid transporters have been observed in *L. japonicus* (Chen *et al*., 2018a).

After N is assimilated into AM fungal hyphae, it is converted to arginine for transport to the arbuscule; however, how N is processed into this form depends upon the original form of N imported (Jin *et al*., 2012).

Nitrate is reduced to nitrite by nitrate reductase before being further reduced to ammonium by nitrite reductase (Jin *et al*., 2012; Figure 1.4a), which is then incorporated into arginine via the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle (Pfeffer *et al*., 2005). In *Rhizophagus irregularis*, GiGS1 and GiGS2 incorporate ammonium into glutamate to produce glutamine, which is then converted into arginine (Tian *et al*., 2010; Figure 1.4a). Organic sources of N also require conversion into arginine for transport to the intraradical mycelium unless direct assimilation of arginine has occurred (Figure 1.4a). However, not all common amino acids are utilized by AM fungi. Cyclic amino acids and amino acids with high bond strengths resist hydrolysis by AM fungi and are consequently not efficient for use, regardless of size, and are thus assimilated less often (Talbot and Treseder, 2010).

Following conversion into arginine by AM fungi, N is transported from the extraradical mycelium into the intraradical mycelium to be exchanged with the plant (Fellbaum *et al*., 2014) (Figure 1.4b). Labelled substrate studies using ^{13}C, ^{14}C and ^{15}N have shown that only N is transferred between the fungus and the plant and that the C skeletons of amino acids synthesised by the fungus remain in the fungus (Pfeffer *et al*., 2005); therefore, the arginine used to transport N to the intraradical mycelium must be broken down to release ammonium. Given that transcript levels of genes with high similarity to ornithine aminotransferase, urease accessory protein, and ammonium transporters are upregulated in the intraradical mycelium of *Rhizophagus irregularis* (Pfeffer *et al*., 2005; Jin *et al*., 2012; Chen *et al*., 2018a), arginine breakdown is likely to occur via the urea cycle, with amino acids recycled via the GS-GOGAT cycle once ammonium has been liberated (Jin *et al*., 2012; Chen *et al*., 2018a). Urease and arginase activity is increased in the mycorrhizal root compartment when compared with the extraradical mycelium, suggesting that the catabolic arm of the urea cycle is more active in the intraradical than the extraradical mycelium (Cruz *et al*., 2007; Jin *et al*., 2012).

1.5 Ectomycorrhizal fungi

Ectomycorrhizal fungi (ECM) are a broad and diverse group of mycorrhizal fungi not strictly categorised by phylogeny but instead by the form of colonisation they take in plants (Read *et al.,* 2004). The evolution of the ectomycorrhizal mode of plant-fungal symbiosis has been identified over 60 times to date across multiple fungal lineages (Tedersoo *et al.,* 2010). Ectomycorrhizal fungi are cosmopolitan in forested areas especially in boral habitats (Read *et al.,* 2004). Contrary to AM fungi, ECM primarily provide host plants with nutrients obtained from organic matter (Rineau *et al.,* 2013; Mahmood *et al.,* 2024). Crucially ECM are not wholly reliant on their host plants wholly for their carbon acquisition, a marked difference from AM fungi (Koide *et al.,* 2008). This reduced reliance on plant partners for direct C acquisition is a trait shared with MFRE (Field *et al.,* 2015a), thus any investigation of MFRE nutrient acquisition would not be complete without an understanding how other partial saprotrophic fungi function.

Given the broad phylogeny and distribution of ECM fungi, there are sure to be a multiplicity of molecular mechanisms and functional traits associated with the varied species within this broad category. Therefore, the following section will discuss the most prominent and generalised traits of ECM fungi and, where relevant, discuss any outliers.

1.5.1 Modes of Ectomycorrhizal colonisation

ECM colonise the lateral roots of their host plants by forming an interlacing mycelial structure, known as the Hartig net, which penetrates between and surrounds the epidermal cells (Stuart and Plett, 2020) and, like the arbuscule, provides a large surface area for nutrient exchange. However, unlike AM fungi arbuscules, plant cell walls are not penetrated during the formation of the Hartig net. This is compensated for by the development of lateral root clusters and tubercles that increase the interface surface area (Smith and Read, 2008).

1.5.2 Ectomycorrhizal nitrogen preference

ECM fungi (Basidiomycota and Ascomycota) acquire both organic and inorganic forms of N, although they are likely to access organic sources more frequently in nature given how abundant (over 95% in some woodlands) organic material is in the woodland ecosystems where ECM are most common (Chalot and Brun, 1998; Nicholás *et al.,* 2019).

Little is known about the processing of N once inside the extraradical mycelium of ECM. Neither the form of N transported to the intraradical mycelium nor the mechanisms behind this are fully known (Stuart and Plett, 2020).

There is some evidence that N transfer to plants occurs via transfer of whole amino acids to host plants. It is also believed that there is exchange of organic compounds in the apoplastic space between plant roots and fungal hyphae, as fungal amino acid exporters are upregulated in mycorrhizal root tips colonized by *Laccaria bicolor* or *Pisolithus microcarpus* (Stuart and Plett, 2020). However, an equally likely mechanism of ERM has been suggested. This mechanism is similar to the arginine breakdown and ammonium release, described in AM (above) has been

proposed for ECM fungi (Nehls & Plassard, 2018). Up-regulation of plant ammonium importer expression in ECM mycorrhizal root tips indicates that ammonium is the principal form of N transferred between the two organisms (Stuart & Plett, 2020).

While the discussion is ongoing (Koide *et al.,* 2008; Lindahl & Tunlid, 2015), this process of organic degradation conducted by ECM is frequently described as saprotrophic in nature, (the process of one organism obtaining nutrition from the deceased organic matter of another) and are often cited as important drivers of organic matter turnover in their native habitats (Rineau *et al.,* 2013). Regardless, ECM are known to utilise a variety of enzymatic and non-enzymatic processes to access organic nutrients.

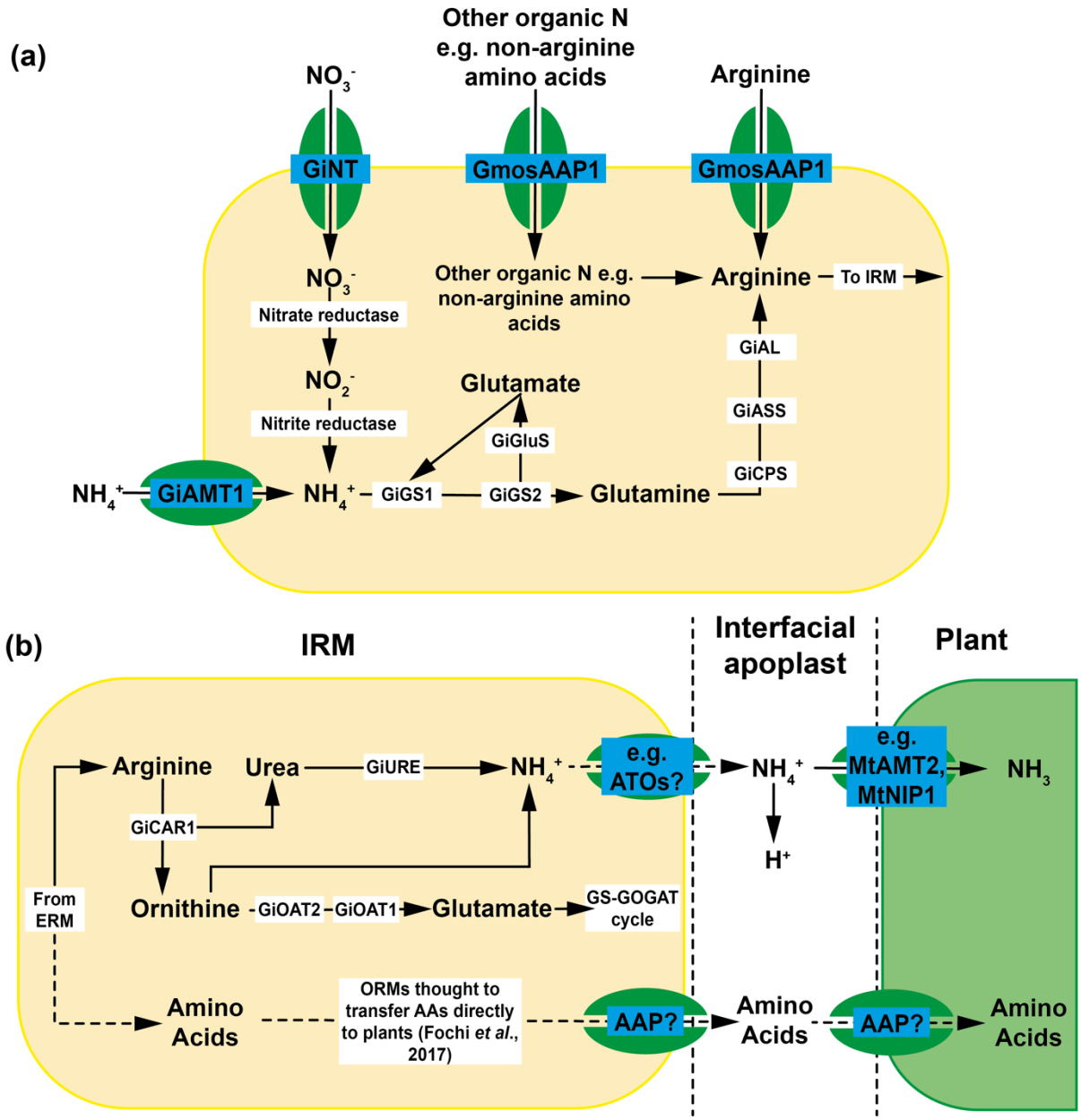

Figure 1.4 N assimilation pathways of mycorrhizas. (a) Pathways by which assimilated N is transformed within ERM of mycorrhizal fungal hyphae before export to plant hosts. (Pfeffer et al.,2005; Tian et al., 2010) **(b)** Pathways by which assimilated N is transferred from IRM of fungal hyphae to plant root cells. Solid arrows indicate known pathways. Dashed arrows indicate potential N assimilation pathway in orchid mycorrhizas (Pfeffer *et al*., 2005; Cruz *et al*., 2007; Jin *et al*., 2012; Fochi *et al*., 2017; Chen *et al*., 2018a; from Howard *et al.,* 2022)

1.6 Mucoromycotina Fine Root Endophytes

One of the lesser-known taxa of mycorrhizal fungi are the Mucoromycotina 'Fine Root Endophytes' (MFRE), an early branching lineage of fungi (Bidartondo *et al.,* 2011; Field *et al.,* 2015b). Recently reclassified members of the subphylum Mucoromycotina (Orchard *et al.,* 2017b), MFRE were previously thought to be encompassed within the Glomeromycotina (Hall, 1977). MFRE potentially consist of several species (Thippayarugs *et al.,* 1999) and have a limited scientific record for a number of reasons.

1.6.1 MFRE History

MFRE have a somewhat obscure and limited recorded history because they have historically been difficult to identify, isolate, and culture. Endophytic fungi likely to have been MFRE were first identified in association with the evergreen tree species *Griselinia littoralis* by Greenall (1963) and named *Rhizophagus tenuis* as they had similar morphological characteristics to *R. populinus,* namely arbuscules but it was noted that the vesicles and hyphae were both smaller than those of *R. populinus*. The new endophyte also resisted attempts at culturing on agar.

In the decade after it was described, there was not much activity in the study of MFRE as distinct from AM fungi. References to MFRE (or "FRE") in the literature only serve to announce its potential presence in a sample (Baylis, 1967; Mosse & Hayman, 1971; Crush, 1973a). This was until Crush (1973b) investigated the effects of *R. tenuis* infection on growth of three grass species under low phosphorus conditions. Under low P the endophyte was seen to improve plant biomass, an effect that was reversed on fertile soils. This effect was confirmed by Johnson (1976), with endophyte colonisation of *Griselinia littoralis* (Kapuka, New Zealand broadleaf or Pāpāuma) and *Leptospermum scoparium* (Mānuka or tea tree) resulting in higher P concentration in plant dry matter on low-P soils than in uninfected plants.

Since the early studies, MFRE fungi occasionally enter into the literature in both field and lab studies relating to their effects on plants. However, these studies rarely measure the exchange of carbon and nutrients between symbiotic partners,

merely the presence/absence, colonisation, and/or abundance of MFRE based on morphological identification (Daft & Nicholson, 1974; Sainz *et al.,* 1990; Postma *et al.,* 2007).

These limitations have resulted in significant gaps within the (endo)mycorrhizal research.

1.6.2 Classification and Nomenclature

After closer study *R. tenuis* was reclassified as *Glomus tenuis* by Hall (1977) owing to its distinctive morphological differences from other species of *Rhizophagus*. In the same publication, however, Hall notes that the fine endophyte is markedly physically distinct from other species of *Glomus*, however owing to lack of any other evidence, it was designated *G. tenuis*. This morphological distinction, coupled with modern molecular analysis, allowed Orchard *et al.,* (2017a) to determine that MFRE actually belongs within the subphylum Mucoromycotina rather than the Glomeromycotina. Subsequently, a new genus *Planticonsortuim* has been suggested for MFRE (Walker *et al.,* 2018) with the combination *P. tenue*. Additionally, it remains unclear whether MFRE, formerly designated *G. tenue*, actually represents more than one species as suspected by Thippayarugs *et al.* (1999).

Most contemporary literature continues to use some variation of ‘Fine Endophyte’, ‘FRE’, ‘Mucoromycotina Fine Root Endophyte’, ‘MFRE’, ‘MucFRE’ etc. to avoid any ambiguity with an ever-changing official designation. Occasionally, this group of fungi is referred to as ‘Mucoromycotinian arbuscular mycorrhizal fungi’ (M-AM fungi; Albornoz *et al.,* 2022; Kowal *et al.,* 2022). This term, while adding to an already crowded register of names, obscures the nature of this group of fungi in three ways; Firstly, it departs from the conventions of all previous common names which included some reference the morphology (‘fine endophytic’) of these fungi. Were the term M-AM fungi widely adopted, all subsequent work regarding these fungi would be nominatively detached from the preceding work, hindering literature searches and occluding current knowledge from future research. Secondly, the use of the ‘AM fungi’ as part of this new term adds taxonomic confusion as all AM fungal

species (excluding FRE/MFRE/MucFRE etc.) are of the Glomeromycotina subphylum. Referring to the fungi in question using a term already in use for species within a different taxonomic group adds unnecessary opportunities for conflation between the separate fungal groups. Additionally, while arbuscule-like structures are sometimes present in MFRE colonisations of host plants (Sinanaj *et al.,* 2021; Hoysted et al. 2023), they are by no means ubiquitously formed in symbioses, and their function is not yet established. Finally, the use of M-AM fungi necessitates the renaming of all other AM fungi species as G-AM fungi (Albornoz *et al.,* 2022), the wide adoption of which is extremely unlikely to occur in a large and growing field of literature. In line with the above reasons, in this thesis the term Mucoromycotina 'fine root endophyte' (MFRE) is used to refer to the endosymbiotic fungi within the Mucoromycotina clade.

1.6.3 MFRE morphology and colonisation structures

MFRE in angiosperms typically produce fine arbuscules together with fan-like hyphal structures and hyphal ropes (Orchard *et al*., 2017b; Hoysted *et al*., 2019; Albornoz *et al*., 2020) and are distinguished morphologically from the 'coarser' AM fungi (Figure 1.5A, B) by their characteristic finer hyphae (<2 µm in diameter vs. >3 µm in AM fungi) with small intercalary and terminal vesicles or swellings (Orchard *et al*., 2017b; Figure 1.5C). They frequently branch and occasionally form so-called 'ropes' by growing around one another in a twisting pattern (Orchard *et al.,* 2017b). Fine arbuscules (Figure 1.5D) alongside coarser ones have also been observed in liverworts co-colonised by MFRE and AM fungi (Field *et al*., 2016; 2019) but, in this group and in other early-divergent spore-producing lineages, the morphology of colonization by MFRE appears highly plastic (Field and Pressel 2018). Both intra- and intercellular phases of colonisation are present in the earliest divergent Haplomitriopsida liverworts and in the gametophyte and early sporophytic stage (protocorm) of several lycophyte specie s (Schmid and Oberwinkler, 1993; Duckett and Ligrone, 1992; Hoysted *et al*., 2019, 2020). Intracellular colonisation results in a variety of structures, including tightly wound hyphal coils with terminal swelling or "lumps" (Haplomitriopsida and outermost cortical layers of lycophyte gametophyte) (Figures 1.3E and 1.4B) and branched fine hyphae with intercalary and terminal vesicles (lycophyte gametophyte and

protocorm) (Figure 1.6A) but, consistently, no arbuscule-like structures (Carafa *et al*., 2003; Duckett *et al*., 2006; Hoysted *et al*., 2019). During intercellular colonisation, the fine hyphae enlarge (Figure 1.6C), eventually forming masses of swollen pseudoparenchymatous structures lacking vesicles and which soon collapse and degenerate (Figure 1.6D). This short life-span mirrors that of the Haplomitriopsida intracellular fungal 'lumps'. It has been suggested that the collapse and lysis of these structures (Figure 1.5F) may provide a source of nutrients, including N, passed from MFRE to their host liverworts, comparable to the mechanism employed by OrM (discussed above; Duckett *et al*., 2006; Hoysted *et al*., 2020).

MFRE colonisation in lycophytes also varies depending on the plant's life stage. In the adult sporophytes of *Lycopodiella inundata*, colonisation is strictly intracellular and consists solely of fine branching hyphae with intercalary and terminal swellings (Figure 1.5C); as in earlier developmental stages, arbuscule-like structures have never been observed in MFRE-colonised roots of *L. inundata* (Hoysted *et al*., 2019, 2020). Recent demonstrations of nutrients-for-carbon exchange in Haplomitriopsida liverworts (Field *et al*., 2015a) and adult sporophytes of *L. inundata* (Hoysted *et al*., 2019, 2020) by isotope tracer experiments indicate that arbuscules are not required for this exchange to occur and that other MFRE structures must therefore be involved in active metabolic interactions with the host cells.

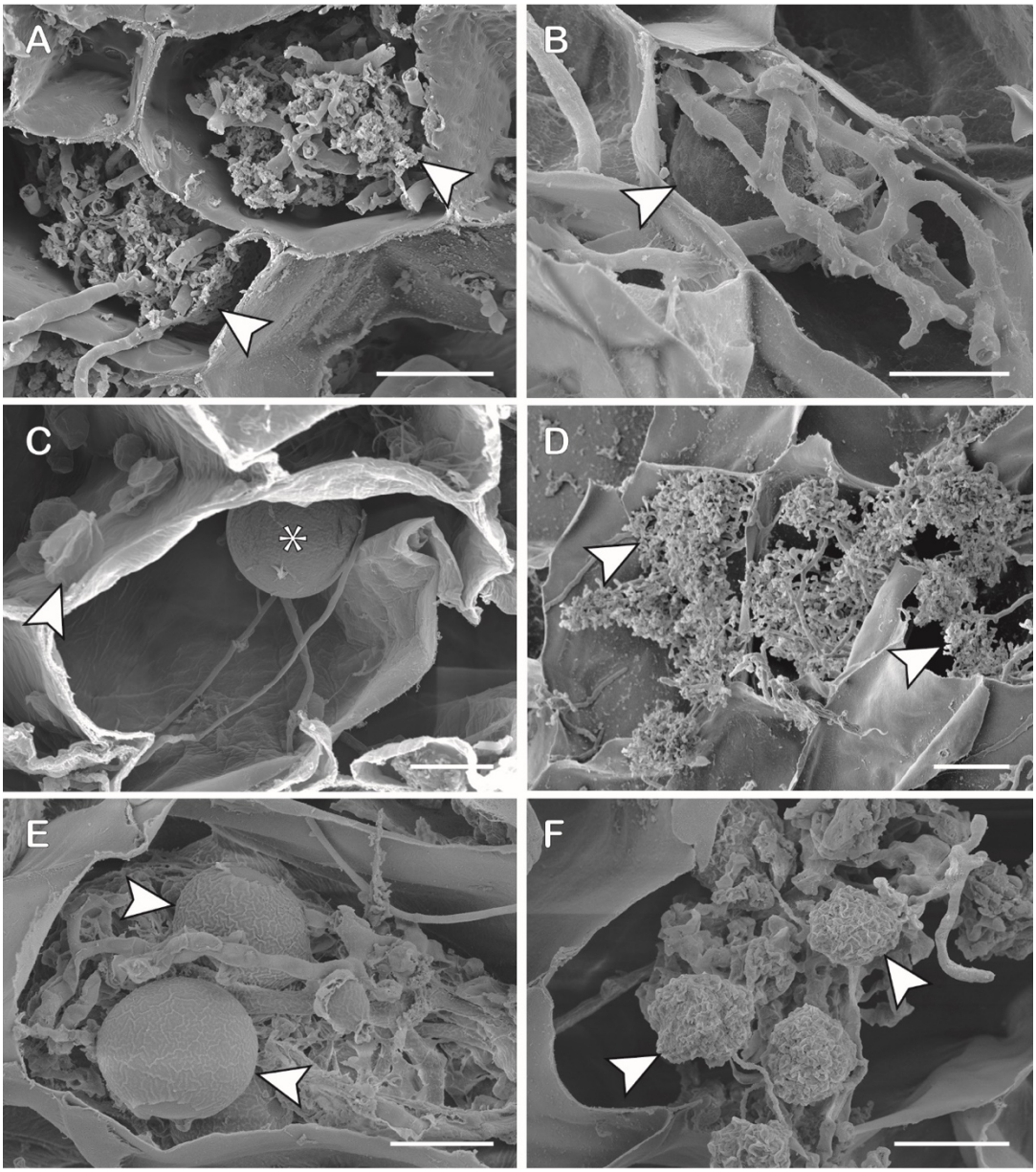

Figure 1.5 Scanning electron micrographs of structures produced by AM fungi (A, B) and MFRE (C-F) during colonisation of host plant tissues. A, B. Typical AM fungi intracellular 'coarse' arbuscules (**A,** arrowed) and large vesicle (**B,** arrowed) with coarse hyphae, shown here in the thallus of the liverwort Neohodgsonia mirabilis. **C.** MFRE intracellular intercalary and terminal small vesicles/swellings, shown here at different stages of development (young*; collapsed, arrowed) with fine hyphae in a root of the vascular plant Lycopodiella inundata. **D.** Intracellular fine arbuscules (arrowed) in the liverwort N. mirabilis. **E, F.** Intracellular tightly wound hyphal coils with young (**E**, arrowed) and collapsed (**F**, arrowed)

'lumps' in the thallus of the Haplomitriopsida liverwort Treubia lacunosa. Scale bars: (**A, B, D**) 20 µm; (**C, E, F**) 10 µm. (From Howard *et al.,* 2022)

1.6.4 Lifestyles of MFRE

Unlike obligately biotrophic AM fungi (Smith and Smith, 2011; Lin *et al*., 2014), MFRE are thought to be facultatively saprotrophic in nature (Lin *et al*., 2014; Field *et al*., 2015a; Field *et al*., 2016), as evidenced by isolation and in vitro culture experiments (Field *et al*., 2015a) whereby MFRE proliferate on synthetic media without a host plant. These assumed saprotrophic capabilities, together with their frequent co-colonisation of host plants with AM fungi, open the possibility that MFRE may play an important role in plant nutrition that is distinct from that of AM fungi. While no studies into the direct mechanisms of N uptake in MFRE have yet been conducted, it is likely that its ability to utilise its saprotrophic capabilities to break down organic N is why it can transport substantially more N to symbiotic plant partners than many AM fungi species.

Recent experimental evidence in both non-vascular liverworts (Field *et al.,* 2019), and in a vascular plant (Hoysted *et al*., 2019; 2021), suggests that there is a degree of complementarity between MFRE and AM fungi function, with MFRE playing a more prominent role in facilitating plant N nutrition alongside AM fungi-acquired P (Field *et al*., 2019). This hitherto unappreciated complementarity may have caused a potential source of confusion so far, whereby fungal-mediated transfer of N to host plants has been misattributed as being wholly due to AM fungi. Thanks to the use of specific MFRE primers (Bidartondo *et al*., 2011; Desirò *et al.,* 2017), consideration of a more cosmopolitan fungal endophyte community is now possible.

1.6.5 Geographic distribution of MFRE

Despite their relative underrepresentation among the literature, recent work has made strides in identifying the distribution of MFRE across the globe. These early studies have shown that MFRE occupy a global distribution across a range of ecosystems, habitats, and land use types. Kowal *et al.,* (2020) investigated the distribution of MFRE among 11 populations of the lycophyte, *Lycopodiella*

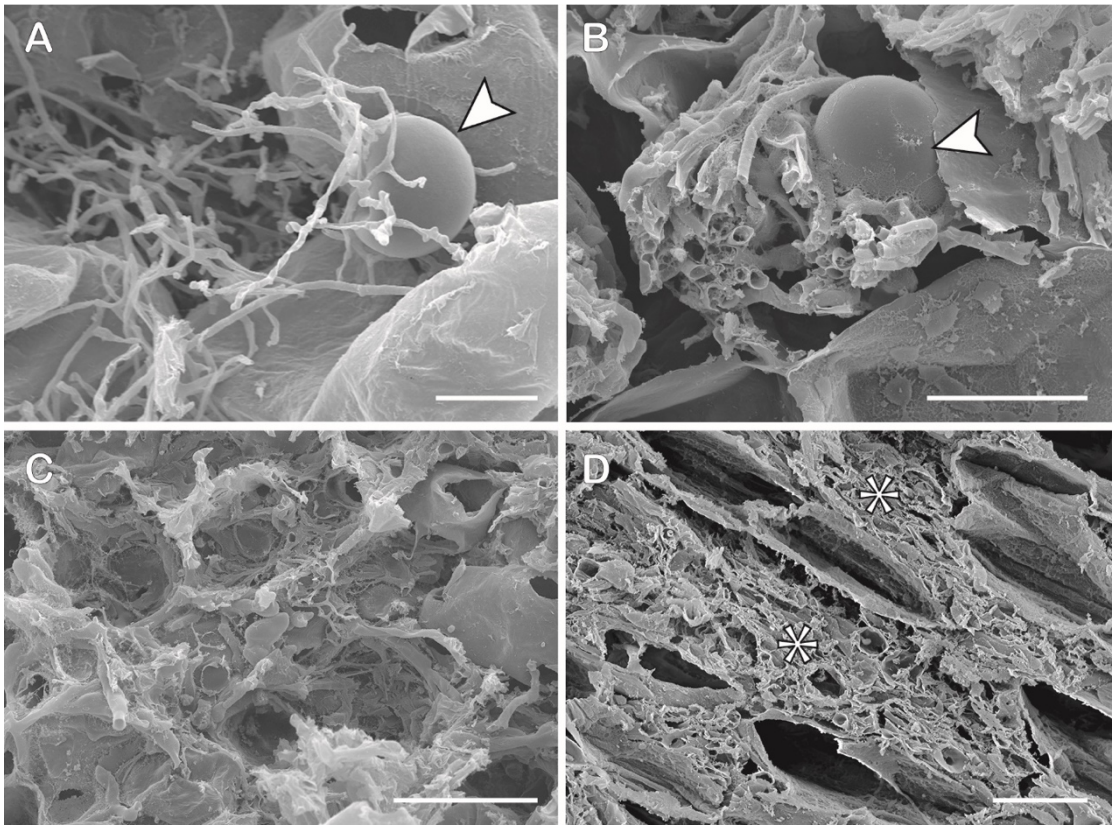

Figure 1.6 Scanning electron micrographs of structures produced by MFRE during colonisation of host plant tissues. A) Intracellular small vesicle with fine, branching hyphae in the sporophytic protocorm of *L. indundata*. **B)** Intracellular tightly wound hyphal coil with larger vesicle in the outermost cortical layers of a gametophyte of *L. indundata.* **C, D.** *L. inundata* sporophytic protocorm. During intercellular colonisation, the fine hyphae enlarge **(C)** until the intercellular spaces are filled with masses of collapsed pseudoparenchymatous hyphae **(D*).** Scale bars: **(B, C, D)** 50 µm; **(A)** 10 µm. (From Howard *et al.,* 2022)

inundata in Great Britain and the Netherlands. They found that season strongly impacted the presence of MFRE fungi in the roots of these host plants. Similarly, MFRE have a wide distribution across plant lineages and ecosystem types, from lycophytes in European heathlands (Kowal et al., 2020) to legumes in Australian pastures (Albornoz *et al.,* 2021).

Rimington *et al.,* (2015) extracted DNA from and visually analysed the roots of wild-collected lycopod and fern sporophytes using the methods described in Bidartondo

et al., (2011). Sequences identified as belonging within the Mucoromycotina were found in five plant species, four lycopod (20% of species sampled) and one fern (5.6% of species sampled). These were distributed across Europe (England, Italy, France), North America (USA), and Australasia (New Zealand). Some plant species and even individual samples were found to be associated with up to three separate accessions of Mucoromycotina. These data, while not demonstrating functional symbiosis between Mucoromycotina fungi and plants, demonstrate colonisation of vascular plants by multiple species of fungi belonging to the Mucoromycotina across a global distribution.

Because of this wide geographic and ecological distribution joined with the fact that these Mucoromycotina fungi colonise vascular plants it is pertinent to investigate what the functions of these fungi are within these host plants. However, the relative ignorance of many mycorrhizal studies to their existence may be resulting in globally important functional traits being ignored. The isolation and axenic culture of one strain of MFRE (Field *et al*., 2015a), allow us to gain new insights into plant-MFRE relationships at community and potentially even agricultural (Albornoz *et al.,* 2022) levels.

1.6.6 Functional symbioses between MFRE and plants

Methods such as isotope tracing studies have been used in concert with molecular genotyping to confirm and quantify the relationships between MFRE fungi and their plant hosts (Field *et al.,* 2016; Field *et al.,* 2019). From this work we now have evidence of nutrient transfer from MFRE to their vascular (Hoysted *et al.,* 2019), including angiosperms (Hoysted *et al.,* 2023), and non-vascular (Field *et al.,* 2019) plant partners and vice-versa. Another interesting development has been the discovery of dual symbiosis of MFRE and species of AM fungi in partnership with the same host plant (Yamamoto *et al.,* 2019), wherein plant hosts derive greater nutrient uptake efficiencies than by partnering with either fungus in a single symbiosis (Field *et al.,* 2016). However, the role of MFRE in nutrient exchange in the presence of different nutrients and availabilities is not currently known, despite a glut of similar research in AM. Understanding the responses of individual plants and their mycorrhizal MFRE partners to changing soil nutrition (e.g. N and P) and

water status is essential to predict plant resilience during more frequent climatic extremes. This branch of fungi has already been suggested to be an important driver of land plant evolution (Rimington *et al.,* 2018) as well as possessing the ability to associate with vascular plants, more than was previously thought (Hoysted *et al.,* 2019). As such, MFRE warrant greater investigation generally as well as part of an integrated effort to protect natural ecosystems and agricultural land from anthropogenic stresses

1.7 Soil nutrition and mycorrhizas

Generally, an increase in soil N content is accompanied by a reduction in the colonisation of plants by AM fungi, as shown in a variety of N addition studies (Bonneau *et al*., 2013; Chen *et al.,* 2018a) as well as over natural soil N gradients (Johnson *et al.,* 2003; Liu *et al.,* 2012; Frater *et al.,* 2018; Klichowska *et al.,* 2019). This trend is likely due to the greater availability of free small inorganic N sources in these soils meaning it is more energetically efficient for plants to directly absorb and assimilate N from the soil rather than exchanging carbon for N with AM fungi. This is a major issue facing commercial agriculture as high levels of inorganic N-containing fertilizer addition to soils reduces mycorrhizal diversity and by extension the other non-nutritional benefits associated with them (Williams *et al.,* 2017; Zeng *et al.,* 2021).

Much of the work around mycorrhizal nutrient uptake from soils has been conducted in an agricultural context (Deepika & Kothamasi, 2015; Zhang *et al.,* 2016; Storer *et al.,* 2017), as the issues of soil nutrition, leaching, and over fertilisation are particularly pressing for food production. The other plant macronutrient, phosphorus, is also important in agriculture as plants cannot readily assimilate a majority (90%) of the phosphate that is applied to these soils due to precipitation reactions that take place between the phosphate and soil particles or organic matter (Plante, 2007). In agricultural systems fertilised with N, P becomes the limiting nutrient to crop plants while simultaneously representing a threat to aquatic ecosystems, in which it is also limiting, which can suffer from eutrophication caused by algal blooms fed on phosphate runoff (Comber *et al.,* 2013).

It is likely that, as partially/facultatively saprotrophic organisms, MFRE have a greater capacity to obtain N from organic sources than AM fungi which allows them to take up and transport more N to plant partners resulting in the trend seen by Field *et al.* (2019). The mechanisms behind this enhanced organic N uptake are unknown but could involve direct uptake or degradation of proteins and other complex organic molecules. It is also possible that MFRE have a greater capacity for uptake of inorganic N, utilising ammonium as the most accessible N source in soil not readily assimilated by plants. Regardless, these mysteries should be teased apart and solved. As a starting point, determining the relative affinity for different N sources in isolation would allow further speculation and development of hypotheses on the mechanisms of N uptake and use by MFRE.

It is unknown in what form MFRE transport N intercellularly, it may be the case that transport occurs in a less complex form than arginine, reducing the need for additional processing, rendering N a more cost-effective resource to take up, allowing greater N exchange with plants than AM fungi are capable of. It may be that MFRE have the capacity to transport N in a range of forms depending on the N source taken up, further alleviating the cost of assimilation and exchange. N tracing experiments such as those performed by Pfeffer *et al.,* (2005) and Tian *et al.,* (2010) using root organ cultures of AM fungi may be an appropriate model for investigating the N metabolism of MFRE.

It is hypothesised that as MFRE are partially saprotrophic, they may utilise similar mechanisms as ectomycorrhizal fungi to break down large N-containing compounds resulting in greater N uptake than we see performed by AM fungi. These organic sources of N must also be converted to compatible amino acids for transport to the IRM, unless of course direct uptake has occurred. However, not all common amino acids are utilised by AM fungi, cyclic AA and AA with high bond strength resist hydrolysis and are consequently not efficient for use by AM fungi regardless of size (Talbot & Treseder, 2010).

The characteristics and mechanisms of N transport and exchange with plant hosts have not yet been investigated in MFRE specifically. Presence or absence of MFRE has been the extent of analysis of MFRE symbiosis for decades (Daft & Nicholson, 1974; Sainz *et al.,* 1990; Postma *et al.,* 2007). However, this is changing

with new experimental techniques, inoculation protocols, molecular identification, and sampling methods as well as the critical new availability of MFRE isolates (Hoysted et al., 2023). As such, we now have the ability to quantify the quantities and efficiency of nutrients exchanged between plants and fungi. Despite the relative dearth of studies on the effects of FRE-plant symbiosis on plants in the past, there have been several investigations relevant to the soil nutrient question. Johnson (1976) for example, demonstrated that FRE have the capacity to increase plant growth on soils with low phosphate content.

However, it remains unknown what forms of N and P are preferred by MFRE as a nutrient source, even down to the coarsest estimates of organic vs inorganic N. While some work has been conducted looking at nutrient exchange between MFRE and plants (Field *et al.,* 2016; Hoysted *et al.,* 2021; 2023), there is thus far no data to answer whether MFRE will assimilate some forms of N more than others, or whether MFRE will selectively assimilate and exchange one form of N over others given the choice.

The effect of MFRE in ecosystems with N deposition remains unknown. Similarly, the interactions between MFRE and AM fungi when colonising the same host plant are largely unknown. Initial work focusing on efficiency of these symbioses under different CO_2 regimes has been conducted (Field *et al.,* 2016; Field *et al.,* 2019), but this has thus far not been continued altering soil nutrient status.

To disentangle this enigmatic clade of fungi, some of the basic features of its biology need to be clarified. The uptake and utilization of different forms of N is one of the more burning questions upon which further experimentation can be based, therefore quantifying the relative uptake, utilisation, and exchange of different forms of N and phosphorus is key to our understanding of the functioning and plasticity of MFRE as well as informing future experimental design in this area. Organic N sources have already been shown to be utilised by MFRE in symbiosis with early-diverging plant species (Field *et al.,* 2019), however the preference of MFRE for any particular N-containing compound has not yet been elucidated.

1.8 Limitations in MFRE research

At present our understanding of the MFRE is severely limited, including the breadth and diversity of species encompassed within the group (Thippayarugs *et al.,* 1999). However, advances have been made in recent years as a concerted effort to demystify this enigmatic mycorrhizal fungus. Chiefly, the development of molecular and radioisotope labelling techniques have enabled research to more accurately identify MFRE where it occurs, and how it interacts with hosts in terms of resource exchange. The current state of mycorrhizal and MFRE research is a good basis for making swift progress in understanding this overlooked fungus, however there are significant gaps that must be addressed before further breakthroughs can be made. The major questions to address focus on distinguishing MFRE from their counterparts, AM fungi. How do MFRE partition nutrients to plant partners? How is this affected by different nutritional contexts and addition biotic interactions? Answering these questions will help us address the twin aims of deepening our knowledge of the functions, development, and host range and assessing how it may be affected by the rapidly changing climate.

MFRE are generally believed to perform similar functions to AM fungi, therefore it is not unreasonable to make hypotheses based on the known functioning AM fungi. MFRE are likely to utilise a similar range of nutrient sources as AM fungi in broadly similar proportions, however this may differ somewhat as MFRE are partially saprotrophic organisms and not obligate symbionts unlike AM fungi (Field *et al.,* 2015a) therefore MFRE may have a greater ability than AM fungi to utilise organic sources of nutrition.

At present these outstanding questions have gone unanswered due to the lack of pure MFRE inocula available for in vitro experiments. Some work has been conducted using sieved soils containing MFRE propagules as an inoculum however, these methods are not rigorous enough to exclude non-MFRE fungi from experimental microcosms (Sinanaj *et al.,* 2021) rendering the results of any experimental work conducted using these methods equivocal. Isolates of MFRE for use in monoxenic nutrient tracing experiments are essential for excluding confounding effects of non-target microbiota. To date MFRE has been isolated and cultured twice, the first isolate of which forms the basis of the following work (Hoysted *et al.,* 2023). This pure culture of MFRE allows us to screen potential host

plants for colonisation rapidly and with high throughput, conduct experiments to understand the fundamental characteristics of MFRE in symbiosis with host plants, and develop inoculation methods for non-sterile pot-based experimental systems.

1.9 Key questions, hypotheses, and objectives

The overall aim of this thesis is to establish the functional relationship between MFRE and host flowering plants, focusing on its role in plant N nutrition. Specifically, the following overarching questions are addressed with corresponding hypotheses:

- Do MFRE exhibit a preference for specific sources of N over others for transfer to *Plantago lanceolata* host plants?
    - *Given the relative simplicity, and therefore lower 'cost', of assimilation that small inorganic N sources present, these may be assimilated and transferred to host plants in greater quantities than small organic molecules.*
- Do MFRE maintain N source preferences under a N concentration gradient?
    - *MFRE may be capable of identification and discretionary assimilation of the most 'efficient' N sources in terms of C gain from plant hosts.*
- How does altered availability of N affect the nutritional relationship between MFRE and plant hosts?
    - *Assuming that nutritional dynamics between MFRE and Plantago are broadly governed in a similar manner to other mycorrhizal fungi, Low N will result in more reliance of plant on MFRE-acquired N*
- Is N assimilation and transfer to host plants by MFRE fungi affected by the presence of a soil microbiome?
    - *Mineralisation and transformation of N compounds in soils by non-mycorrhizal microbes, as well as interactions between mycorrhizal fungi and soil bacteria are known to impact the character of mycorrhizal symbioses. Therefore, the presence of these bacteria in tandem with MFRE is likely to have impacts on the nature of the nutritional mutualism.*

The overall aim of this thesis is to expand our current understanding of N dynamics within MFRE-plant symbioses, to begin to determine which factors influence how mutualistic MFRE fungi are when colonising plant hosts, and to understand how their saprotrophic capabilities interact with their symbiotic lifestyle.

To test the hypotheses described above, I conducted several experiments *in*- and *ex-vitro*. The first (**Chapter 2**) describes development of two experimental systems with the objective of determining the N source range of MFRE fungi in symbiosis with plants in the absence of other microorganisms. The second (**Chapter 3** and **Chapter 4**) focuses on how the nutritional context affects the MFRE-plant symbiosis and begins to uncover how MFRE fungi utilise organic N sources. Next, I developed soil-based systems for investigating MFRE-plant symbioses in the context of additional biotic interactions with bacteria in a more ecologically relevant setting (**Chapter 5**). Finally, I discuss the results of these experiments in the context of one another and suggest future directions for research into MFRE both with and without plant hosts (**Chapter 6**).

Chapter 2: Development of monoxenic and soil-based systems to investigate MFRE-plant symbioses

2.1 Introduction

Before a systematic investigation of MFRE-plant nutritional symbioses can begin, it is necessary to first ensure that the experimental systems employed for these purposes can facilitate a functional symbiosis that can be investigated. Previous investigations into MFRE-plant symbioses have been conducted in both monoxenic (two species present in systems only, in this case fungus and plant) microcosms (Hoysted *et al.,* 2023) and soil-based (Field *et al.,* 2015a, 2015b) mesocosms. Both systems require suitable MFRE inoculants that allow the establishment of functional mycorrhizal relationships between MFRE and host plants. Currently, owing to the technical difficulties associated with isolation and pure culture of the fungus, MFRE inoculation methods and experimental systems are extremely limited.

2.1.1 Monoxenic systems

The study of MFRE is a relatively young field, with much of its experimental methodology adapted from research techniques developed for AM fungi including monoxenic systems. One of the major limitations of these systems, however, is the inability of AM fungi to exist in pure culture (Smith & Read, 2008). As obligate biotrophs, AM fungi must associate with live plants from which they gain C. In monoxenic culture, this usually involves culture of AM fungi in association with transformed plant roots ('hair root' transformations using agrobacteria; St-Arnaud *et al.,* 1996) that do not photosynthesise or produce shoots or leaves. This method of fungal culture has a relatively long growth period for full colonisation of a plate (St-Arnaud *et al.,* 1996; Hawkins *et al.,* 2000) to occur. Despite the drawbacks of using non-photosynthetic plant material, these systems have become routine in research into AM symbiosis, particularly mechanistic studies (Goh *et al.,* 2022).

MFRE, as facultative saprotrophs, are not restricted to root organ culture techniques as viable pure isolates can be cultured axenically (Field *et al.,* 2015a; Hoysted et al., 2023). This is an advantage in experiments as un-transformed, fast

growing, photosynthetic plants can be germinated and inoculated with MFRE under sterile conditions. Systems established in this manner are highly controlled, isolating the effects of MFRE colonisation on host plants. To date, the only published example of experimental systems making use of MFRE isolates and monoxenic culture is that of Hoysted et al., (2023). In this study, white clover (*Trifolium repens*) seedlings were cultured with the *Lyc-1* isolate of MFRE in monoxenic systems on modified MSR media (Declerck *et al.,* 1998; Table 2.1) at 27ºC with a 16hr photoperiod.

In order to address the central aims of this thesis investigating the functional relationship between plants and MFRE with nutrient tracing studies, it was necessary to refine the methods of Hoysted et al. (2023) and develop novel monoxenic experimental systems using *Plantago lanceolata* (ribwort plantain) as the focal plant species and the MFRE isolate Lyc1.

Table 2.1 Composition of MSR nutrient media used by Hoysted et al., (2023)

Compound	M.Wt	$mg.L^{-1}$
$MgSO_4.7H_2O$	246.48	739
KNO_3	101.10	76
KH_2PO_4	136.086	4.1
$Ca(NO_3)_2.4H_2O$	236.15	359
NaFeEDTA	367.047	8
KCl	74.5513	65
$MnSO_4.4H_2O$	151.001	2.45
$ZnSO_4.7H_2O$	287.54	0.29
H_3BO_3	61.83	1.86
$CuSO_4.5H_2O$	250	0.24
$(NH_4)_6Mo_7O_{24}.4H_2O$	1235.86	0.035
$Na_2MoO_4.2H_2O$	205.92	0.0024

2.1.2 Soil-based systems

Despite the practical and scientific advantages of monoxenic systems, they are limited in their ecological relevance. Therefore, the development of reliable soil-based inoculation methods is another key step in generating consistent and reliable experimental systems to allow direct comparisons between datasets generated from experiments. Previous experiments using MFRE-colonised plants in soil-based systems have provided invaluable early insights into the functionality of these symbioses (Field *et al.,* 2015a, 2015b; Hoysted *et al.,* 2019). However, these experiments made use of wild-collected plants replete with MFRE symbionts and rhizosphere soil. The collection of adult plants from the wild is labour-intensive, costly, and time consuming. There are also ethical considerations that must be taken into account, including the duty of the scientific community to minimise the disturbance and potential contamination of natural habitats (E.g. Thursley common, UK; Hoysted *et al.,* 2023) with foreign species. With the increasing research interest surrounding MFRE, the collection without replacement of wild adult populations of MFRE-colonised plants should be minimised to avoid damaging these populations through over-collection.

There are many protocols for the production of sterile and non-sterile AM fungal inocula (Walker & Vesberg, 1994; Brundrett *et al.,* 1996; Vimard *et al.,* 1999). Many of these make use of pre-colonised root fragments from plants inoculated with one or multiple strains of AM fungi. These protocols were initially developed to study AM fungal symbioses with plants as monoxenic culture methods had not yet been developed, with the first monoxenic method developed by St-Arnaud *et al.* (1996). These systems are also useful as experimental mesocosms in themselves and can be modified as needed to simulate many different ecological contexts (Mueller *et al.,* 2004). These systems are still widely used in AM fungal research for the same reasons (Yadav *et al.,* 2021; Pandit *et al.,* 2022; Zhang *et al.,* 2023). Therefore, to generate data from investigations into MFRE-plant symbioses that can be directly compared to the existing AM literature, it is imperative that soil-based cultures of MFRE be developed.

I used a combination of existing methods for the production of non-sterile soil-based AM fungal inoculum (Brundrett *et al.,* 1996; Vimard *et al.,* 1999) to develop pot cultures of MFRE from which to generate a non-sterile root inoculum. I then verified the viability of this inoculum by establishing a colonisation time series using a commonly studied cereal crop. Wheat was chosen as the model species for this trial as wheat is known to be colonised by AM fungi (Pellegrino *et al.,* 2015) and has high economic and strategic importance (UKRI, 2023). Additionally, MFRE have been identified in arable soils, including those where wheat had been cultivated (Albornoz *et al.,* 2021). Wheat was also used here as an example to investigate whether a common crop species is susceptible to MFRE colonisation.

2.3 Aims and research questions

The aims of this chapter are to develop viable experimental systems to investigate plant interactions with MFRE fungi. I addressed the following key questions:

- Can MFRE colonise *Plantago lanceolata* in agar-based monoxenic experimental systems?
- Can axenic MFRE plates be used to inoculate plants in soil-based systems and does this allow establishment of pot cultures of MFRE-colonised plants?
- Can pot cultures be used to inoculate non-sterile soil-based experiments?

2.4 Methods

2.4.1 Axenic culture of MFRE fungi

The MFRE isolate *Lyc-1* used throughout this thesis is the same isolate as used by Hoysted et al. (2023), that was originally isolated from *Lycopodiela inundata* (shown in Figure 2.1a-c). Cultures were maintained axenically on the same MSR media used by Hoysted *et al.* (2023). These cultures were maintained under lab conditions, covered with black netting to reduce light penetration and simulate the light conditions of soil. Growth of MFRE hyphae on MSR media (Hoysted *et al.,*

2023; formula in table 2.1) solidified with 0.4% Phytagel (Sigma-Aldrich) was relatively slow, and it was hypothesised that the nutrient-poor conditions were the cause, and that using a more nutrient-rich medium would improve axenic growth. In addition, the greater concentration of C-containing agar in ½GB5, compared to the 0.4% phytagel (Sigma-Aldrich) in MSR may provide a greater C source for MFRE growth.

Table 2.2 Nutrient composition of ½GB5 nutrient solution.

Compound	M.Wt	mg.L^{-1}
$CaCl_2$	110.98	56.62
$MgSO_4.7H_2O$	246.47	125.38
$MnSO_4.H_2O$	151.00	5
$FeSO_4 \cdot 7H_2O$	278.01	13.9
EDTA disodium salt.$2H_2O$	372.24	18.65
$ZnSO_4.7H_2O$	287.60	1
H_3BO_3	61.84	1.5
KI	166.00	0.38
$Na_2MoO_4.2H_2O$	241.95	0.13
$CuSO_4.5H_2O$	249.69	0.01
$CoCl_2.6H_2O$	237.93	0.01
myo-Inositol	180.16	50
Nictotinic acid (free acid)	123.11	0.5
Pyridoxine.HCl	205.64	0.5
Thiamine.HCl	337.27	5
NaH_2PO_4	119.98	65.25
KNO_3	101.10	166.25
$(NH4)_2SO_4$	132.14	8.911

The growth medium, Gamborg B5 basal medium (Sigma-Aldrich; full nutrient composition in table 2.2) at ½ the recommended concentration (1.6 g L^{-1}; Sigma-Aldrich) was buffered with 0.5 g L^{-1} MES (Sigma-Aldrich). The pH of the solution was adjusted to 5.5 using 1M NaOH and was solidified with 1% agar and finally

sterilised in an autoclave. This formulation (hereafter referred to as ½GB5) was used as it facilitated vigorous growth of MFRE cultures.

Under sterile conditions a flame-sterilised scalpel was used to cut small (~0.5-1 cm^3) sections of agar from growing cultures. These were transferred onto solid ½GB5 in 14.5 cm triple-vented petri dishes with 3-5 agar sections per dish. Dishes were sealed with Parafilm® and maintained at 25ºC, covered with netting to reduce light penetration, under a 16hr photoperiod. After 8-12 weeks, where hyphae were visibly colonising a large area of agar, the same technique was used to subculture viable MFRE onto new media under sterile conditions (Figure 2.1 c, d).

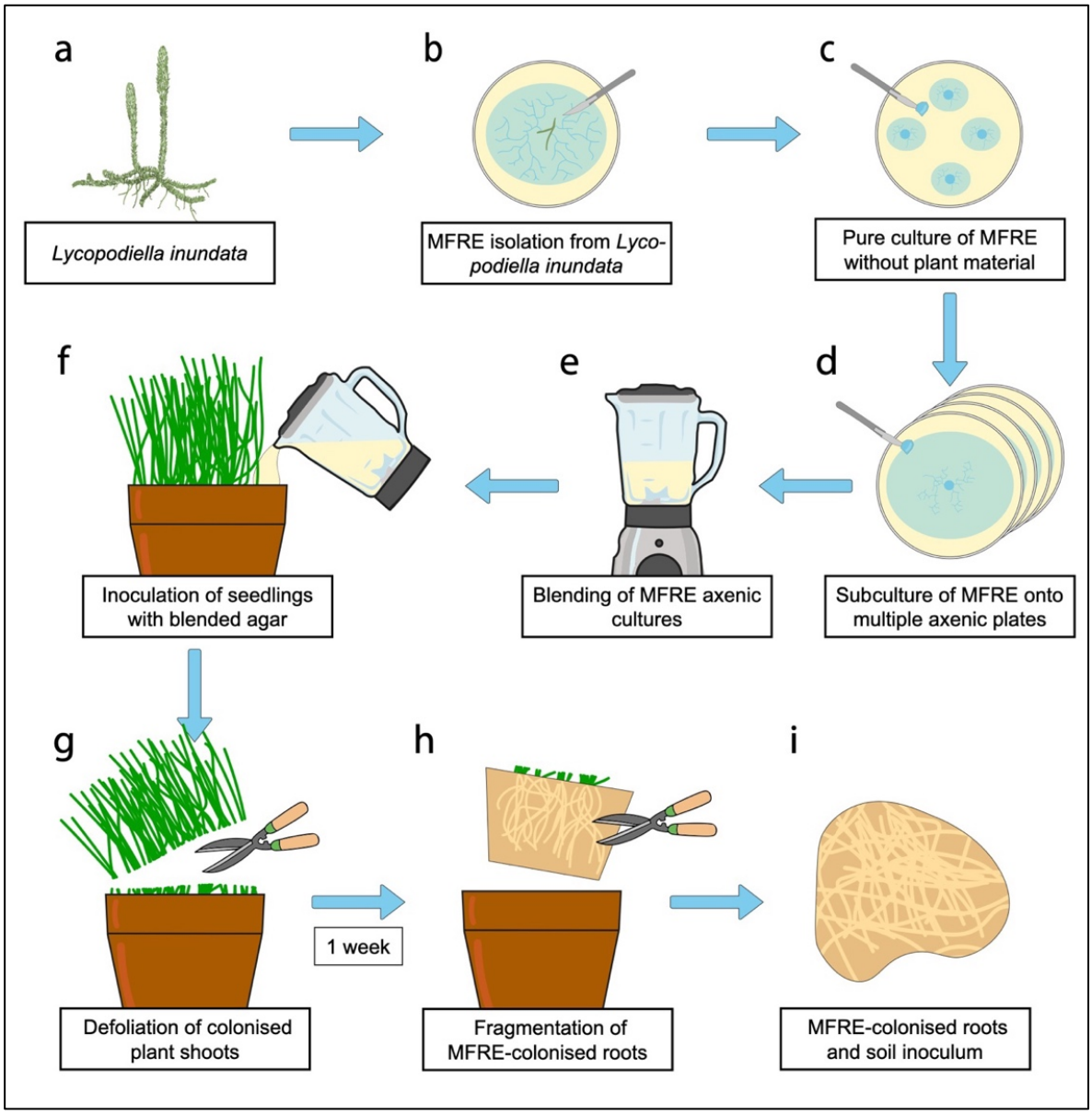

Figure 2.1 Flow diagram of isolation of MFRE and subsequent preparation of non-sterile inoculum. a–c) Isolation of MFRE from *L. inundata* and generation of pure axenic cultures. **d)** Production of multiple MFRE stocks by sub-culturing. **e, f)** Preparation and inoculation of pot culture plants with blended axenic MFRE cultures. **g–i)** Preparation of non-sterile root inoculum from pot MFRE cultures.

2.4.2 Monoxenic agar-based microcosms

Experimental microcosms were established using the same method as Hoysted *et al.,* (2023). *Plantago lanceolata* seeds (Yellow Flag Wildflowers, Gloucester, UK) were sterilised in a 4.5% bleach solution (Lindsey *et al.,* 2017) and transferred to ½GB5 dishes under sterile conditions (in a laminar flow hood using flame-sterilised tools), then maintained at 25ºC with 16:8hr (light:dark; 300 µmol m^{-2} s^{-1}) for 7 days. In the meantime, 50ml autoclaved agar was poured into sterile 14.5cm triple-vented petri dishes placed at an angle so that only 50% of the surface of the dishes were in contact with agar.

7 days after seed sterilisation, individual seedlings were transferred under sterile conditions, to the top edge of the agar in slanted ½GB5 plates with tap root pointing downward in their final growth orientation. Immediately after this, three ~0.5-1 cm^3 sections of MFRE-colonised agar from axenic cultures were placed in close proximity to the seedling taproot. Microcosms were then sealed with Parafilm® and the agar portion of each dish was covered with aluminium foil to reduce light penetration to the plant roots and MFRE hyphae. They were then stacked so that the shoots were growing upright and maintained at 25ºC with 16:8hr (light:dark; 300 µmol m^{-2} s^{-1}). As the seedlings grew, MFRE hyphae spread out from the colonised sub-cultured agar across and below the surface of the agar (Figure 2.2).

2.4.3 Monoxenic colonisation time series

To determine the timepoint at which colonisation is achieved in inoculated monoxenic systems, I conducted a time series experiment. I established 21 monoxenic microcosms using the method described above and with the same light and temperature conditions. Starting one week after microcosms were established,

three were randomly selected to be harvested. This was repeated every week for seven weeks. Microcosms were randomly moved within the growth space weekly to control for positional effects. During each successive harvest, plates were opened and *Plantago lanceolata* shoots were separated from the roots using a scalpel. After this, roots were removed gently from the agar, taking care to remove any severed fragments for staining. Roots fragments had any excess agar removed gently using forceps and were placed into individual histology cassettes for staining.

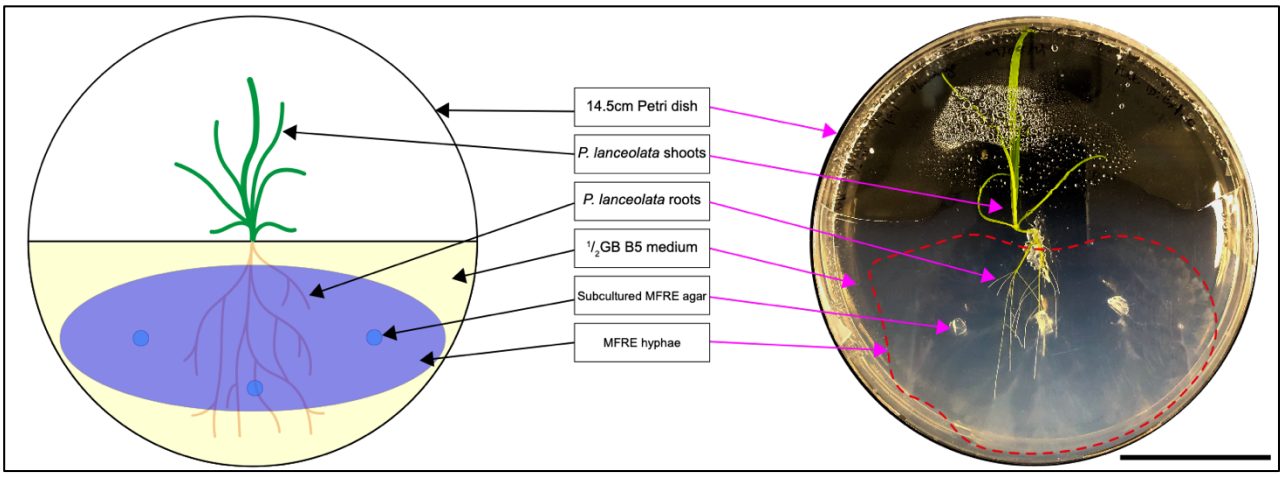

Figure 2.2 Monoxenic microcosms used throughout this thesis. Schematic diagram of experimental microcosms (left) compared with photograph of microcosm (right) with boundary of MFRE hyphal growth indicated (dotted line). Both are labelled with corresponding features. Scale bar = 5 cm.

2.4.4 Preparation of non-sterile inoculum

Seeds of *Plantago lanceolata, Trifolium repens,* and *Holcus lanatus*, known MFRE host plant species (Hoysted *et al*., 2022; Sinanaj *et al.,* 2024; Howard *et al*., 2024), were sterilised according to a method adapted from Lindsey *et al.* (2017). Briefly, seeds were placed in a 4.5% bleach solution for ten minutes. These were then rinsed in sterile deionised water five times by pipetting and then placed in separate petri dishes on damp filter paper under benchtop conditions for seven days.

The growing medium was 4L of a 50:50 (vol:vol) mixture of sand and perlite. These were autoclave sterilised three times with at least one day between autoclaving sessions. The sand:perlite potting medium was placed into a 5 litre circular pot previously soaked in Virkon® overnight. 20-30 pre-germinated seedlings of each species were sown 1cm below the potting medium surface.

A blended agar inoculum was prepared. It consisted of two well-colonised axenic cultures of MFRE on 14.5 cm petri dishes. The agar contents of these cultures (200ml total) were placed into the chamber of a kitchen blender previously soaked in Virkon® to remove microbial contaminants. 200 ml deionised water was added to this and the contents pulse blended to a relatively consistent texture without large (> 2 cm) chunks of agar visible (Figure 2.1e).

The inoculum was poured onto the surface of the sand:perlite medium, taking care not to disturb the seedlings too much (Figure 2.1f). The pot was covered with polythene kitchen film until seedlings were well established (1-2 weeks). The pot was placed into glasshouse conditions (18ºC, 16hr photoperiod), and top watered twice weekly with 200ml deionised water, increasing to 300ml when plants were well established. Watering was adjusted according to the surface moisture level, ensuring not to let it become dry to the touch. The culture was fed with 50ml of liquid ½ Gamborg B5 solution, buffered with 0.5g.L^{-1} MES (Sigma-Aldrich) pH adjusted to 5.5, which was added before watering once a week.

This culture was maintained under these conditions for four months. Plant shoots were checked regularly for signs of disease or pests, flowers were removed by hand to prevent senescence.

To generate an inoculum, plants were defoliated using secateurs sterilised with 70% ethanol, ensuring to remove as much shoot material as possible (Figure 2.1g). After a one-week period to encourage fungal sporulation (Quilliam *et al.,* 2010), the plant roots and medium were removed from the pot and placed in a sterilised tray where roots were cut by hand using secateurs into ~1-2 cm long pieces (Figure 2.1h, i). At this point, sub samples of root material were taken, cleaned of sand in tap water, and placed in a histology cassette for staining. Once homogenised, the

mixture of sand, perlite, and plant roots were used to inoculate seedlings in new pots.

2.4.5 Inoculation of wheat using non-sterile inoculum

To test the efficacy of the non-sterile inoculum, I established a colonisation time series, wherein three wheat cultivars (Skyfall, Avalon, and Cadenza) were inoculated using colonised pot culture roots. These were destructively harvested after eight, ten, and twelve weeks post inoculation.

Seeds of three wheat varieties (Skyfall, Avalon, and Cadenza; cultivars known to form nutritionally functional – albeit variable between cultivars in terms of % colonisation and amount of C-for-N/P exchange - AM symbioses; Thirkell *et al*., 2019) were surface sterilised by immersion in 4.5% bleach solution for 10 minutes (Lindsey *et al.,* 2017). After this time seeds were rinsed in sterile deionised water five times by pipetting. They were then germinated on damp filter paper under bench conditions for seven days prior to planting.

Pot culture inoculum was prepared as above on the same day as the experiment was established. 21 1-litre pots were filled with a potting mixture comprising 500g triple autoclaved 50:50 (vol:vol) sand:perlite combined with 150g (fresh weight) of pot culture root MFRE inoculum (root fragments and sand/perlite mixture). Germinated seedlings were planted one per pot with seed 1-2 cm below the potting medium surface.

Wheat plants were then maintained under glasshouse conditions (18ºC, 16hr photoperiod) with positions in the growth chamber regularly changed at random. Plans were watered from the top three times weekly with 50 ml deionised water, and fertilised once weekly with 10ml liquid ½GB5 (buffered with 0.5 $g.L^{-1}$ MES; Sigma-Aldrich; pH adjusted to 5.5, without agar added).

At each harvest time point (8, 10, and 12 weeks post establishment), three replicates per cultivar were randomly selected for harvest. Whole plants were excised from potting medium and all sand and perlite washed from the roots in tap water. 2-3 cm root samples were taken from the growing tips, middle, and stem base of the root system to gain data on a representative sample of the whole root

system. These samples were placed in individual histology cassettes, stained, and colonisation quantified.

2.4.6 Root staining and colonisation counting

Following the methods of Hoysted *et al.* (2023), root samples were placed into individual histology cassettes and immersed in 10% KOH for 1hr at 70ºC. They were then rinsed in tap water and placed into ink-vinegar stain (5% Pelikan Brilliant Black 4001 ink, 5 % acetic acid, 90 % dH_2O; Vierheilig *et al.,* 1998) for 1hr. After this, roots were washed in tap water and placed into 1% acetic acid overnight to allow excess stain to leach out from root tissues to ensure only fungal biomass was stained.

Stained roots were sectioned into ~1cm lengths which were then mounted on slides in polyvinyl lacto-glycerol (PVLG; ~20 1cm root sections per slide, 1 slide per microcosm) and gently compressed so that all cell layers were visible when magnified. Colonisation counts were conducted at 100x magnification according to McGonigle (1990). In brief, 100 unique fields of view per slide were assessed viewed on a compound microscope (Ceti Max II; Medline Scientific, Chalgrove, UK) with a hairline eyepiece graticule for incidence of hyphae and hyphal swellings. Where fungal structures intersected with the graticule, their presence was recorded.

2.4.7 Statistical Analyses

All statistical analyses were conducted using (R Development Core Team, 2023) and R (v2023.3.0.386, R Studio team, 2023) [using packages ‘dplyr’, ‘car’, ‘rosetta’, ‘stats’, ‘agricolae’]. Colonisation data were analysed using 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 S2.1). Figures were created in R (v2023.3.0.386, R Studio team, 2023) using the ‘ggplot2’ package.

2.5 Results

2.5.1 *Plantago lanceolata* colonisation in monoxenic culture time series

After incubation, *Plantago lanceolata* roots were assessed for the presence of MFRE colonisation structures (Figure 2.3). Initial statistical testing failed to identify any differences between mean colonisation based on time point (ANOVA: $F_{6,14}$ = 2.4512, $p > 0.05$). However, post hoc testing revealed a significant increase in total colonisation between one- and two-weeks post inoculation (Tukey's HSD: $p < 0.05$). All plants harvested at time points after week 2 were not significantly more colonised than either week one or two. There is greater variation in mean colonisation until week 4, beyond this time point the mean colonisation stabilises at 25-35%.

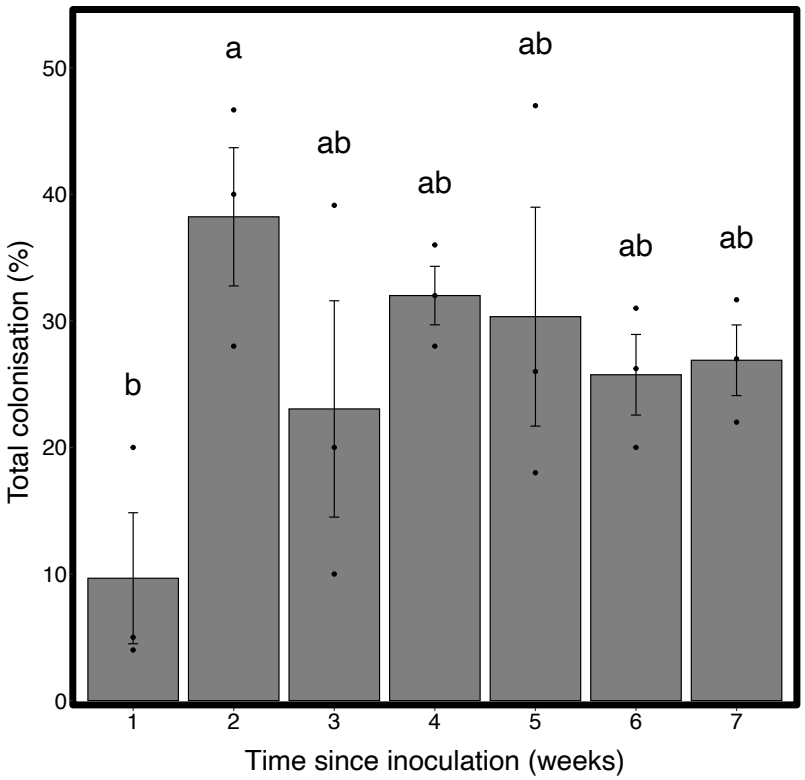

Figure 2.3 Comparison of mean total colonisation (%) of *Plantago lanceolata* roots on subsequent weeks after inoculation. Different letters denote significantly different means (Tukey's HSD: $p < 0.05$; n = 3 per time point).

2.5.2 MFRE colonisation of pot culture roots

Samples of roots were taken from pot inoculum after sectioning into ~1-2cm pieces. These were stained and images captured under 100 or 400x as indicated (Figure 2.4) (Leica DM6; Leica Microsystems, Wetzlar, Germany). Characteristic MFRE structures such as fine branching hyphae can be observed colonising soil-grown roots.

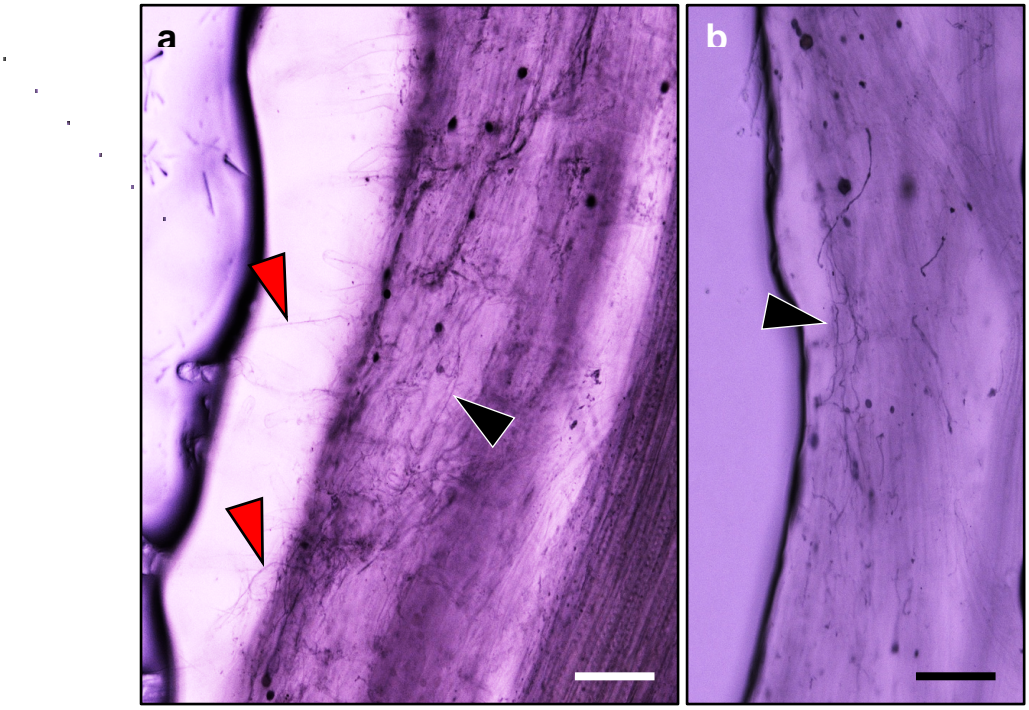

Figure 2.4 Micrographs of MFRE colonising roots from pot culture after 4 months. MFRE Hyphae is visible within plant root epidermal cells (**a, b**; black wedges) and root hairs (**a,** red wedges). 100x objective magnification. Scale bars = 100 µm.

2.5.3 Colonisation of wheat plants with non-sterile MFRE inoculum

Colonisation of wheat plants by MFRE structures was observed at all time points. After ink-vinegar staining, characteristic MFRE structures were observed in plant root epidermal cells and root hairs (Figure 2.5). Initial statistical testing indicated that cultivar was a significant driver of the level of colonisation (Table S2.1; ANOVA: $F_{2,9}$= 6.3304, $p < 0.05$), however, there was no significant interaction was observed between cultivar and tome point (ANOVA: F4,9 = 2.0764)

post-hoc testing revealed no significant differences in colonisation between plants harvested at the same time (Figure S2.1; Tukey's HSD: $p > 0.05$). Therefore, data for different cultivars were pooled to increase replicates at each time point.

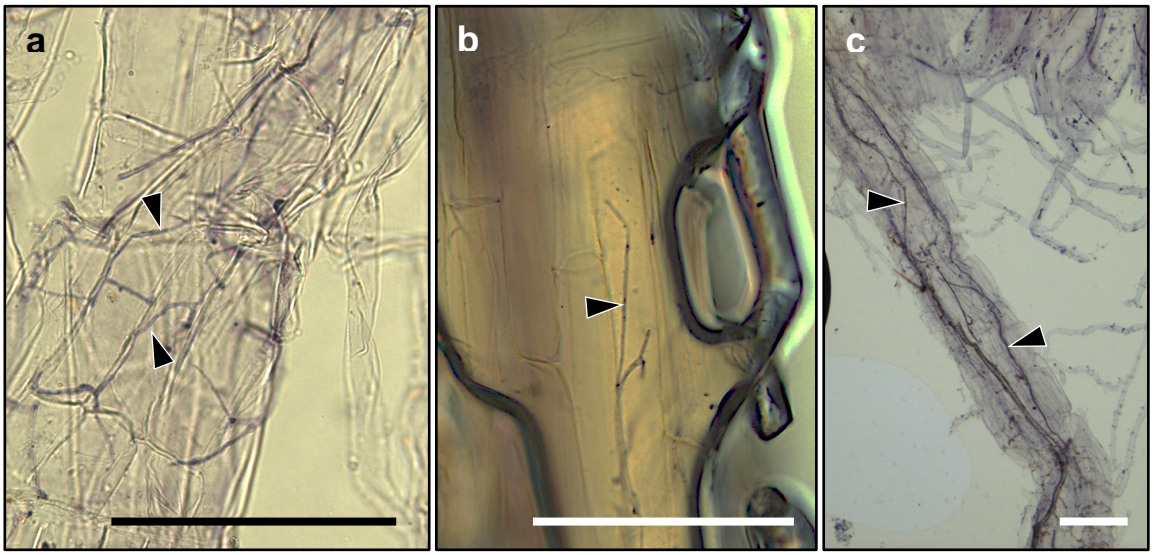

Figure 2.5 Micrographs of MFRE structures colonising wheat roots. Branching hyphae can be seen within root epidermal cells (black wedges). **a, b)** 400x objective magnification. **c)** 100x objective magnification. Scale bars = 100 µm.

This pooled data reveals time as a significant driver of colonisation (ANOVA: $F_{2,15} = 24.076$, $p < 0.001$), with increased colonisation after ten and twelve weeks than after only eight (Figure 2.6; Tukey's HSD: $p > 0.05$). The mean total colonisation at twelve weeks after inoculation is 60.5%, much greater than in monoxenic systems, which was 26.9% after seven weeks. This is much more similar however to the level of wheat colonisation in soil systems after eight weeks which was 34.1% (Figure 2.6).

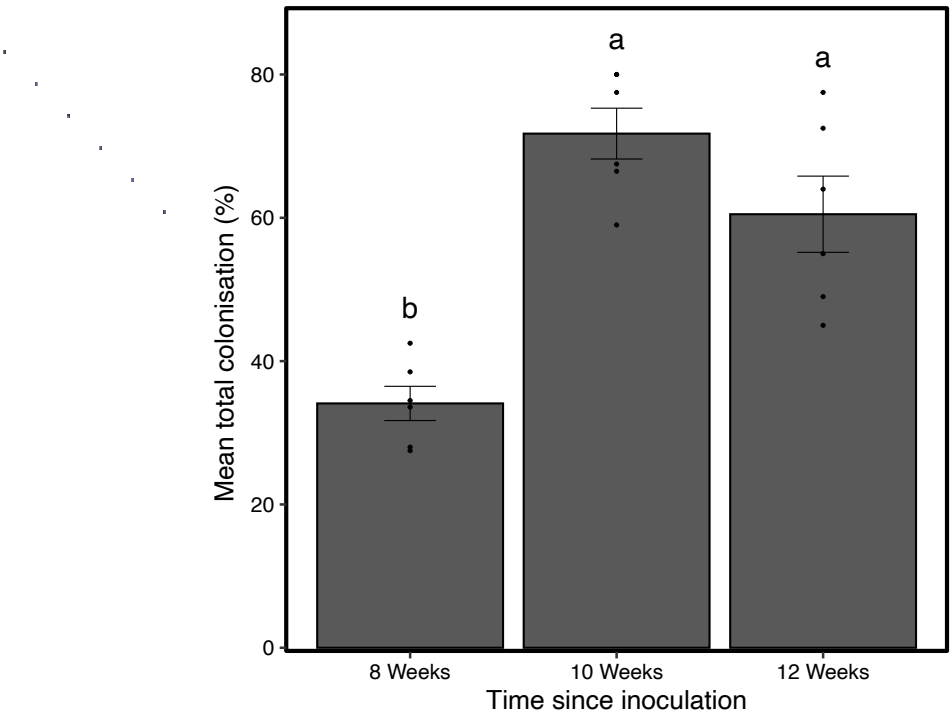

Figure 2.6 Comparison of mean total colonisation (%) of wheat roots at 8, 10, and 12 weeks after inoculation with non-sterile inoculum. Different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 6 per time point.

2.6 Discussion

The aim of this chapter was to further develop existing experimental systems and methods of inoculating plants with MFRE for experimental purposes. This was met through the establishment of two colonisation time series experiments and ink-vinegar staining to visualise and quantify MFRE structures in plant roots. I have demonstrated the successful colonisation of plants with MFRE in monoxenic systems. I have also successfully established pot cultures to inoculate plants in soil-based systems.

The levels of colonisation I observed differed between the systems utilised. In the monoxenic systems the final level of colonisation achieved in *Plantago lanceolata* roots after seven weeks was 31.7%. This is lower than the final observed colonisation in wheat plants after twelve weeks using pot culture inoculum (60.5%).

This discrepancy is potentially due to the different periods of time these systems were allowed to grow for. Additionally, there may be impacts of species tested; the wheat plants harvested at the same time were no different in terms of colonisation based on cultivar, were colonised much more thoroughly than *Plantago lanceolata* seedlings.

The colonisation data obtained from the monoxenic time series (Figure 2.3) reveals the presence of MFRE hyphae in plant roots after only two weeks, albeit at a low (~20%) level. This increases over subsequent time points to peak at two weeks and to plateau at an average of 31.7% colonisation at seven weeks post inoculation. This rapid and stable colonisation is comparable to that seen in soil-based studies of AM fungi (Ordoñez *et al.,* 2016) and falls within the range of total colonisation observed in some monoxenic studies of AM fungi (Kirk *et al.,* 2005; Labidi *et al.,* 2011).

I developed a method of inoculating sterilised seedlings and growth media with blended agar inoculum (Figure 2.1e, f). MFRE structures were observed in the roots of adult plants after 4 months (Figure 2.4). This method was used to produce an inoculum which was used to inoculate wheat plants over the course of a time series from 8 weeks to 12 weeks, the growth period previously used in soil-based studies of MFRE symbiosis (Field *et al.,* 2015a, 2015b; Hoysted *et al.,* 2019).

The data from this time series reveals that wheat plants are susceptible to colonisation by typical MFRE structures (Figure 2.5). Plant roots contained significantly increased colonisation by MFRE hyphae between 8 and 10 weeks after mesocosms were established (Figure 2.6).

Both monoxenic and soil-based systems have individual drawbacks. Monoxenic systems are not representative of natural systems as they cannot account for the biotic and abiotic complexities of soil systems. Conversely, while soil-based systems address many of the issues with ecological relevance that monoxenic systems have, they have other drawbacks such as higher maintenance requirements, longer experimental time periods, and greater labour of harvesting and processing experimental samples.

To fully validate the results from each of these systems a combined approach is needed, utilising both monoxenic systems and soil-based mesocosms, to draw in all aspects of mycorrhizal research to investigate MFRE in symbiosis with plants. The work presented here represents a significant step in experimental MFRE inoculation techniques. The two systems I have tested bring the systems available for studying MFRE-plant symbioses close to parity with AM fungal research. I have developed two methods that generate well-colonised plants in relatively short time periods. Both of the methods described in this chapter allow greater control over MFRE inoculation than previously described methods.

The monoxenic systems using ½GB5 medium are used in **Chapter 3** and **4** of this thesis to conduct nutrient tracing experiments. I conduct a soil-based experiment in **Chapter 5** using the same blended plate inoculation method used to inoculate the pot cultures.

Chapter 3: Nitrogen source preference and transfer to host plants by MFRE

3.1 Introduction

The co-colonisation of plants by MFRE and AM fungi appears to occur regularly (Field *et al.,* 2016; Albornoz *et al.,* 2021). Because of this co-localisation *in* and *ex planta,* there is strong potential for functional role sharing between the two groups, potentially resulting in complementary nitrogen (N) and phosphorus (P) transfer to plant hosts. When dual MFRE-AM fungal associations are formed by the liverworts *Allisonia* and *Neohodgsonia*, MFRE and AM fungi supply host plants with greater amounts of N and P than the other respectively compared to when in single colonisation in other liverwort species (Field *et al*., 2016). MFRE promotion of plant N uptake is not limited to non-vascular plants; MFRE transfer ammonium-N to the host plant *Lycopodiella inundata*, in return for photosynthetically fixed carbon (C; Hoysted *et al*., 2019; 2021). Likewise, flowering plants also gain nutritional benefits directly from MFRE associations; *Trifolium repens* (white clover) for instance forms mycorrhizal associations with MFRE, assimilating fungal-acquired N and P in return for plant-fixed C resources in monoxenic cultures (Hoysted *et al.,* 2023). This demonstration of direct involvement of MFRE in plant N nutrition provides a basis on which to expand our knowledge of how MFRE functions as a nutritional symbiont of vascular plants.

Experimental evidence for MFRE involvement in plant N acquisition is currently limited to a single source of inorganic N, ammonium chloride (Hoysted *et al.,* 2023). Ammonium (NH_4^+)-N is preferentially transferred to host plants by AM fungi over other N-containing compounds (Johansen *et al*., 1996; Toussaint *et al*., 2004) and, thus, is often used in experiments (e.g. Ames *et al.,* 1983; Yang *et al.,* 2014; Field *et al.,* 2015a; Hoysted *et al.,* 2023). Although some organic sources are also assimilated by some AM fungi (see **1.4.1**)

Considering the putative saprotrophic capabilities of MFRE (Field *et al*., 2015a, 2019; Hoysted *et al.,* 2023) and the recent indication that these fungi may access, assimilate, and transfer N derived from complex organic matter to plant hosts (Field *et al*., 2019), the possibility that MFRE access and assimilate N from a variety of sources, which could feasibly be transferred to host plants, remains open.

Therefore, the dual colonisation of plants by both MFRE and AM fungi and dual roles in host nutrient acquisition may not be limited to transfer of different macronutrients to hosts but could potentially take the form of different symbionts both accessing and transferring N to the host but from different sources in the soil.

The apparent preference of AM fungi for assimilating inorganic sources, such as NH_4^+ and nitrate (NO_3^-; Johansen *et al*., 1996; Toussaint *et al*., 2004) is likely due to the relative simplicity and therefore low energetic cost of assimilating these molecules compared to organic N-containing compounds. Similarly, NH_4^+ is favoured over NO_3^- likely due to the higher energetic cost of NO_3^- reduction than direct NH_4^+ uptake (Johansen *et al*., 1996; Marzluf, 1997; Toussaint *et al*., 2004). The cost of N assimilation is met through supply of hexoses and lipids to AM fungi by their host plants (Shachar-Hill *et al*., 1995; Keymer *et al.,* 2017). However, given that MFRE are putatively facultative saprotrophs (Field *et al*., 2015a), it is possible that at least some of the energetic cost of assimilation of N from sources in the soil may be ameliorated through saprotrophic C acquisition. Hence, MFRE may have developed a niche by offsetting fungal demand on host plant C resources, while providing plants access to a wider pool of soil organic N from sources that AM fungi alone would be incapable of accessing.

Organic compounds are a significant component of soil N; amino acids comprise a significant component of plant N content (Inselsbacher *et al.,* 2011), specifically glycine is one of the most abundant free amino acids found in soils (Moe*,* 2013). Crucially, AM fungi transfer N derived from glycine to plant in both monoxenic and pot-based systems (Hawkins *et al.,* 2000). Therefore, I hypothesise that MFRE can transfer multiple N-sources, organic and inorganic, to hosts in return for plant C. Additionally, I hypothesise that MFRE will preferentially assimilate N from inorganic sources when they are also supplied with organic ones due to the extra C cost of transferring organic N resulting from the production of enzymes necessary for the degradation of organic molecules. Using *Plantago lanceolata* colonised by an MFRE isolate (*Lyc-1*; Hoysted et al., 2023) in monoxenic microcosms, we investigated the ability of MFRE to access, assimilate, and transfer ^{15}N from a selection of inorganic and organic compounds commonly found in soils (Pankoke *et al.,* 2015; Kang *et al.,* 2015).

With a series of single 'N source' and 'fungal choice' experiments, we simultaneously quantified the allocation of host plant photosynthates to MFRE mycelium, and N transfer to the host, for multiple N sources.

3.2 Key research questions

- What sources of nitrogen do MFRE take up and transfer to *Plantago lanceolata* hosts?
- How much plant-fixed C is provided to MFRE in return for N sources?
- Is there a preference by MFRE for N sources based on whether they are organic or inorganic?
- Does MFRE discriminate between N sources for transfer to host plants when there are multiple present?

3.3 Methods

3.3.1 Fungal inoculum

MFRE isolate *Lyc-1,* initially from *Lycopodiella inundata* (Hoysted et al., 2019; see **chapter 2**), was maintained on Gamborg B5 basal medium at 50% concentration (1.6g.L^{-1}; Sigma-Aldrich) buffered with 0.5g.L^{-1} MES (Sigma-Aldrich) solidified with 1% agar (referred to hereafter as ½GB5). Cultures were kept in the dark and incubated at 25ºC. Subcultures were regularly established under sterile conditions to fresh media at 8–12-week intervals.

3.3.2 Experimental microcosms

Using monoxenic *Plantago lanceolata*-MFRE microcosms (below), two experiments were established:

(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 compounds abundant in soils and transferred to plants by AM fungi (Hawkins *et al.,* 2000; Reay *et al.,* 2019) (ammonium, nitrate, urea, glycine)

were supplied to MFRE mycelium and traced into plant tissues. Simultaneously, plant C was traced into MFRE mycelium (Fig 3.1a).

(ii) To investigate MFRE ^{15}N source preference, we conducted a ***'fungal choice'*** experiment whereby microcosms were simultaneously labelled with the same four sources of N as the 'N source experiment', providing a choice of N source to MFRE mycelium (Fig. 3.1b).

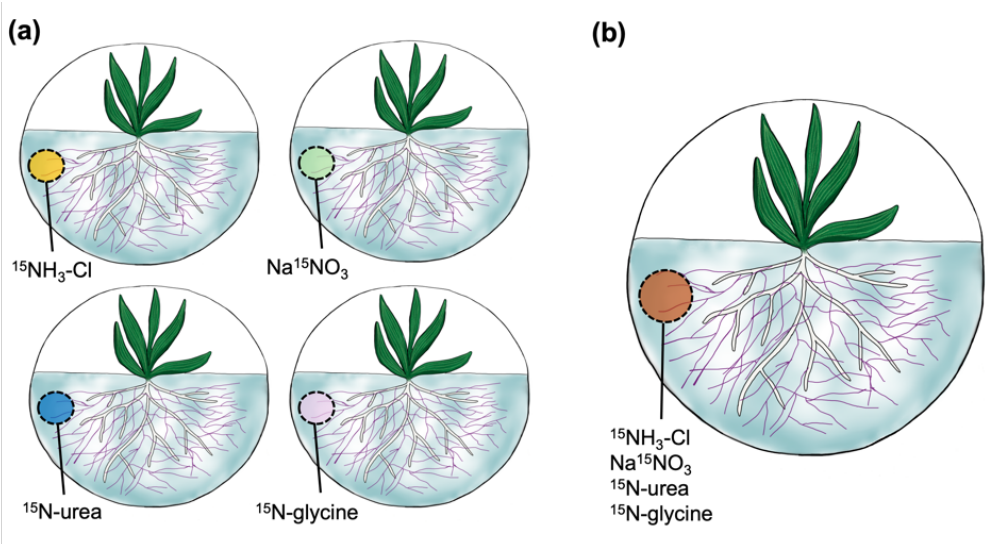

Figure 3.1 Monoxenic microcosm experiments for tracing movement of C and N between Mucoromycotina fine root endophyte fungi and *Plantago lanceolata* symbionts. (a) *'N source'* experiments where N addition sites contain one of $^{15}NH_3Cl$, $Na^{15}NO_3$, ^{15}N-urea or ^{15}N-glycine. **(b)** *'Fungal choice*' experiments where well contains a mixture of all sources used in **(a).**

Monoxenic microcosms (Fig. 3.1) were established using *Plantago lanceolata,* a commonly used plant model for AM studies (Pankoke *et al.,* 2015). These seedlings (Yellow Flag Wildflowers, Gloucester, UK) were colonised with MFRE (*Lyc-1)* hyphae introduced from axenically-grown stocks (see **2.4.1**). In brief, 140 mm sterile triple vented Petri-dishes were filled with ~60 mL of ½GB5 (containing 187.4 µg g^- total N; See table 2.2 for full nutrient breakdown) poured on a gradient that allows for plant development in an upright position. *Plantago lanceolata* seeds were sterilised in a 4.5% bleach solution for ten minutes (Lindsey *et al.,* 2017)

before being rinsed five time in sterile deionised water. These were transferred to flat 'nursery' plates of ½GB5 under sterile conditions and maintained under 16:8hr (day:night) at room temperature to germinate. 7 days after sterilisation, individual germinated seedlings were transferred to experimental microcosms under sterile conditions. At the same time, 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.

3.3.3 ^{15}N and ^{14}C isotope tracing

To investigate the capability of MFRE to assimilate and transfer N to the host plant from diverse sources we conducted the '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 ($^{15}NH_4Cl$, 27.53µg^{15}N; Sigma-Aldrich), sodium nitrate ($Na^{15}NO_3$, 17.44µg^{15}N; Sigma-Aldrich), glycine ($C_2H_5{}^{15}NO_2$, 19.72µg^{15}N; Sigma-Aldrich) and Urea ($CH_4{}^{15}N_2O$, 48.36µg^{15}N; Sigma-Aldrich) and solidified 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. These consisted of uninoculated plants (n = 10 for each ^{15}N treatment apart from ^{15}N-ammonium chloride which n = 9 due to microbial contamination).

In the 'fungal choice' experiment, seven weeks after inoculation with MFRE, wells were filled with 25µL of a 4mg.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 (0.1mg compound per source, 0.4mg compound in total per microcosm). Each well was backfilled with ½GB5 as previously described. In total the fungal

'choice' experiment comprised 10 fungal experimental microcosms with 10 non-fungal control microcosms per treatment. To control for any diffusion of isotope through the agar medium, non-fungal microcosms were established (n = 10 per treatment).

For both sets of microcosms, plant shoots were harvested and freeze-dried after 24 hrs of incubation post introduction of the ^{15}N source. Between 0.1 and 5mg freeze-dried and homogenised shoot tissue were measured into tin capsules (Sercon, Crewe, UK) and the relative 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. Plant tissue concentration of ^{15}N was calculated using the following equations from Cameron *et al.* (2006):

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

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, 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.

In addition, in microcosms where MFRE-mediated transfer of ^{15}N to plant hosts was observed, the total mass of ^{15}N in the plant shoots was divided by the total mass of ^{15}N supplied to obtain the percentage of ^{15}N transferred to plant hosts.

For the 'N source' microcosms only, immediately after ^{15}N addition the surface of the agar was covered with clear PVC sealed with anhydrous lanolin and a 0.25 MBq $^{14}CO_2$ pulse was liberated into the headspace of sealed plates from 6.75µl ^{14}C-labelled sodium bicarbonate (2.14 GBq/mmol) by the addition of and 2 ml 90% lactic acid. Microcosms were incubated for 24 hrs to allow for $^{14}CO_2$ fixation and

movement of ^{15}N and ^{14}C between plants and MFRE. At the end of the labelling period, 2M KOH was introduced into small containers within the microcosms to absorb any remaining $^{14}CO_2$. After an hour, all plant materials were removed carefully from the agar, separating plant shoots from roots, and removing as much excess agar from root material as possible prior to freeze-drying. The agar (containing MFRE fungal mycelium) 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 C (^{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 C between fungal and non-fungal plants is equivalent to the total C transferred from plant to MFRE within the fungal microcosms. Total C assimilated by the plant was calculated using the following equations:

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

Where T_{pf} = Transfer of C from plant to fungus, A = radioactivity of the 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 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.

3.3.4 Colonisation of *Plantago lanceolata* roots by MFRE

A subset of freeze-dried root material was sampled and stained using 5% ink-vinegar according to a modified method from (Vierheilig *et al.,* 1998). Briefly, roots were places 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_20) 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.

3.3.5 Statistical Analyses

All statistical analyses were conducted using (R Development Core Team, 2023) and R (v2023.3.0.386, R Studio team, 2023). [using packages 'dplyr', 'car', 'rosetta', 'stats', 'agricolae'] 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 S3.1), or a non-parametric test Kruskal-Wallis with Dunn's post hoc test (as indicated) was conducted. Plant biomasses were 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. Figures were created in R (v2023.3.0.386, R Studio team, 2023) using the 'ggplot2' package.

3.4 Results

3.4.1 Plant growth

In single 'N source' microcosms, plant roots colonised by MFRE were not significantly greater in biomass than those without MFRE (Fig. 3.2a; Table S3.1). However, shoot biomass (Fig. 3.2b) was significantly impacted by inoculation with MFRE (ANOVA; $F_{1,110}$=7.05, p<0.01) and by the ^{15}N treatment applied (ANOVA; $F_{3,110}$=3.29, p<0.05), although there was no significant interaction (Table S3.1). However, post-hoc testing revealed no significant differences in mean shoot biomass based on MFRE inoculation of plants treated with the same ^{15}N source (Tukey's HSD; p > 0.05). In the 'fungal choice' microcosms, no significant

differences were observed in the dry masses of plant roots (Fig. 3.2c) or shoots (Fig. 3.2d; Table S3.1)

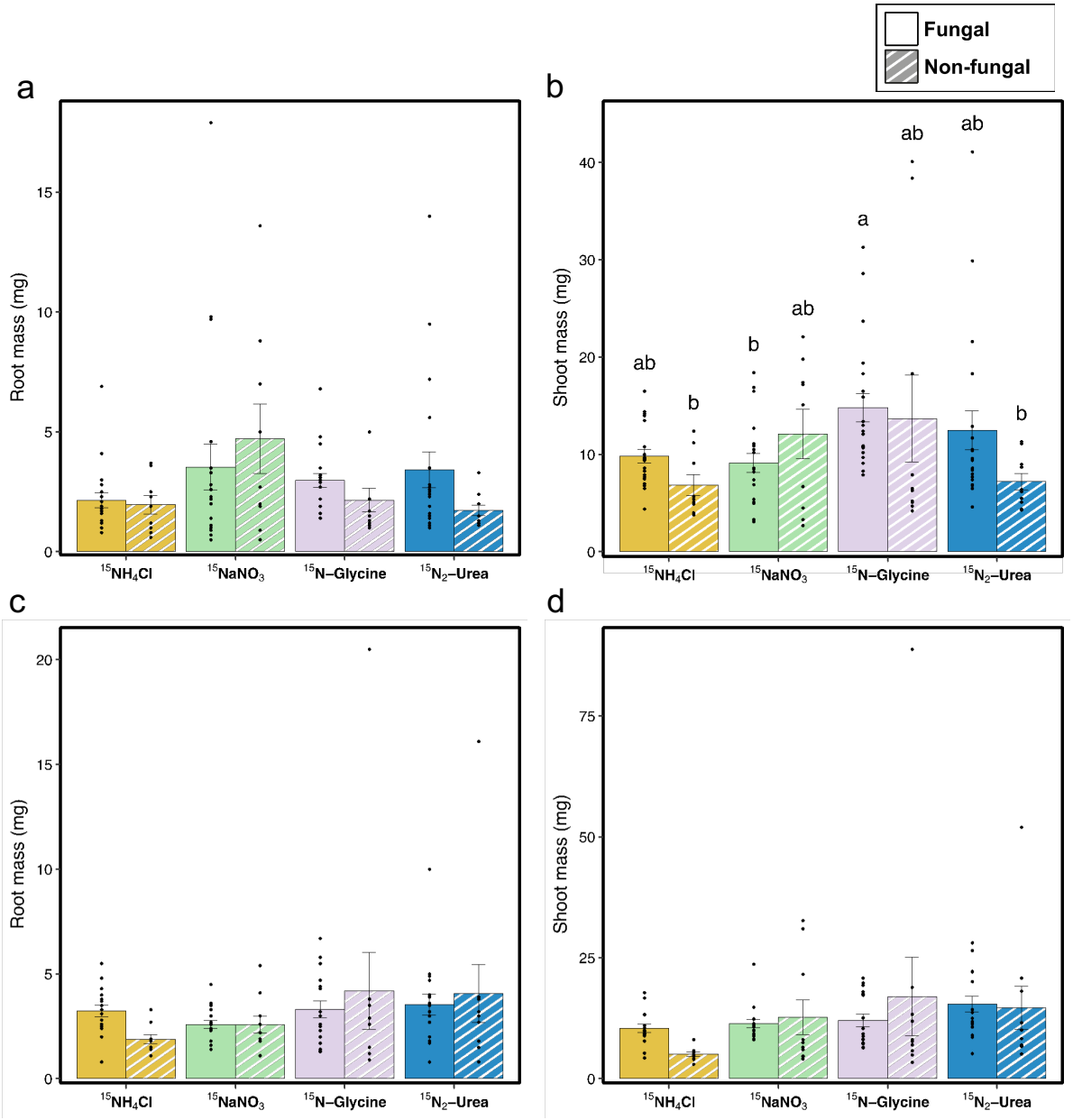

Figure 3.2 Biomass (mg) of plant roots (a & c) and shoots (b, d) in single 'N source' experiment (a & b) and 'fungal choice' (c & d) experiment compared between fungal (solid bars) and non-fungal (hatched bars) microcosms. Error bars indicate ±SE, different letters denote significant difference (Tukey's HSD: $p<0.05$). **a & b;** n = 20 per fungal treatment, n = 10 per non-fungal treatment apart from $^{15}NH_4$ which n = 9) and **c & d** (n = 17 per fungal treatment apart from $^{15}NH_4$ which n = 16, n = 10 per non-fungal treatment).

3.4.2 Plant shoot N concentration

In the 'N source' microcosms, significant effects on total N concentration (Fig. 3.3a) was only observed based on fungal inoculation (ANOVA; $F_{3,109}$=7.56, p<0.001), with both inoculated and uninoculated plants treated with $Na^{15}NO_3$ having greater ^{15}N concentrations than inoculated plants treated with $^{15}NH_4Cl$ (Tukey's HSD: p<0.05). Total N concentration in 'fungal choice' microcosms was driven by ^{15}N treatment applied (ANOVA; $F_{3,99}$=7.55, p<0.001; Fig. 3.3b) with both inoculated and uninoculated plants treated with $Na^{15}NO_3$ having greater N concentrations than inoculated plants treated with $^{15}NH_4Cl$ (Tukey's HSD: p<0.05).

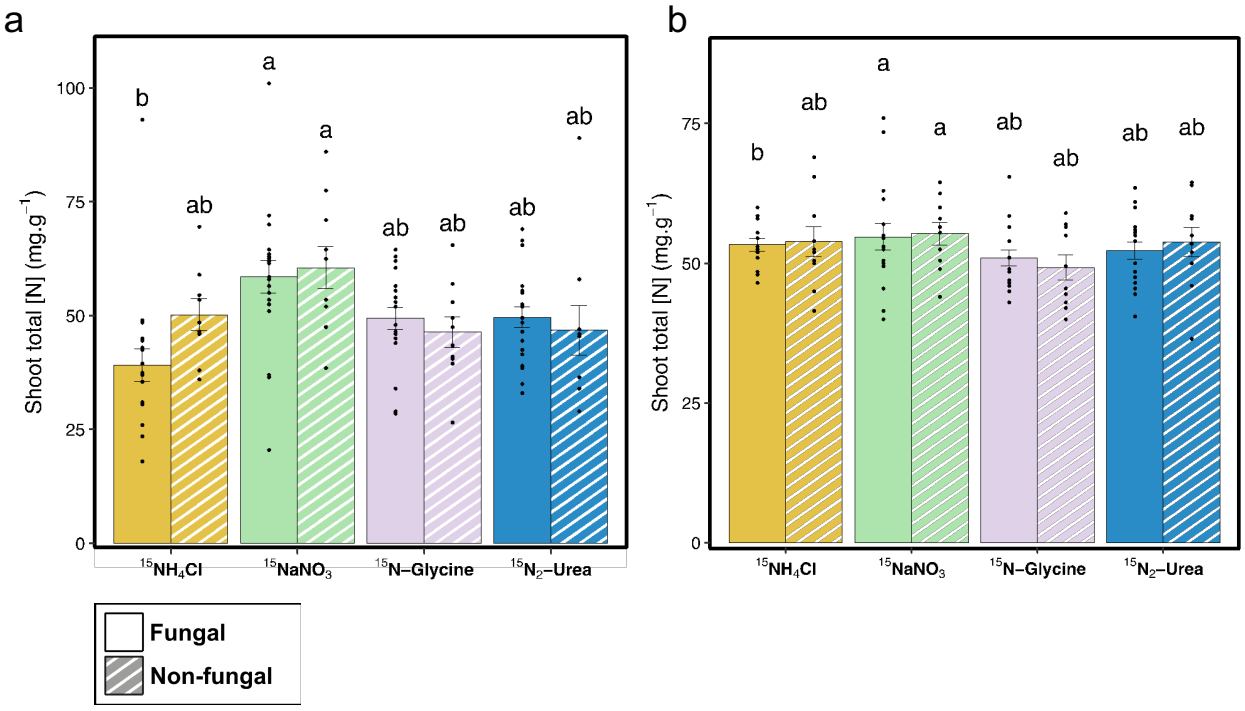

Figure 3.3 Total N concentration of plant shoots in 'N source' (a), 'fungal choice' (b) experiments. a) n = 20 per fungal treatment, n = 10 per non-fungal treatment apart from $^{15}NH_4$ which n = 9). **b)** n = 17 per fungal treatment apart from $^{15}NH_4$ which n = 16, n = 10 per non-fungal treatment. Hatched bars indicate microcosms not inoculated with MFRE. Solid bars indicate inoculation with MFRE. Error bars indicate ±SE, different letters denote significant difference (Tukey's HSD: p<0.05).

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

Net fungal-mediated ^{15}N transfer was determined by subtracting the mean ^{15}N of non-fungal plant shoots from the ^{15}N of individual microcosms inoculated with MFRE. The recovery of ^{15}N in plant shoots is presented as a percentage of the total ^{15}N that was available to the microcosms.

In 'N source' microcosms ^{15}N source was a driver of ^{15}N concentration in plant shoots (Kruskal-Wallis: d.f.=3, X^2=9.54, p<0.05; Fig. 3.4a) with microcosms treated with ^{15}N-glycine resulting in greater MFRE-mediated ^{15}N in plant shoots than microcosms treated with either ^{15}N-sodium nitrate or $^{15}N_2$-urea (Dunn test: p<0.05.

In the 'fungal choice' experiment, the type of 15N source influenced 15N concentration observed in plant shoots (ANOVA: F3,18=5.91, p<0.01; Fig. 3.4b), with microcosms containing 15N-glycine or 15N-ammonium chloride accumulating more 15N in plant shoots than microcosms where the labelled ^{15}N source was sodium nitrate (Tukey's HSD: p<0.05).

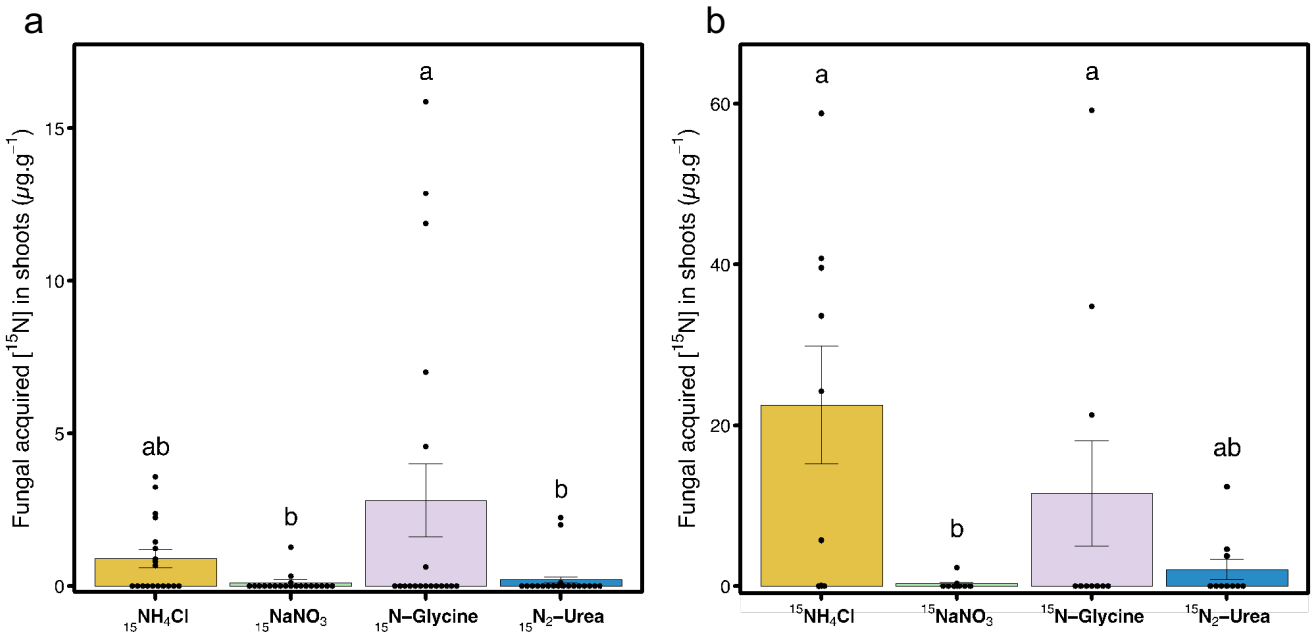

Figure 3.4 MFRE-mediated ^{15}N concentration in 'N source' (a) and 'fungal choice' (b) experiment. a) n = 20 ($Na^{15}NO_3$, $^{15}N_2$-urea), n = 19 ($^{15}NH_4Cl$, ^{15}N-glycine). **b)** n = 10 per treatment apart from $^{15}NH_4$ which n = 9. Error bars indicate ±SE. Different letters denote significant difference (Tukey's HSD: p<0.05).

3.4.4 Plant-to-fungus C transfer

The trends observed in plant-to-MFRE C transfer (Fig. 3.5) mirror those observed for MFRE-mediated ^{15}N transfer to plants (Fig. 3.4a, b). ^{15}N source is a significant driver of the amount of plant C transferred to MFRE (Kruskal-Wallis: d.f.=3, X^2=20.256, p<0.001), with microcosms treated with ^{15}N-glycine or $^{15}NH_4Cl$ returning significantly more C to MFRE than microcosms treated with either $^{15}N_2$-urea or $Na^{15}NO_3$.

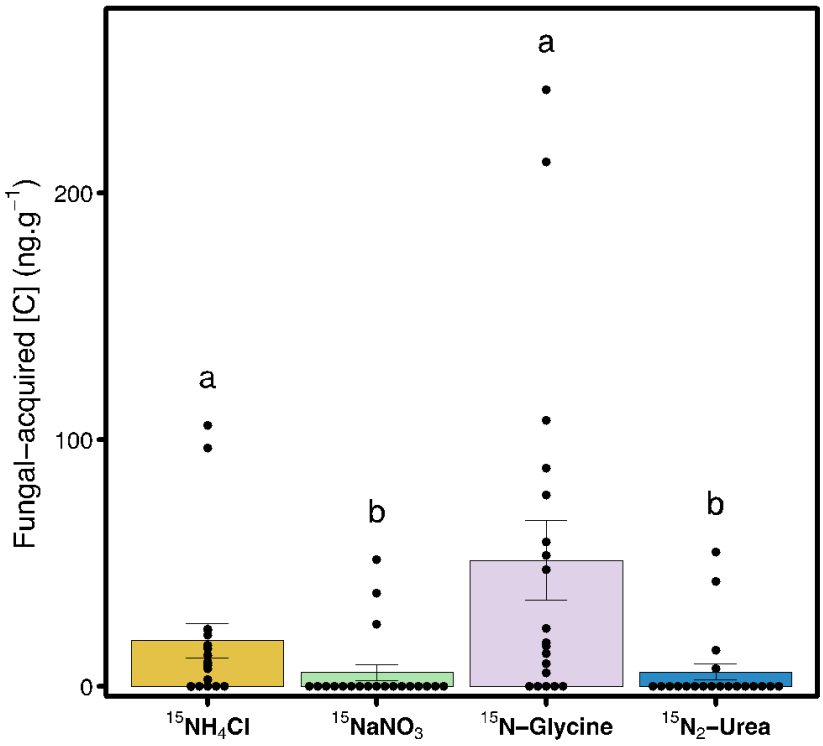

Figure 3.5 Plant-derived [C] in MFRE hyphae. n = 20 ($Na^{15}NO_3$, $^{15}N_2$-urea), n = 20 ($^{15}NH_4Cl$, ^{15}N-glycine). Different letters denote significant difference (Dunn's post hoc: p<0.05). Error bars indicate ±SE.

3.5 Discussion

Using the commonly occurring and well-characterised AM host plant *Plantago lanceolata* (Stewart, 1996; Pankoke *et al.,* 2015; Pel *et al.,* 2018), these experiments provide unequivocal evidence of C-for-N exchange between the *lyc-1* MFRE isolate and *P. lanceolata* in *in vitro* microcosms, from which other soil microbiota were excluded. Of the four nitrogen sources applied, there was a greater transfer of glycine-N and ammonium-N than N derived from nitrate or urea (Fig. 3.5a, b), corresponding to enhanced photosynthate allocation to the fungus (Fig. 3.6). This is the first time MFRE have been shown to engage in C-for-N exchange with *Plantago lanceolata* and the first demonstration that MFRE preferentially provide N from specific sources, with proportional C exchange. Despite the observed C-for-N exchange, there were no significant differences in biomass (Fig. 3.3) or shoot total N concentration (Fig. 3.4b, d) between fungal and non-fungal plants of the same N treatment. This was perhaps to be expected given the plants' young age and relative confinement within the systems used. The relatively greater MFRE-mediated assimilation of glycine- and ammonium-N by *Plantago* occurred regardless of whether the ^{15}N-ammonium or ^{15}N-glycine tracer was the only N source provided to the MFRE mycelium or part of a mixture of four N sources (Fig. 3.5c, d). MFRE's preference for glycine-derived N in our experimental systems contrasts with the tendency of many AM fungal species to assimilate N from inorganic sources for transfer to host plants (Johansen *et al.,* 1996; Toussaint *et al*., 2004). 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 (Hodge, 2001). Having a strictly biotrophic lifestyle, AM fungi lack the saprotrophy-associated molecular toolkit required to degrade organic matter, such as extracellular proteases that are commonly found in saprotrophic ericaceous and ectomycorrhizal fungi (Jin *et al.,* 2012). In contrast, MFRE can be isolated from host plants and maintained in a free-living state in axenic culture (Field *et al*., 2015a; Hoysted *et al*., 2023), indicating that MFRE possess at least some degradative capabilities to maintain mycelial growth in the absence of a host plant. My key finding that MFRE preferentially assimilate and transfer glycine-derived N to the host plant supports there being at least partial saprotrophic capabilities of MFRE, even when associated with a living host plant.

To date, demonstration of resource exchange between MFRE and host plants has been limited to a handful of plant species, the only other example using monoxenic experimental systems being with *Trifolium repens* (Hoysted *et al*., 2023). In agreement with the results presented here, MFRE provided N to *T. repens* hosts in return for plant-fixed C resources in the absence of other soil microbes; however, in Hoysted *et al*. (2023) the N source was limited to the readily plant-available, inorganic NH_4Cl. The data presented here build on this, demonstrating symbiosis between MFRE and *Plantago lanceolata* and expanding the range of N sources MFRE are known to utilise. However, the possibility of functional complementarity with AM symbionts, where AM fungi primarily support host plant P and N nutrition from inorganic sources (Johansen *et al.,* 1996; Toussaint *et al*., 2004) while MFRE supply hosts with organic-derived N (Field *et al*., 2019; Hoysted *et al*., 2019, 2023), remains untested.

There was very little-to-no transfer of nitrate-derived ^{15}N to host plants by MFRE in all 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 (Fig. 3.5). This contrasts with AM-mediated plant N uptake which frequently utilise nitrate-N in the symbiosis (Toussaint *et al.,* 2004; Calabrese *et al*., 2016), in line with the presence of nitrate reductases in the genomes of many AM species (Kaldorf *et al.,* 1994). The relative bioavailability of nitrate-N to both plants (Noguero & Lacombe, 2016) and AM fungi (Bago *et al.,* 1996; Tian *et al.,* 2010) may provide a reason for the lack of MFRE-mediated nitrate-N transfer to host plants as in natural environments, assimilation of nitrate N would represent a relatively more competitive niche for MFRE to exploit. Therefore, it is plausible that MFRE have not evolved the capability to exploit soil nitrate pools, instead developing the capacity to acquire N from organic sources.

Although MFRE transferred glycine-derived ^{15}N 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 could be due to the nature of urea itself; urea is a relatively stable molecule, with a half-life of over three years in solution during which time ~50% of present urea will have degraded to produce ammonium ions (Amtul *et al.,* 2002). Additionally, urease enzymes are produced by a range of

soil microbes (Rana *et al.,* 2021) including AM fungi (Jin *et al.,* 2012). It may be that MFRE do not produce these enzymes to utilise this competitive nutritional niche, instead potentially scavenging ammonium-N – which MFRE readily utilises (Fig. 3.5) – from decomposition of urea by other microorganisms. The addition of urease-producing soil bacteria to these MFRE-only systems, or the development of soil-based systems to investigate MFRE-plant symbioses is needed to determine whether this is the case.

Our experiments show that MFRE are able to utilise organic N from glycine to engage in nutritional symbiosis with host plants (Fig. 3.5). This contrasts to our hypothesis that inorganic N would be preferred owing to its relative ease of metabolism by comparison. However, a preference for glycine-N is consistent with the putative saprotrophic capacity of MFRE; access to nutrients bound up in organic compounds is essential for when MFRE do not have a ready supply of plant-derived C while also providing access to a broader N pool when in symbiosis with host plants. This ability is consistent with other members of the Mucoromycotina which often occupy saprotrophic roles (Chang *et al.,* 2022). It is possible that MFRE preferentially utilises organic compounds as these provide not only N, an exchangeable commodity, but also C which can be used by the fungus in addition to plant-fixed C. If this is the case, then assimilation of organic compounds would provide more benefit to MFRE than an inorganic N source. Developing this line of enquiry with dual isotopically labelled glycine would allow the determination of whether/how MFRE utilise the C components of organic molecules when in symbiosis with plants.

By measuring the flow of C into extraradical MFRE mycelium across the range of N sources in our experiments (Fig. 3.6), we assessed the relative ‘cost’ of assimilation and transfer for each of the ^{15}N sources the MFRE isolate was supplied with. We found greatest allocation of photosynthetic C to MFRE mycelium where ammonium chloride or glycine were present as N sources, correlating with the amounts of N transferred to host plants from those sources. Following the reciprocal rewards model of mycorrhiza resource exchange (Kiers *et al*., 2011), it could be expected that MFRE would gain the most C in return for the N it provides in the greatest quantity to its plant host. However, it should be noted that the

systems presented here represent only the most basal level of ecological complexity; in nature, these symbioses occur in most scenarios, encompassing many other abiotic factors including variable light conditions, nutrient availability and access to water, as well as interactions with other soil microorganisms. As such our findings should not be generalised to all MFRE-plant symbioses in all environmental scenarios but as a starting point for exploration of their broader ecological significance.

The mechanisms of N acquisition by MFRE remain unknown. However, we now have evidence demonstrating an enhanced capacity for organic N acquisition compared to inorganic N sources which tend to be favoured by AM fungi. In return for this MFRE-mediated N, plants allocate photosynthetically acquired C differentially based on the amount of fungal N supplied. We have also demonstrated that MFRE discriminates between forms of N when multiple sources are present. Our data add nuance to what is known about dual symbioses between MFRE and AMF in host plants; we have demonstrated that MFRE possess the ability to utilise N sources AM fungi are less capable of assimilating. This may indicate that there is less resource competition between the two symbionts than might otherwise be expected. Furthermore, as we did not quantify plant fecundity, resilience to nutrient stress, or fitness within adverse environments, the ability of MFRE symbioses to provide additional benefits outside of nutrient acquisition are yet to be determined. Our research lays the foundation for further targeted assays using sterile and non-sterile systems to develop a more holistic understanding of MFRE-plant symbioses encompassing soil microbe interactions, soil nutrient dynamics, and further investigation of the mechanisms underpinning C-for-nutrient exchange between MFRE and host plants.

Chapter 4: Substrate nitrogen concentration affects C-for-N exchange in Mucoromycotina 'fine root endophyte' symbiosis with host plants

4.1 Introduction

Soils are dynamic environments; moisture, temperature, pH, and nutrient balance all vary over geographical gradients and time. N, an essential macronutrient for plants, is present in soil in many forms, both organic and inorganic, and at variable concentrations (Matsumoto *et al.,* 2000). Soil N availability is affected by a number of factors – natural and anthropogenic – including atmospheric deposition of nitrogenous compounds (Chen *et al.,* 2018b; Moore *et al.,* 2020), direct application of inorganic fertilisers for agricultural purposes (Suzuki *et al.,* 2017), deposition of organic molecules during the natural processes of animal and microbial defecation, death, and decay (Greenfield, 2001; Keenan *et al.,* 2023). Such heterogeneity in soil nutrient availability has repercussions for many soil biotic processes, from the production of microbial N-degrading enzymes (Fujita *et al.,* 2018) to the symbiosis between plants and their mycorrhizal fungi. Soil N concentration can affect the frequency of root colonisation and efficiency of nutrient transfer in mycorrhizal relationships (Johnson *et al.,* 2005, Bonneau *et al.,* 2013) with greater N availability in soils reducing the amount of AM fungal colonisation of roots (Johnson *et al.*, 2005; Solaiman *et al.,* 2010; Bonneau *et al.* 2013) and N limitation driving increased AM-mediated plant N assimilation from soil (Johnson *et al*., 2010). This suggests that soil fertility, including N availability, can be a key environmental control on the mycorrhizal benefits derived by host plants either directly by influencing plant C for N exchange, or indirectly by impacting photosynthetic capacity. Globally, soil N impacts the mycorrhizal colonisation in plants along with other soil factors such as pH, moisture content, and temperature (Soudzilovskaia *et al.,* 2015; Mickan *et al.,* 2019). Johnson *et al.* (2005) found fertilisation with inorganic N and lime increased root length colonised by plants significantly, although nutrient exchange was not quantified. Addition of organic compounds as fertilisers also affects mycorrhizal colonisation of plants, increasing the total root length colonised (Solaiman *et al.,* 2010).

There is a general assumption in the literature that the increased growth of host plants is proportional to total root colonisation by mycorrhizal fungi, and that this is caused by equally proportionate gain in mycorrhizal-acquired nutrients. this is not always supported by data; Treseder (2013) found variation in the correlation between root colonisation by AMF and plant P content. The data I have presented in this chapter contributes to a growing body of evidence demonstrating context-dependent symbiosis between plants and mycorrhizal fungi (Treseder & Allen, 2002; Hoeksema, 2010).

While the distribution of MFRE across habitats has begun to be characterised, the impact of different environmental factors on the functional significance of MFRE-plant symbioses has not yet been determined experimentally. MFRE occupy a wide range of habitats from western Europe (Field *et al.,* 2015a) to Australasia (Albornoz *et al.,* 2022); regions which encompass wide diversity in many edaphic factors including moisture content (Deng *et al.,* 2020), organic matter content (Stockmann *et al.,* 2015) and nutrient status (Zhang *et al.,* 2019). Identifying the broad impacts of nutrient availabilities on MFRE-plant symbioses is an important step in clarifying their importance in both natural and agricultural ecosystems. As MFRE are at least partially saprotrophic (Field *et al*., 2015a) and, given that even obligately biotrophic AM fungi can become parasitic under some soil conditions (Solaiman *et al.,* 2010), determining whether the N status of soil significantly alters the MFRE-plant symbiosis is important for assessing the role of MFRE in plant nutrition in both natural and managed ecosystems.

I established in **chapter 3** that MFRE preferentially assimilate glycine-N to exchange with *P. Lanceolata* hosts for C. This, in combination with other work demonstrating growth of MFRE without plant hosts (Field *et al.,* 2015a) adds to the evidence for MFRE functioning as a facultative saprotroph. Accordingly, I hypothesise that MFRE utilises organic compounds in greater amounts than inorganic ones to make use of the C contained in these molecules. This could potentially offset the C ‘cost’ of association with MFRE to host plants, potentially leading to potential functional complementarity between MFRE and AM fungi (Field *et al.,* 2019).

Here, using stable and radio-isotope tracers, I investigated how plant-MFRE bi-directional C-for-N exchange is affected by the concentration of N in the plant growth media, paying particular attention to the fates of glycine-derived C and N within monoxenic systems.

4.2 Key research questions

- Do the amounts of C-for-N exchange differ when N is available in different amounts?
- Dow do different nutritional contexts affect the growth rate of extraradical MFRE hyphae?
- How is C from organic molecules utilised by MFRE when in symbiosis with plants?
- How does MFRE colonisation affect plant growth across an N availability gradient?

4.3 Methods

4.3.1 Fungal Inoculum

MFRE isolate *Lyc-1,* was isolated from *Lycopodiella inundata*, as described in Hoysted et al., 2023 and **chapter 2**. Sub-cultures of MFRE were maintained on Gamborg B5 basal medium at ½ the recommended concentration (1.6 g L^{-1}; Sigma-Aldrich). This was buffered with 0.5 g L^{-1} MES (Sigma-Aldrich) and solidified with 1% agar (hereafter referred to as ½GB5). MFRE cultures were incubated at 25ºC in the dark and sub-cultured on to new media under sterile conditions every 8–12 weeks.

4.3.2 Experimental microcosms

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'. 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 conducted in chapter 3 is equivalent to the 'High N' treatment in this chapter) as well as a limestone grassland in the Peak district (Horswill *et al.,* 2008; 'Low N'). Treatments comprised: 'High N' (187.4 $mg.g^{-1}N$), 'Medium N' (93.7 $mg.g^{-1}N$), and 'Low N' (25 $mg.g^{-1}N$) (Full nutrient composition in table 4.1).

Plantago lanceolata seeds (Yellow Flag Wildflowers, Gloucester, UK) were sterilised in a 4.5% bleach solution (Lindsey *et al.,* 2017) and germinated on ½GB5. After 7 days, individual seedlings were transferred to sterile experiment al microcosms. At the same time, small (approximately 1.25 cm^3) sections of ½GB5 agar containing abundant MFRE hyphae and spores were placed adjacent to emerging roots. Each microcosm was sealed with Parafilm and the 'belowground' agar portion of each plate was wrapped in aluminium foil to reduce light penetration. These microcosms were maintained in 16:8 hr, day:night conditions at a constant temperature of 25°C, and non-viable microcosms containing dead plants or other contamination were removed and destroyed (See **chapter 2** for detailed setup).

Table 4.1 Composition of modified Gamborg B5 nutrient medium used in the 'N concentration' experiment

Compound	M.Wt	g.L^{-1} (High N)	g.L^{-1} (Medium N)	g.L^{-1} (Low N)
Calcium chloride	110.98	0.05662	0.05662	0.05662
Magnesium sulfate.7H_2O	246.47	0.12538	0.12538	0.12538
Manganese sulphate.H_2O	151.00	0.00500	0.00500	0.00500
Ferrous sulphate.7H_2O	278.01	0.01390	0.01390	0.01390
EDTA disodium salt.2H_2O	372.24	0.01865	0.01865	0.01865
Zinc sulphate.7H_2O	287.60	0.00100	0.00100	0.00100
Boric acid	61.84	0.00150	0.00150	0.00150
Potassium iodide	166.00	0.00038	0.00038	0.00038
Molybdic acid (sodium salt).2H_2O	241.95	0.00013	0.00013	0.00013
Copper sulphate.5H_2O	249.69	0.00001	0.00001	0.00001
Cobalt chloride.6H_2O	237.93	0.00001	0.00001	0.00001
myo-Inositol	180.16	0.05000	0.05000	0.05000
Nictotinic acid (free acid)	123.11	0.00050	0.00050	0.00050
Pyridoxine • HCl	205.64	0.00050	0.00050	0.00050
Thiamine • HCl	337.27	0.00500	0.00500	0.00500
Sodium phosphate monobasic	119.98	0.06525	0.06525	0.06525
Potassium nitrate (KNO_3)	101.10	0.166250	0.62500	1.25
Ammonium sulphate ($(NH4)_2$ SO4)	132.14	0.008911	0.03350	0.067

4.3.3 ^{15}N, ^{13}C, and ^{14}C isotope tracing

7 weeks after *Plantago lanceolata* plants were inoculated with MFRE, stable and radioisotope labelled nutrients were presented to the plant and fungus simultaneously. To determine MFRE transfer of N to plant hosts a ~0.5 ml well was excavated in the agar of the microcosms in proximity (~1-2 mm) to the growing edge of the MFRE mycelium (Fig. 4.1a). Into these wells, 100 µl of a 1 mg.ml^{-1} solution of ^{15}N-glycine ($C_2H_5{}^{15}NO_2$) resulting in a total of 19.72 µg ^{15}N was added

to each microcosm before the well was backfilled with liquid ½GB5 medium. To account for diffusion and direct uptake of ^{15}N by plant roots, control microcosms consisting of *Plantago lanceolata* without MFRE inoculation were also established. To determine the fate of C from organic compounds in MFRE-plant symbioses, 100µl of 1 mg.mg^{-1} ^{13}C-glycine (^{13}C-CH_5NO) was applied into the same well concurrently with the ^{15}N tracer. In total 17.09 µg ^{13}C was added to each microcosm individually.

After the experiment was completed, plant shoots were freeze dried and 0.1-5 mg was weighed into tin capsules (Sercon, Crewe, UK). The abundance of ^{15}N and ^{13}C in samples was 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/%^{13}C and as %N/%C using un-labelled control plants for background detection. Shoot tissue ^{15}N and ^{13}C concentration was determined using equations adapted from Cameron *et al.* (2006):

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

Here, M_{Ex} = mass (excess) of ^{15}N in samples in grams , At_{lab} = atom percentage of $^{15}N/^{13}C$ in the experimental microcosms, At_{cont} = atom percentage of $^{15}N/^{13}C$ in unlabelled plant material, M = sample biomass in grams g, and $\%E$ = total percentage of N/C. M_{Ex} was converted to µg and divided by total mass of plant shoots to obtain concentration of $^{15}N/^{13}C$ in plant tissue ($[^{15}N]/[^{13}C]$). The average $[^{15}N]/[^{13}C]$ of non-fungal control microcosms of each N concentration treatment was then subtracted from the $[^{15}N]/[^{13}C]$ for individual experimental microcosms within that treatment.

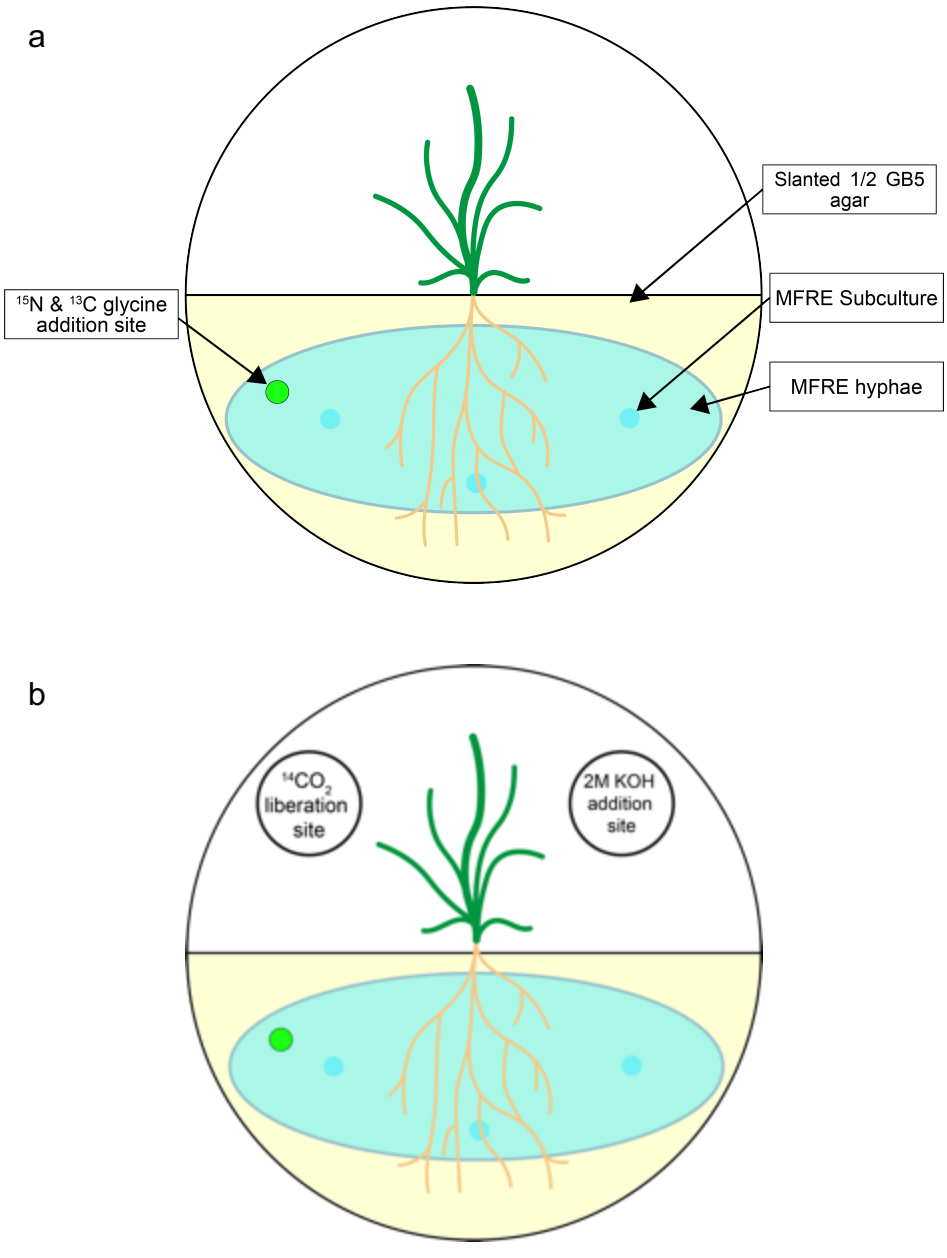

Figure 4.1 Schematic diagram of experimental microcosms showing a) *Plantago* seedling growing on ½ gamborg B5 medium solidified with 1% agar, inoculation site of MFRE-containing agar plugs, as well as the addition site of ^{15}N and ^{13}C isotope tracers. **b)** Experimental microcosm with labelled sites of $^{14}CO_2$ release and sequestration at the beginning and end of labelling period respectively.

To determine how availability of N affects allocation of plant photosynthate to MFRE, immediately after ^{15}N and ^{13}C were introduced into microcosms, the surface of the agar portion of the microcosm was covered with a clear PVC sheet and sealed with anhydrous lanolin. A 0.2 MBq pulse of $^{14}CO_2$ was released into the headspace of sealed microcosms by adding 2 ml 90% lactic acid to 5.4µl ^{14}C-sodium bicarbonate (2.14 GBq/mmol), as per Hoysted *et al.* (2023) (see Fig. 4.1b). Microcosms were incubated for 24 hrs to allow movement of ^{15}N, ^{13}C and ^{14}C to occur. After this period, 2M KOH was injected into small containers within the microcosms (Fig. 4.1b) to absorb any remaining $^{14}CO_2$ gas that had not been fixed by the plants. All plant materials were removed carefully from the agar after 1 hour. Plant shoots were separated from roots which had as much agar removed from them as possible. Shoots and agar (containing MFRE fungal mycelium) were all freeze-dried. 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).^{14}C was quantified via liquid scintillation counting (Packard Tri-Carb 4910TR, Perkin Elmer, Beaconsfield, UK). The amount of C transfer to MFRE hyphae alone was determined by subtracting the average concentration of ^{14}C in non-fungal controls from the ^{14}C concentration in individual experimental microcosms.

4.3.4 Staining, Colonisation counts, & Hyphal growth

After the labelling period was complete, whole root systems were placed into individual histology cassettes and immersed in 10% KOH for 1hr at 70ºC. They were then rinsed in tap water and placed into ink-vinegar stain (5% Pelikan Brilliant Black 4001 ink, 5 % acetic acid, 90 % dH_2O; Vierheilig *et al.,* 1998) for 1hr. After this, roots were washed in tap water and placed into 1% acetic acid overnight to

allow excess stain to leach out from root samples. Stained roots were sectioned into ~1cm lengths which were then mounted on slides in polyvinyl lacto-glycerol (PVLG; ~20cm root material per slide, 1 slide per microcosm) and gently compressed so that all cell layers were visible. Colonisation counts were conducted at 100x magnification according to McGonigle (1990). In brief, 100 unique fields of view per slide were assessed viewed on a compound microscope (Ceti Max II; Medline Scientific, Chalgrove, UK) with a hairline eyepiece graticule for incidence of hyphae and hyphal swellings. Where fungal structures intersected with the graticule, their presence was recorded.

The area of MFRE hyphae beyond plant roots in the microcosm was measured by tracing the outline MFRE hyphae at its greatest (Figure. 4.2) extent at weekly intervals from the initial inoculation. Traced areas were digitised and areas determined using ImageJ (Schneider *et al.,* 2012).

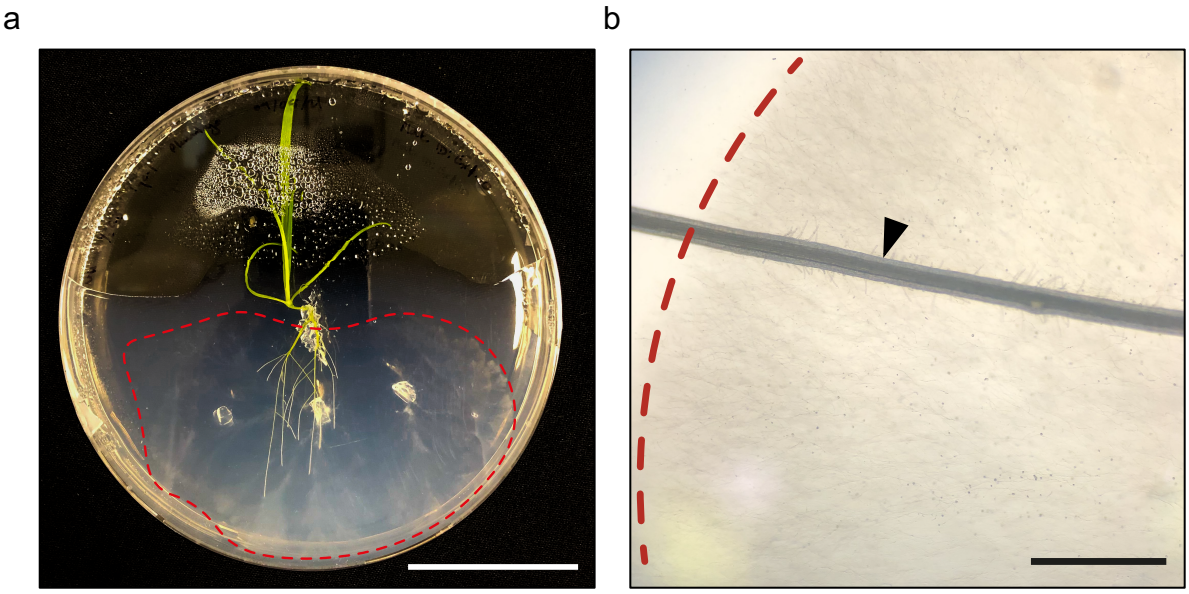

Figure 4.2 Images showing MFRE hyphal area. a) Whole microcosm with outer boundary of MFRE hyphae indicated (red dotted line). The area within this bound was recorded as the area of MFRE. Scale bar = 5 cm. **b)** Close up image showing the densely branching hyphal growth along *Plantago lanceolata* root (black wedge). Red dotted line indicated boundary of branching hyphae. Scale bar = 500 µm.

4.3.5 Statistical Analyses

All analyses performed in R studio (v. 2023.03.0+386; R core team, 2023) using the programming language R (v. 4.3.0 “Already Tomorrow”) with the packages “dplyr”, “car”, “rstatix”, “ggpubr”, and “tidyverse”. All graphs were produced using the “ggplot2” package.

Biomass, colonisation, and Isotope tracing data were checked for conformity with assumptions of normality and homogeneity of variance. Where these assumptions were met, they were analysed using analysis of variance (ANOVA) with post hoc Tukey testing (as indicate); where assumptions were not met data were log or square root transformed (as indicated). Hyphal growth data were analysed using a repeated measures ANOVA with post hoc pairwise t-tests.

4.4 Results

4.4.1 Shoot biomass

When plants were colonised by MFRE, the concentration of N in the growth media made no difference to plant shoot biomass (Figure. 4.3). However, in non-fungal colonised plants, greatest shoot biomass was recorded in plants grown in media containing the least N of those tested. As such, it appears that colonisation by MFRE had a strong effect on the biomass of host plants, causing reduced biomass in plants associated with MFRE compared to those without fungal partners (ANOVA: $F_{1,50}$=16.0256, $p<0.001$). This effect is particularly pronounced in the low N treatment where the presence of MFRE had a suppressive effect on plant shoot masses (Figure. 4.3; Tukey’s HSD: $p<0.05$) with uninoculated plants grown on media containing 25$\mu g.g^{-1}$ N having a biomass more than twice as large as those grown in the presence of MFRE.

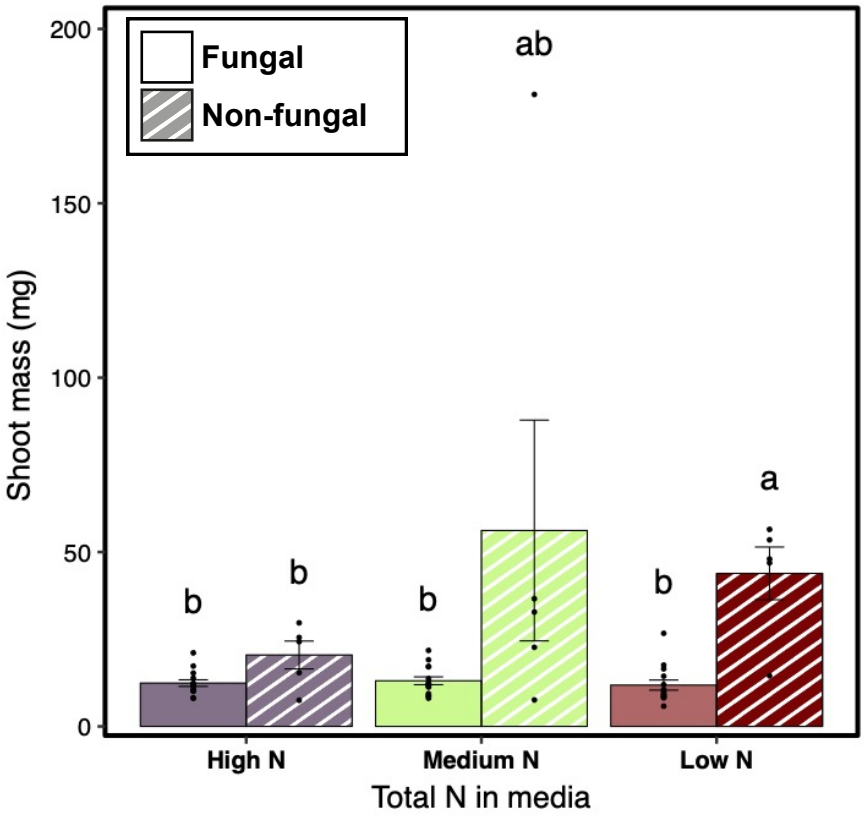

Figure 4.3 Shoot biomass compared between fungal and non-fungal plants under different media total N concentrations. Data were log transformed to meet assumptions of normality and heteroscedasticity (n = 14 per treatment for fungal microcosms (solid bars), n = 5 for non-fungal (hatched bars) microcosms at all treatments apart from the "medium N" treatment which n = 4).

4.4.2 Colonisation and mycelial growth

Whole root systems of plants were stained post-harvest to assess colonisation. Hyphae and spherical structures were observed colonising both the surface of roots (Figure. 4.4c, d; S1.1a), and entering cells with characteristic fine branching hyphae (Figure S4.1b-e), as well as forming hyphal terminal swellings (Figure S4.1e,f). Total colonisation of *Plantago lanceolata* roots by MFRE was impacted by

N concentration in the growth media (Figure. 4.4a; ANOVA: $F_{2,39} = 6.5694$, $p<0.01$) with significantly less colonisation of MFRE at 25 µg g^{-1} N than in the higher N concentrations (Tukey's HSD: $p<0.05$). This trend is mirrored by the abundance of spherical "vesicular" structures observed in roots (Figures. 4.4b, d; ANOVA: $F_{2,39}=10.561$, $p<0.001$) with significantly fewer vesicles produced in roots of plants grown on 25µg.g^{-1}N compared to the higher N treatments (Tukey's HSD: $p<0.05$)

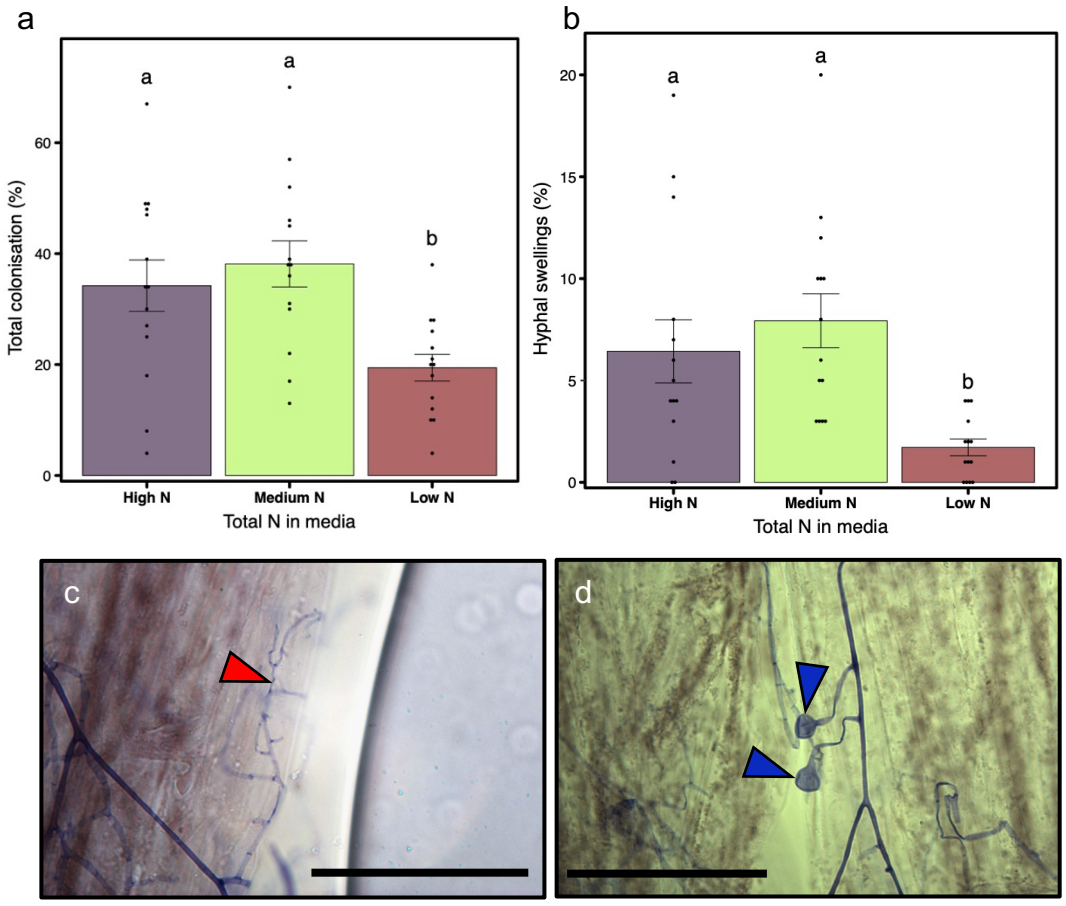

Figure 4.4 Colonisation of *Plantago* roots by MFRE. a) Mean total colonisation by fungal structures as a % of total root length. (n = 14 per N treatment). **b)** Mean colonisation by vesicular structures as a % of total root length. (n = 14 per N treatment). **c, d)** Fine branching hyphae within *Plantago* root epidermal cells (red arrows; 100x magnification). **d)** Branching hyphae with terminal swellings (Blue arrows; 100x magnification). Scale bars = 50µm.

The surface area of MFRE mycelium extending beyond the root (Figure. 4.5) was strongly influenced by both N treatment (ANOVA: $F_{2,39}$= 20.186, p<0.001) and time (ANOVA: $F_{1.91,74.37}$= 350.927, p<0.001), with a significant interaction between both factors (ANOVA: $F_{3.81,74.37}$=14.856, p<0.001). From weeks 1 to 4 of inoculation, the hyphal area of MFRE on 25µg.g^{-1}N is significantly higher than those of the other two treatments (Pairwise T-test; Table S4.2), however from weeks 5 to 7 the area at 25 µg g^{-1}N and 93.7 µg g^{-1}N are not significantly different from each other, however both are significantly greater than MFRE grown at 187.4 µg g^{-1}N (Pairwise T-test; Table S4.2).

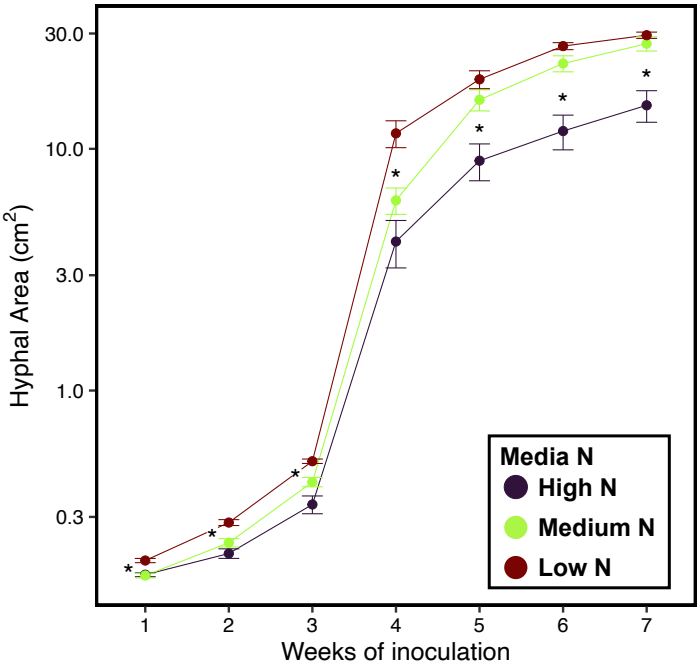

Figure 4.5 Growth of extraradical MFRE hyphal mycelium. Stars between points indicate statistical significance between treatments above and below (Weeks 1-3 stars offset left for clarity) (n=14 per N treatment per point).

4.4.3 Total N in plant shoots

When normalised to shoot biomass, total shoot N (Figure. 4.6) reflects the N treatment applied with the greatest shoot concentrations of N being in 187.4 µg g^{-1} N treatment followed by 93.7 and 25 µg g^{-1} N treatments, regardless of the presence of MFRE or not (ANOVA: $F_{2,51}$=15.7688, p<0.001). Presence of MFRE associates also had a significant effect on plant shoot N concentration (ANOVA: $F_{1,51}$=15.5000, p<0.001) however, within each N treatment no significant difference between total [N] based on MFRE inoculation was observed (Tukey's HSD: p<0.05).

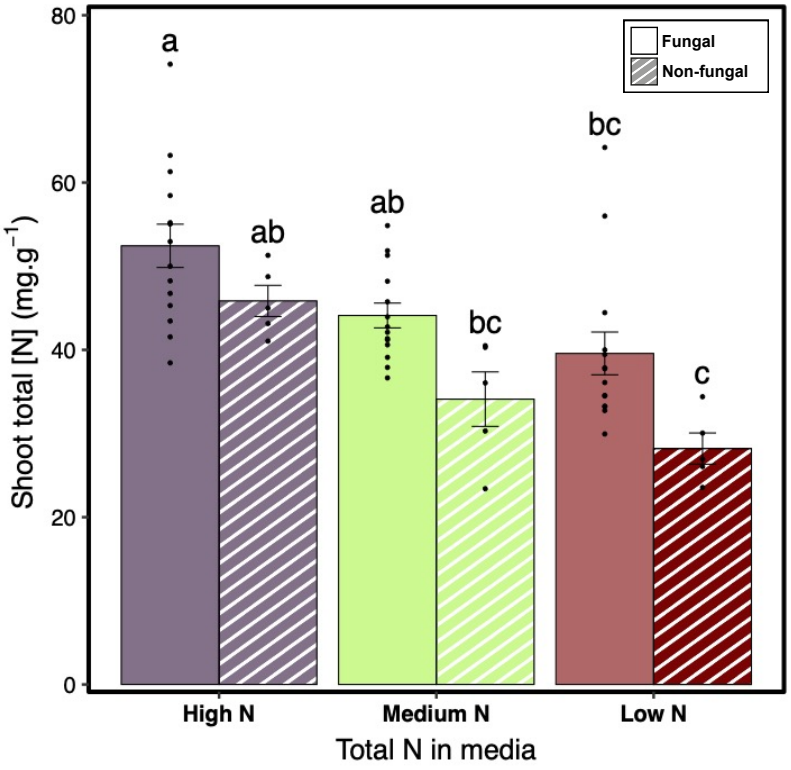

Figure 4.6 Mean concentration of N in plant shoots in media of different N concentrations. Means are compared between microcosms with (n=14 per treatment; solid bars) and without (n=5 per treatment; hatched bars) MFRE symbiosis. Different letters denote significantly different means (Tukey's HSD; p<0.05)

4.4.4 ^{15}N in plant shoots

^{15}N in plant shoots was significantly impacted by fungal inoculation (ANOVA: $F_{1,51}$=1.0786, p<0.05) with significantly greater concentrations of ^{15}N in shoots of plants inoculated with MFRE at 25µg g^{-1} N than non-fungal plants grown on the same N concentration (Tukey's HSD: p<0.05). While there is a slight decrease in shoot ^{15}N concentration in non-fungal plants at lower N concentrations, this effect was not statistically significant (Table S4.1). However, shoot ^{15}N concentration was greater in plants associated with MFRE growing on media with lower N concentration (Tukey's HSD: p<0.05; Figure. 4.7).

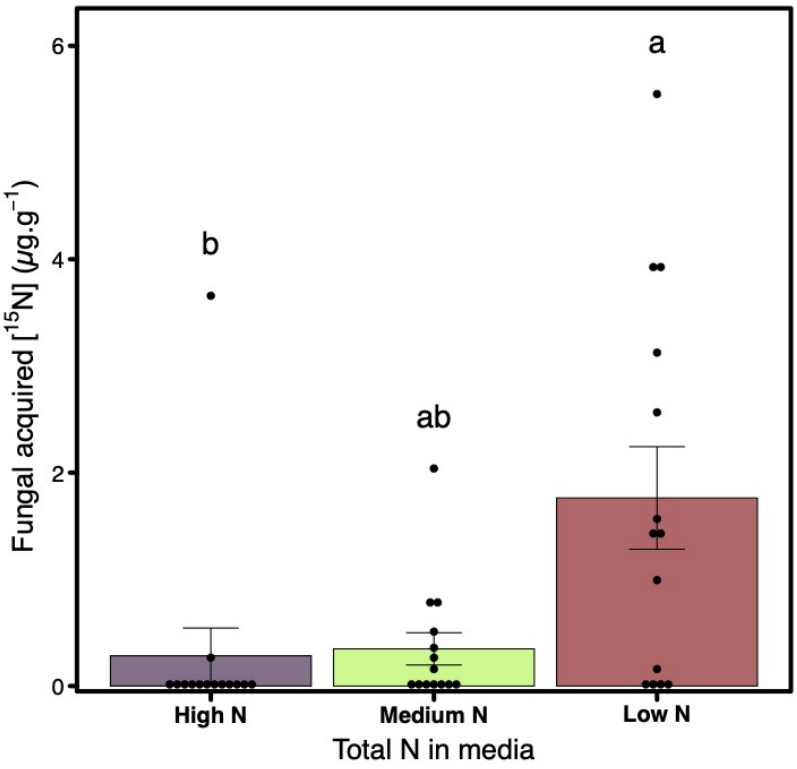

Figure 4.7 ^{15}N in shoots. Concentration of ^{15}N derived from MFRE fungi in plant shoots. Different letters denote significantly different means (Tukey's HSD: p<0.05). n = 14 per treatment.

4.4.5 ^{14}C in MFRE

Similarly to the trends in ^{15}N transfer to plants, differences in plant-fixed [C] (ng.g^{-1}) transferred to MFRE hyphae (Figure. 4.8) were significantly driven by the concentration of N available (ANOVA: $F_{2,50}$=5.0497, p < 0.01; Table S4.2).

However, the pattern is reversed, with increased [C] in MFRE hyphae of microcosms under the high N treatment (Tukey's HSD: $p < 0.05$).

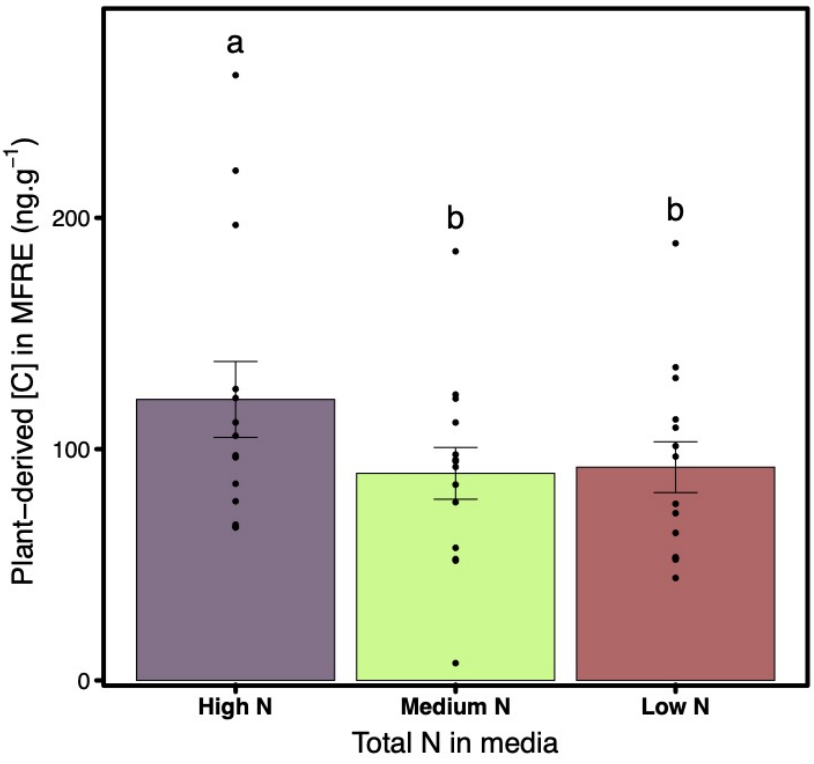

Figure 4.8 Plant-derived C concentration in MFRE hyphae. Different letters denote significantly different means (Tukey's HSD: $p<0.05$). n = 14 per treatment, Error bars indicate ±SE.

4.4.6 *Plantago* shoot ^{13}C

I observed a significant effect of fungal inoculation on the ^{13}C concentration of plants (ANOVA: $F_{1,50}=20.6347$, $p<0.001$)**.** Under all N treatments, the average ^{13}C concentration of plants associated with MFRE is less than that of asymbiotic plants (Figure. 4.9; Tukey's HSD: $p<0.05$). In MFRE-colonised plants, shoot [^{13}C] decreases in line with decreasing N content of the (Tukey's HSD: $p<0.05$). This is not the case in asymbiotic plants which maintain shoot [^{13}C] across all treatments.

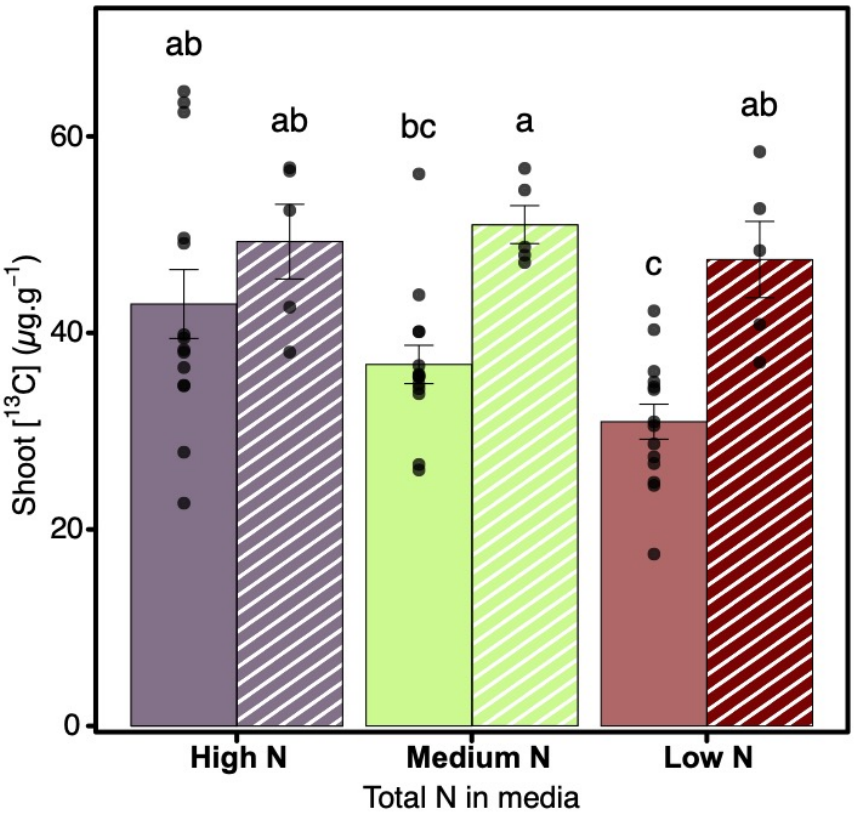

Figure 4.9 Plant glycine-derived ^{13}C concentration compared between fungal (solid bars) and non-fungal (hatched bars) microcosms. Different letters denote significant difference (Tukey's HSD: $p<0.05$). n = 14 per fungal treatment, n = 5 per non-fungal treatment. Error bars indicate ±SE.

4.5 Discussion

From my experiments it appears that MFRE allocates greater N to host plants under N limiting conditions (Figure. 4.7) while significantly altering its morphology (Figure. 4.5) and production of colonisation structures within plant roots (Figure. 4.4). I have also demonstrated that the utilisation of glycine-C and N by MFRE are distinct from one another, with MFRE exchanging glycine-N for plant fixed C (Figure. 4.7, 1.7) while retaining organically derived glycine-C (Figure. 4.9).

The interactions between MFRE and host plants have been studied in increasing detail in recent years (Hoysted *et al.,* 2019; Field *et al.,* 2019; Hoysted *et al.,* 2022).

These studies focused on MFRE nutrient transfer to hosts under uniform conditions (Field *et al*., 2016; Hoysted *et al.,* 2019; Field *et al.,* 2019; Hoysted *et al.,* 2022) finding that MFRE take a greater role in plant N nutrition than AM fungi (Field *et al*., 2016). This work has also shown MFRE assimilate N from organic sources for host plant exchange. However, the impacts of variability in nutrient availability have not previously been investigated. MFRE can function as a saprotroph (Field *et al.,* 2015a) therefore, may function differently under variable conditions to AM fungi which, as obligate biotrophs (Smith & Read, 2008), necessarily must engage in mutualism with photosynthetic plants in order to obtain C.

I show here that MFRE colonisation and production of intracellular structures is reduced under low N conditions (Figure. 4.4a, b). This observation is striking as there is a body of literature that supports the assumption of greater mycorrhizal colonisation under nutrient limitation (Bonneau *et al.,* 2013; Soudzilovskaia *et al.,* 2015) or indeed, reduction of AM fungal colonisation under nutrient addition (Frater *et al.,* 2018). However, this is not universal. Johnson et al. (2005) found fertilisation with inorganic N and lime increased root length colonised by plants significantly, although nutrient exchange was not quantified. These studies generally assume that lower colonisation corresponds with reduced mycorrhizal benefit to plants, however the data I present here challenges this notion. This illustrates the need for direct indicators of symbiotic benefit such as nutrient tracing respectively to become more common.

To assess mycorrhizal functionality, I applied ^{15}N and ^{13}C labelled glycine to MFRE and radioactively labelled $^{14}CO_2$ to *Plantago lanceolata* hosts to determine how the exchange of carbon for nutrients is altered between symbionts under three N conditions. ^{15}N transfer to host plants was elevated under the lowest N treatment compared to both higher N treatments (Figure. 4.7). This was expected based on previous work conducted using AM fungi (Thirkell *et al.,* 2019; Kiers *et al.,* 2011). Thirkell et al. (2019) found that elevated soil nitrate resulted in greater transfer of N to barley by AM fungi with no difference in root colonisation. This contrasts with my finding that reduced nitrate N in the growth media increased MFRE-mediated transfer of N to host plants, however, it highlights the importance of soil nutrient form and availability on mycorrhizal symbioses.

This fungal-mediated N acquisition by plants also seems to marginally ameliorate reduced N in the growth media, leading in a slightly greater concentration of N in plant shoots under low (25μg.g^{-1}N) media N (Figure. 4.6).

An unexpected result from my study was the consistent ^{14}C transfer to MFRE from plants regardless of available N (Figure. 4.9). This maintenance of C transfer to MFRE was despite not receiving like-for-like N in exchange as in previous experiments (**Chapter 3**) under homogeneous N.

I found that host plants relied on MFRE for a greater proportion of their ^{15}N assimilation when grown on reduced N availability media (Figure. 4.7). The low N media stimulated greater surface area of extraradical MFRE mycelium (Figure. 4.5) and reduced presence of colonisation structures within host roots (Figure. 4.4) than the other media, suggestive of an explorative, foraging 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. 4.8). However, there was a corresponding decrease in plant acquisition of ^{13}C from glycine in low N conditions (Figure. 4.9), indicating assimilation and sequestration of ^{13}C (and ^{12}C) from glycine by MFRE. This C acquisition by MFRE from an exogenous source could offset 'costs' associated with increased transfer of N by MFRE to hosts (Kiers *et al.,* 2011) in low-N environments, potentially explaining maintenance of plant C transfer to MFRE mycelium. 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 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 colonisation being recorded.

I presented MFRE with glycine, a small organic molecule common in soils (Hawkins *et al.,* 2000), based on previous work which found MFRE preferentially transfers glycine-N to hosts over other N sources, organic or inorganic. The preference for organic N sources raises the hypothesis that, as a partial saprotroph, MFRE can offset the greater N cost of symbiosis under low

N conditions by utilising both plant-fixed C and organic C from glycine. My data supports this hypothesis as the presence of MFRE inhibits plant acquisition of glycine-^{13}C, especially under low N conditions (Figure. 4.9). In combination with the finding that MFRE transfer greater ^{15}N to hosts under low N, I have demonstrated evidence that MFRE may be able to offset the greater cost of N transfer to hosts under low N by utilising glycine-^{13}C, therefore requiring less plant-fixed C in return (Figure 4.10).

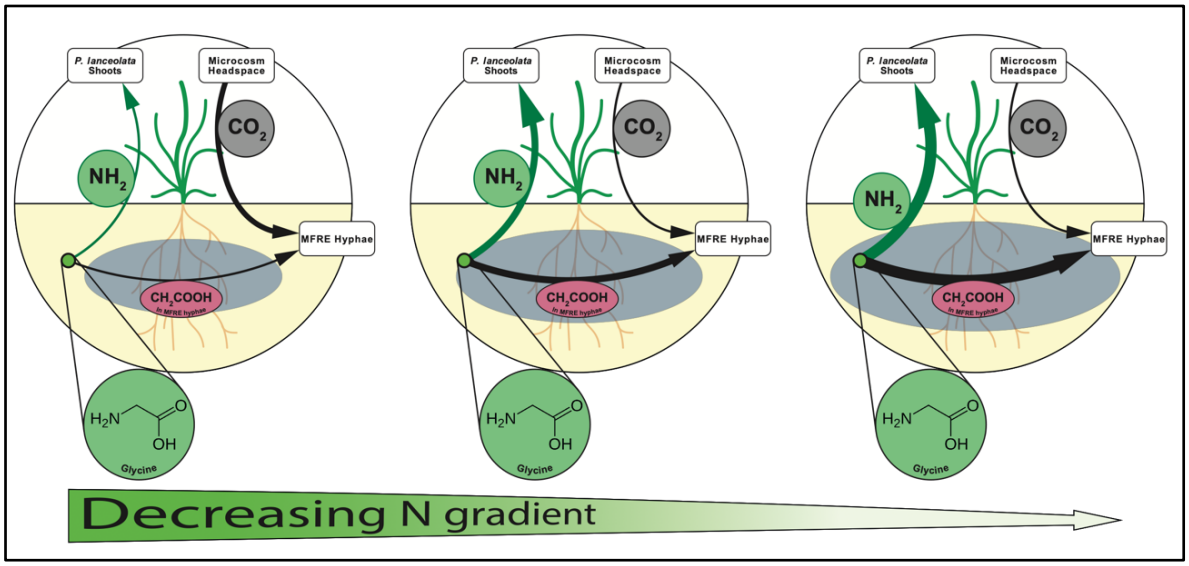

Figure 4.10 Schematic diagram showing proposed MFRE N and C dynamics under a decreasing media N gradient. Thicker arrows indicate Increased nutrient transfer. Under all N treatments, C derived from plant photosynthesis is transferred to MFRE hyphae. As N in media is reduced, organic N is transferred to plant shoots via MFRE. In conjunction, there is increased interception of organic C derived from glycine which is retained in MFRE hyphae.

I have previously shown MFRE can assimilate and transfer N in substantial amounts from organic sources to host plants (**Chapter 3**). MFRE also take on a greater role in the N nutrition of host plants than AM fungi when both are present in symbiosis with the same host plant (Field *et al.*, 2019). This functional niche MFRE occupy when in symbiosis with host plants is potentially a result of the dual lifestyle they lead whereby MFRE act as saprotrophs in the absence of plant host and symbionts when plant hosts are present (Field *et al.*, 2015a). When living as a

saprotroph, the degradation of organic molecules is essential to obtain C that would be otherwise unavailable. The ability to degrade organic molecules would place MFRE at an advantage when in symbiosis with plants, allowing them to access N from an otherwise underutilised pool, given that the endosymbionts they are competing with – AM fungi – have a generally lower capacity to assimilate nutrients from organic sources (Leigh *et al.,* 2009; Xie *et al.,* 2022).

To bring this work into greater relevance, the wider ecology of MFRE in soils should be brought into consideration; interactions between MFRE and other soil microorganisms should be investigated as soil bacteria interact with other mycorrhizal fungi in many complex ways with implications on the nutritional relationship between them. Close associations between AM fungi and bacteria have been found, with AM fungi facilitating the growth of certain species of phosphate solubilising bacteria (PSB) via exudation of carbon compounds, in return for greater access to soil P pools (Zhang *et al.,* 2014; Jiang *et al.,* 2021). This multipartite symbiosis between plants, AM fungi, and soil bacteria also has additive effects on plant N acquisition and is similarly impacted significantly by changes in soil N status (Hestrin *et al.,* 2019). However, this study also demonstrates competition between AM fungi and bacteria for soil N. These interactions do not occur by chance, AM fungi have been shown to significantly alter soil microbial communities in the hyphosphere, potentially by modulating N secretion (Nuccio *et al.,* 2013).

Chapter 5: The Effects of non-MFRE Soil Microbiota on MFRE-to-*Plantago lanceolata* nutrient transfer

5.1 Introduction

Thus far my investigation of the symbiosis between mycorrhiza-forming Mucoromycotina 'fine root endophyte' (MFRE) fungi and plant hosts has been conducted using axenic agar plate-based systems. These aim to isolate the interaction between the fungus and plant to characterise effects of MFRE without confounding effects of nutrient cycling and metabolism of other soil microbes. There have been studies conducted in non-sterile soil-based systems. These investigations (e.g. Field *et al.,* 2016; Hoysted *et al.,* 2019) used wild-collected plant specimens complete with rhizosphere soil in a nutrient experiment. The presence of native bacteria, transferred within the rhizosphere soil, cannot be accounted for. Limiting fungal interactions with soil bacteria and other mycorrhizal fungi can be useful to investigate direct effects of fungal acquired soil nutrients on host plant nutrition, however, these fungi occur in soils replete with other microbes (Kowal *et al.,* 2020, Albornoz *et al.,* 2021) which may aid, hinder, or alter the nutrient uptake by MFRE, altering its real-world effects on plant hosts.

In **Chapters 2** and **3**, I used monoxenic agar plate systems to show increased transfer of N from an organic nitrogen-containing compound (glycine) to plant hosts by MFRE in much greater amounts than from inorganic compounds (ammonium chloride and sodium nitrate). However, the other organic source used in these experiments, urea was not transferred to host plants by MFRE. I hypothesised that in natural ecosystems soil bacteria facilitate the hydrolysis of urea, precluding the direct assimilation of urea by MFRE.

5.1.1 Recruitment of microbes by mycorrhizal fungi

Interspecies interactions occur between plant hosts and mycorrhizal fungi and in turn between these fungi and other soil microbes. The recruitment of different taxa of bacteria by AM fungi is thought to be functionally necessary due to AM fungi having a more limited or non-existent suite of carbohydrate-degrading or organic phosphate-mineralising genes, rendering associations with bacteria with

these functions highly beneficial to AM fungal-plant symbioses (Zhang *et al.,* 2021).

Mycorrhizal organic phosphorus (P) uptake is facilitated by hyphosphere bacteria capable of mineralising organic P, known as 'phosphate solubilising bacteria' (PSB) (Zhang *et al*., 2016). In return for breakdown of complex P-containing substances into simple inorganic ones by hyphosphere bacteria, AM fungal hyphae secrete carbon-rich compounds which simultaneously increase the growth rate of PSB (Zhang *et al*., 2016) while stimulating bacterial P acquisition (Charakas & Khokhani, 2024). Experiments investigating the effect of mycorrhizal hyphae on organic and inorganic P mineralization and uptake, found soil P concentrations significantly reduced while phytase and phosphatase were elevated in soil plots with plant roots excluded but fungal hyphae allowed to penetrate soil compartments (Zhang *et al.,* 2018a). This, in combination with previous experiments showing AM fungi are unable to obtain organic P on their own (Zhang *et al*., 2016) indicates that for P uptake by AM fungi to occur, symbioses with PSB must be formed.

The presence of *Glomus hoi* in leaflitter was found to affect the bacterial community, enhancing occurrence of *Firmicutes*, *Gemmatimonadetes*, *Deltaproteobacteria* and *Planctomycetes* while also decreasing abundance of some bacterial taxa such as the *Actinobacteria*, at the same time, increasing uptake of N by the plant host (Nuccio *et al.,* 2013). Crucially, bacteria in the taxa *Firmicutes* are decomposers of organic material while *Actinobacteria* secrete a wide range of metabolites including antibiotics (Bérdy, 2005) that could negatively affect *Firmicutes sp.,* reducing the N available to *G. hoi* for exchange with host plants. This suggests that some AM fungi shape the bacterial community composition of the hyphosphere in favour of maximising soil nutrient acquisition by providing suppressing antagonistic taxa while facilitating the growth of beneficial taxa (Zhang *et al.,* 2022).

There have been several mechanisms proposed by which AM fungi may tailor the hyphosphere bacterial community by wither recruiting beneficial bacteria or suppressing antagonistic bacteria.

One of the methods of recruitment of various beneficial microorganisms by AM fungi is via the secretion of carbon-rich compounds such as glucose, galactose, trehalose, and fructose (Zhang *et al.,* 2016; Zhang *et al.,* 2018b) which are assimilated by PSB to fuel the degradation of organic material (Williams *et al.,* 2024). In addition to this, some studies have shown AM fungi facilitate the translocation of PSB to sites rich in organic P through the water film on the outer hyphal surface (Jiang *et al.,* 2021), however this work only confirmed the movement of a single species of flagellate bacteria, *Rahnella aquatilis,* on *Rhizophagus irregularis* hyphae. However, the same phenomenon was not observed between the non-flagellate bacteria *Micrococcus luteus* and *R. irregularis* (Jiang *et al.,* 2021).

Possible mechanisms for the growth suppression of pathogens by AM fungal-associated hyphosphere bacteria include increased competition for nutrients, and potentially direct secretion of antibacterial and antifungal compounds by the bacteria (Bharadwaj *et al.,* 2008). These compounds included cellulases, chitinases, and proteases, as well as phosphate solubilising compounds.

5.1.2 MFRE and plant nutrition

The assimilation of soil N by MFRE fungi has been shown in both monoxenic (**Chapter 3, 4**; Hoysted *et al.,* 2023) and soil-based (Field *et al.,* 2016; Hoysted *et al.,* 2019) systems. MFRE can also access and transfer inorganic N to host plants in much greater quantities than AM fungi (Field *et al.,* 2019). It has even been suggested that MFRE and other mycorrhizal fungi that occupy the sublphylum Glomeromycotina (AM fungi) form dual symbiosis in plant roots and perform separate but complementary functions, with MFRE supplying the host plant with mainly N while AM fungi supply mainly P (Field *et al.,* 2019). This is consistent with **Chapter 4** where I show that MFRE utilise glycine, an organic that can be utilised by AM fungi (Hawkins *et al.,* 2000) albeit at lower rates than inorganic N sources (Talbot & Treseder, 2010).

In previous chapters, I have demonstrated that MFRE in the absence of other microorganisms can obtain and transfer organic N to plant hosts in monoxenic

microcosms. However, a number of factors are not considered in these axenic experiments that make interpretation of the results within an ecological context difficult. These include how the action of organic N breakdown by other soil microorganisms including bacteria and soil fungi, and how recruitment of a specific microbiome by mycorrhizal fungi to the hyphosphere impact their function in relation to host plant nutrition.

5.1.3 MFRE and soil microbes

While the reductive nature of the monoxenic experimental systems, such as those used in **chapters 3** and **4** of this thesis, is undoubtedly useful for investigating the fundamental biology of MFRE. More complex systems, as explored in **chapter 2** are required when questions of ecology are raised. AM fungi are known to associate with soil bacteria in ways that impact nutrient assimilation and symbiosis with plants. For example, phosphate solubilising bacteria (PSB) enhance the growth of both plants and AM fungi (Nacoon *et al.,* 2021). It has recently been shown that PSB receive C compounds from AM fungal hyphae in return for bacterial-assimilated P and are physically transported to patches of P in soils (Jiang *et al.,* 2021). In this manner, the AM fungi act as intermediaries between plants and bacterial P.

Therefore, I wanted to test whether inclusion of a relevant soil microbiome from ecosystems known to harbour MFRE affects the assimilation and transfer of N to host plants. As MFRE and AM fungi have been observed to occupy similar habitats (Ryan & Kirkegaard, 2012), and even co-colonise the roots of the same plant (Rimington *et al.,* 2015; Field *et al.,* 2016), it is logical that MFRE could engage in similar interactions and that these interactions affect the nutritional symbioses between MFRE and plants. There is also the possibility that additional soil bacteria could not act synergistically with MFRE but present as competitors, scavenging available N before MFRE is able to.

5.2 Questions and hypotheses

- Is MFRE colonisation of host plant roots affected by the soil microbiome?

    - If MFRE recruitment and utilise soil bacteria to enhance assimilation of soil nutrients, the theoretical cost of symbiosis to MFRE fungi may be reduced, resulting in greater colonisation of plant roots.
- Are there synergistic effects of interactions between MFRE and the soil microbiome on plant growth?
    - Based on the observed effects of AM fungi and soil microbiota on host plants, I hypothesise that plant biomass will be significantly increased by both MFRE and the soil microbiome.
- Does the soil microbiome influence nitrogen assimilation and transfer to host plants by MFRE?
    - Given the extensive literature cited above wherein AM fungi access more N and P via the formation of symbioses with bacterial communities, I hypothesise MFRE utilise similar methods to enhance their own transfer of N to host plants.

5.3 Methods

5.3.1 Experimental design

The experimental design was established to discriminate the effects soil microbes on MFRE-*Plantago* symbiosis. Three replicate blocks of 48 mesocosms each (Figure 5.1) were established up to 14 days apart (see table S5.2). Each block contained 12 individual mesocosms of a distinct treatment. In total, 144 1-litre experimental mesocosms were established.

The treatments were as follows: control treatments ('Control') were not amended with MFRE or a bacteria-containing soil wash. Only an MFRE inoculum was added to 'MFRE' treatments. In contrast, only a bacteria-containing soil wash was added to 'SW' treatments. Finally, both an MFRE inoculum and soil were added to 'MFRE+SWSW' treatments.

12 weeks after these mesocosms were established, ^{15}N and ^{33}P tracers were injected into the hyphal ingrowth cores to (Figure 5.1) investigate the impact of

MFRE-to-plant nutrient transfer with and without the presence of a soil microbiome. Approximately three weeks (see Table S5.2) after this labelling when radiation monitors showed increased activity of plants, the headspace around *Plantago lanceolata* plants was fumigated with $^{14}CO_2$ for 24 hours to determine transfer of plant-derived C to soil containing mycorrhizal fungi.

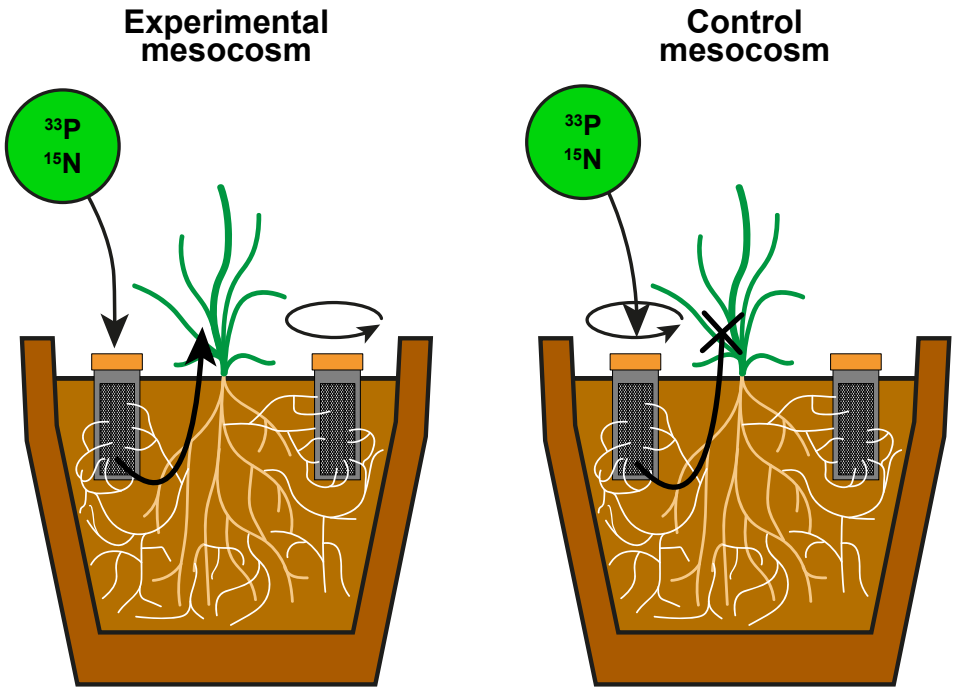

Figure 5.1 Schematic diagram of soil-based mesocosms showing rotated and static hyphal ingrowth cores.

5.3.2 Plant and fungus culture

Seeds of *Plantago lanceolata* were sterilised by immersion in a 4.5 % bleach solution for ten minutes before being rinsed in sterile deionised water five times. These were then germinated on filter paper (Whatman no. 1) under bench conditions for 7 days prior to sowing into pots. They were then sown into 1L pots containing 80:20 sand:topsoil (v:v) mix. Sand was washed prior to sterilisation, both sand and soil were autoclaved twice 3 days apart to prevent contamination by fungal propagules prior to experimental initiation.

After seedlings were sown, pots were placed in a greenhouse chamber at 25ºC under long daylight (16:8hr: day:night) conditions. Mesocosms were watered with 100ml distilled H_2O initially after sowing, then 3x weekly with 50ml distilled H_2O for the duration of the experiment. This was done to prevent the potting media from fully drying as the MFRE isolate used, *Lyc-1* (Hoysted *et al.,* 2023) was isolated from *Lycopodiella inundata,* a liverwort native to flooded conditions (Hoysted *et al.,* 2023). Additionally, colonisation of plant roots by MFRE were found by Albornoz et al. (2021) to increase under increased soil moisture.

Individual pots were moved to a different location within the greenhouse chamber at random once a week to minimise any effects of greenhouse location on plant growth.

24 hrs after seedlings were sown into pots, a soil wash (see methods below) was applied to microcosms. 20ml of soil wash inoculum was syringed onto the soil surface before watering. This was done on half of the pots (the '+' treatment), 20ml 2.5mM MES (the extraction buffer used to generate the soil wash) added to the other half (the '-' treatment).

5.3.3 Fungal inoculum

An inoculum containing MFRE hyphae was prepared by blending 18 axenic cultures of MFRE colonies (grown on ½ GB5; ~990ml) with 10ml distilled H_2O to a homogenous consistency. 20ml of this blended inoculum was mixed through the substrate of each pot prior to seedling transplant. Mesocosms in "Control" and "SW" treatments had 20ml of freshly prepared ½ GB5 mixed into soil prior to seedling inoculation.

5.3.4 Soil wash

A soil sample was taken from a heathland site at the Bradfield Environment Laboratory research station in the peak district national park, UK on (11/4/22) and stored outside until 11/05/22 when the initial replicate experimental block was established. This site was selected as it has previously been shown to harbour

MFRE within plant roots (unpublished data). Soil was removed from the turf manually and passed through a 5mm sieve to remove any stones or large particles. 500g of sieved soil was taken for the initial replicate block and the rest was stored at 4ºC until further use.

To prepare the soil inoculum, 500g of sieved soil was mixed with 750ml 2.5mM MES (pH 5.7). This mixture was then sieved through 1mm mesh initially before being passed through progressively finer mesh down to 45µm (Walker, 2013; Boyno *et al.,* 2023) to exclude fungal propagules. Filtrate was then diluted to 1L with 2.5mM MES. 20ml of filtrate was added to the soil surface of mesocosms in the "MFRE+SW" and "SW" treatments 24hrs after seedling transplantation to pots. At the same time, 20ml 2.25mM MES without soil filtrate was added to mesocosms in the "MFRE" and "Control" treatments. This process was repeated for both subsequent replicate blocks.

5.3.5 Nutrient tracing

For stable- and radio-labelled nutrient tracing, meshed cores per pot were utilised according to the methods of Johnson *et al.* (2001). 2 polyurethane cores with windows covered by fine mesh (30µm) were placed into the soil of each microcosm and filled with the same substrate as the bulk soil. A third meshed core was placed into a subset of pots. This contained fiberglass wool to allow below-ground respiration sampling during the ^{14}C labelling period (Figure 5.1).

12 weeks (table S5.2) after seedling transplantation, a 100µl solution containing ^{15}N and ^{33}P labelled sources was injected to one of the open cores per pot. ^{15}N was presented in one of three sources (NH_4Cl; 27.533 µg, glycine; 19.721 µg, or urea; 48.356 µg; 1 mg.ml). ^{33}P was present as ^{33}P-orthophosphate (111 TBq $mmol^{-1}$ SA, 14.86 mg ^{33}P supplied, Hartmann Analytics). Pots were maintained for 3 weeks for assimilation and nutrient transfer to occur. During this time half of the pots had the core that the isotope was added to rotated 90º 3x weekly (Control mesocosms; Table S5.2). The other half of pots had the core where isotope was not added rotated at the same frequency (Experimental mesocosms). This was done to

account for and direct assimilation of isotopes by plants due to diffusion and bulk flow of isotopes out of cores.

After the $^{15}N/^{33}P$ assimilation period, ^{14}C fumigation was conducted. The cores filled with glass wool had a rubber seal placed in the opening to prevent infusion of aboveground $^{14}CO_2$. Pots were sealed in an airtight chamber (2.8 L vol) containing a cuvette filled with 13.5 µl $Na^{14}CO_3$ (0.5MBq per mesocosm). The cuvette then had 10% lactic acid (80%; ~2ml) added to it, releasing $^{14}CO_2$ into the mesocosm headspace. Gas samples were taken immediately after $^{14}CO_2$ fumigation according to Thirkell et al. (2020). Briefly, 1 ml of headspace gas was taken using a 1ml syringe with a needle attached. The headspace gas was then ejected from the syringe into a sealed scintillation vial filled with 10ml each of Carbosorb® (Revvity), a carbon-trapping compound and Permafluor® scin- tillation cocktail (Revvity). The ^{14}C content of these gas samples was determined by liquid scintillation counting (Tricarb 3100TR scintillation counter; Revvity)

After a 24-hour photoperiod, Excess KOH (2M ~2ml) was injected into a second container within the sealed mesocosm to absorb remaining $^{14}CO_2$. After one hour, mesocosms were harvested. During the harvest, root samples from each plant were taken for staining and fungal colonisation quantification. In addition, shoot, root, and bulk soil samples were taken for ^{15}N, ^{33}P, and ^{14}C analysis (below). Soil samples were taken from in the bulk soil for DNA extraction and bacterial community size analysis using qPCR.

5.3.6 Root Staining and Colonisation Count

Root samples were removed during harvest, and stained following a method modified from Vierheilig *et al.,* 1998. Roots were washed in tap water to remove soil debris and placed in 10% KOH for 60 mins at 70º. Samples were rinsed with tap water before being staining (see **Chapter 2** for full method). Slides were mounted and MFRE colonisation counted at 100x magnification using the method of McGonigle (1990).

5.3.7 ^{15}N quantification

Leaf and root samples were freeze dried after harvest. Dry mass of all samples was recorded before the ^{15}N content of *Plantago* leaves and roots was be determined using continuous flow isotope ratio mass spectrometry (IRMS). 0.1-5 mg was weighed into tin capsules (Sercon, Crewe, UK). The abundance of ^{15}N in samples was 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 $\%^{15}N/\%^{13}C$ and as %N/%C using un-labelled control plants for background detection. Shoot tissue ^{15}N concentration was determined using equations adapted from Cameron *et al.* (2006):

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

Here, M_{Ex} = mass (excess) of ^{15}N in samples in grams , At_{lab} = atom percentage of ^{15}N in the experimental microcosms, At_{cont} = atom percentage of ^{15}N in unlabelled plant material, M = sample biomass in grams g, and $\%E$ = total percentage of N/C. M_{Ex} was converted to µg and divided by total mass of plant shoots to obtain concentration of ^{15}N in plant tissue ($[^{15}N]$). The average $[^{15}N]$ of non-fungal control microcosms of each N concentration treatment was then subtracted from the $[^{15}N]$ for individual experimental microcosms within that treatment.

5.3.8 ^{14}C quantification

Bulk soil samples (containing MFRE fungal mycelium) were freeze-dried after harvest. 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).^{14}C was quantified via liquid scintillation counting (Packard Tri-Carb 4910TR, Perkin Elmer, Beaconsfield, UK). The amount of C transfer to MFRE hyphae alone was determined by subtracting the average concentration of ^{14}C in non-fungal controls from the ^{14}C concentration in individual experimental microcosms.

Total carbon assimilated by the plant was calculated using the following equations modified from Hoysted *et al.* (2023):

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

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.

5.3.9 ^{33}P quantification

Soil and shoot samples were freeze dried, homogenised, and weighed. 10-30mg of homogenised samples were taken and digested in 1ml conc. H_2SO_4. These were heated to 365°C for 15 min, before the addition of 100 µl H_2O_2 to each sample when cool. Samples were then reheated to 365 °C, and each clear digest solution diluted to 10 ml with distilled water. Two ml of each diluted digest was then added to 10 ml of the scintillation cocktail Emulsify-safe (Perkin Elmer, Beaconsfield, UK) and quantified through liquid scintillation. ^{33}P transferred to the plant via fungal mycelium was then calculated by subtracting the concentration of ^{33}P in plants where the isotope was injected into rotated cores from the ^{33}P concentration of those where the isotope was added to static cores.

5.3.10 Fungal and bacterial community abundance

To identify the bacterial and fungal load present in mesocosm soil at two major stages of the experiment, after soil wash addition and after harvest, freeze dried samples of homogenised bulk soil had DNA extracted (DNeasy PowerSoil DNA extraction kit, Qiagen).

To determine the fungal community size a 351 bp region of the fungal 18S rRNA was amplified using FungiQuant primers (Liu *et al.,* 2012). The V4 region of the bacterial 16S SSU rRNA gene was amplified using 515F/806R (Muyzer & de Waal, 1994; Apparill *et al.,* 2015; Parada *et al.,* 2016) primers to quantify bacterial abundance in soil samples. All amplification steps were completed using a Lightcycler® 480 (F. Hoffmann-La Roche Ltd, Basel, Switzerland). The following PCR conditions were used: 3 min at 50°C for UNG treatment, 10 min at 95°C for Taq activation, 15 s at 95°C for denaturation and 1 min at 65°C for annealing and extension. This was done for 50 cycles.

To control for variation in DNA extraction, a known quantity of a marker DNA sequence was added to each sample prior to DNA extraction. The number of copies of this sequence in each sample after amplification was compared to amplification of a series of concentrations of the same sequence (Harshitha & Arunraj, 2021). This was then expressed per gram of soil.

5.3.11 Statistical Analyses

All statistical analyses were conducted using R (R Core tram, 2023) and R Studio (v2023.3.0.386, R Core team, 2023). [using packages ‘dplyr’ (v1.1.2; Wickham *et al.,* 2023), ‘rosetta’ (v0.3.12; Peters & Verboon*,* 2023), ‘stats’ (v4.3.0; R Core team), and ‘agricolae’ (v1.3-5; Mendiburu & Yaseen, 2020)] Isotope tracing data were analysed using analysis of variance (ANOVA) with post hoc Tukey testing (as indicated in Table S5.1). Data were checked for normality and homogeneity of variance. Where assumptions were not met, either a square root or logarithmic transformation was performed (Table S5.1). Figures were created in R (v2023.3.0.386, R Core team, 2023) using the ‘ggplot2’ package (v3.4.2; Wickham, 2016).

5.4 Results

5.4.1 Plant growth

Plant biomass was weighed after freeze drying post-harvest. Shoot masses (Figure 5.2) had no significant differences in mass between treatments within replicate blocks (ANOVA: $F_{3,132}$= 0.0289, $p > 0.05$). However, there were significant differences in shoot biomass between replicate blocks (ANOVA: $F_{2,132}$ = 153.5916, $p < 0.001$). Shoots in the second replicate block were significantly heavier than the two other blocks (Tukey's HSD: $p < 0.05$). Root masses followed a similar pattern (Figure 5.3) with no difference based on soil treatment applied (ANOVA: $F_{3,132}$ = 0.2230, $p > 0.05$) but significant differences based on replicate block (ANOVA: $F_{2,132}$ = 67.5191, $p < 0.001$) with replicate block 2 having overall more biomass than either blocks 1 or 3 (Tukey's HSD: $p < 0.05$).

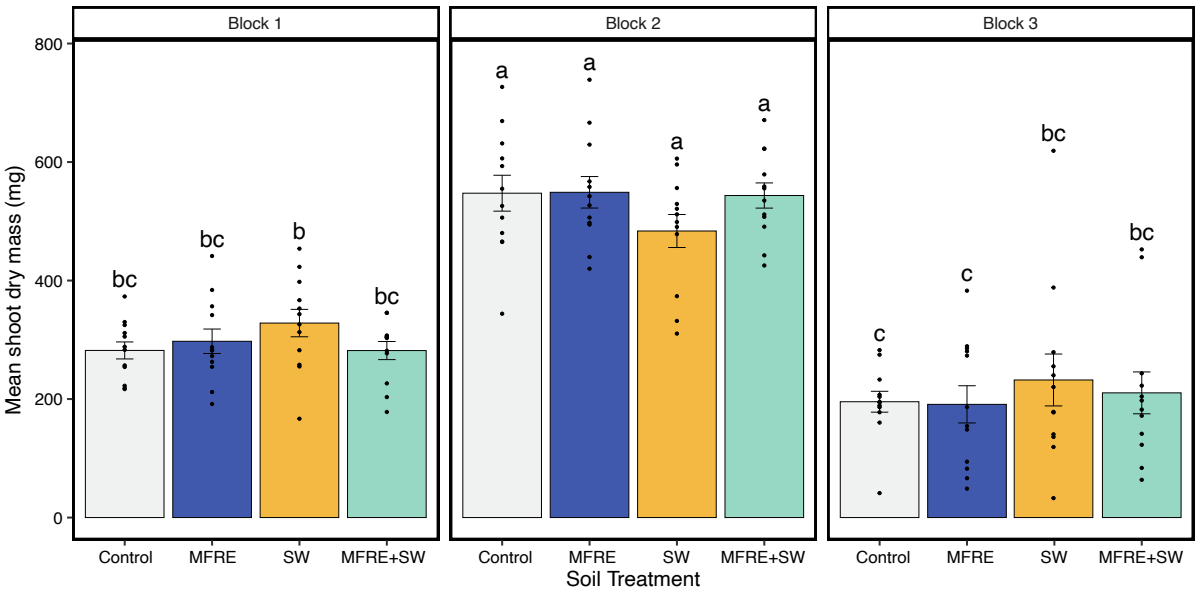

Figure 5.2 Mean Shoot Mass compared between soil treatments and replicate blocks. Data are compared between replicate blocks and soil amendments. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 12 per soil treatment.

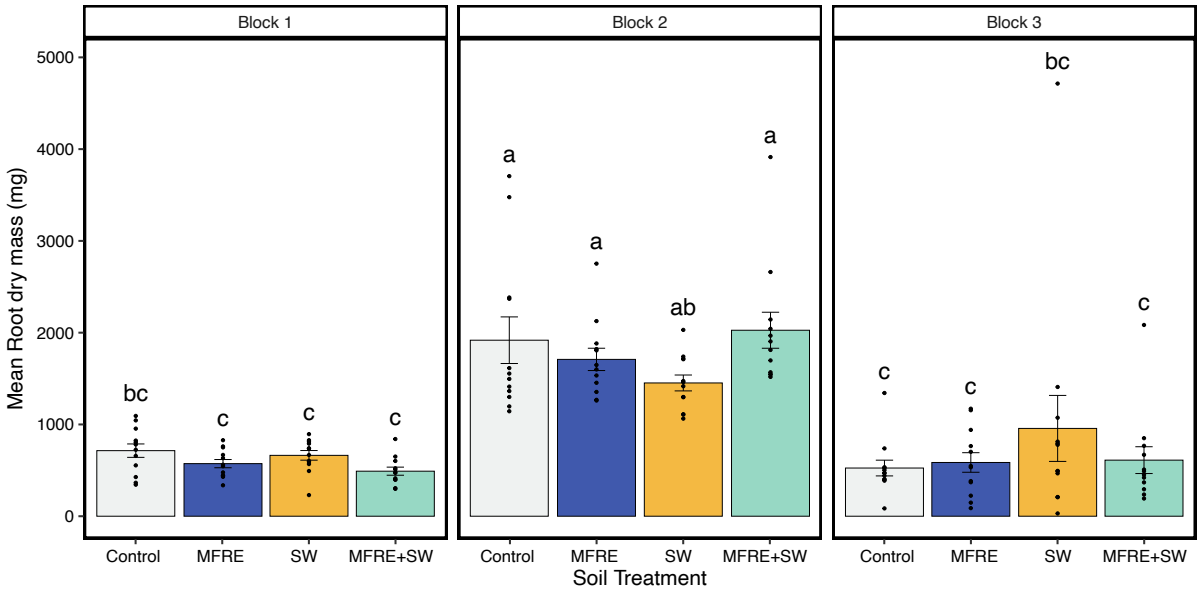

Figure 5.3 Mean Root mass compared between soil treatments and replicate blocks. Data are compared between replicate blocks and soil amendments. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 12 per treatment.

5.4.2 Colonisation

There were no differences in plant root colonisation by MFRE between replicate blocks (ANOVA: $F_{2,132} = 0.1108$, $p > 0.05$; Figure 5.4), however soil inoculation was a significant driver with MFRE-inoculated soils ("MFRE+SW" and "MFRE") producing more heavily colonised plant roots than non-inoculated soils (ANOVA: $F_{3,132} = 114.5991$, $p < 0.001$) as well as a combination of the two factors interacting (ANOVA: $F_{6,132} = 3.8985$, $p < 0.01$). Despite this interaction, similar trends in colonisation were observed across all three blocks (Tukey's HSD: $p < 0.05$; Table S5.1) with significantly less colonisation in "Control" and "SW" treatments than in MFRE-inoculated systems.

The only major difference in trends between blocks is between block 1 and the other two. In block one SW and MFRE- are not significantly different (Tukey's HSD: $p > 0.05$) from one another. In the other two blocks, both the "SW" and "Control" treatments both were not differently colonised from one another (Tukey's HSD: $p > 0.05$).

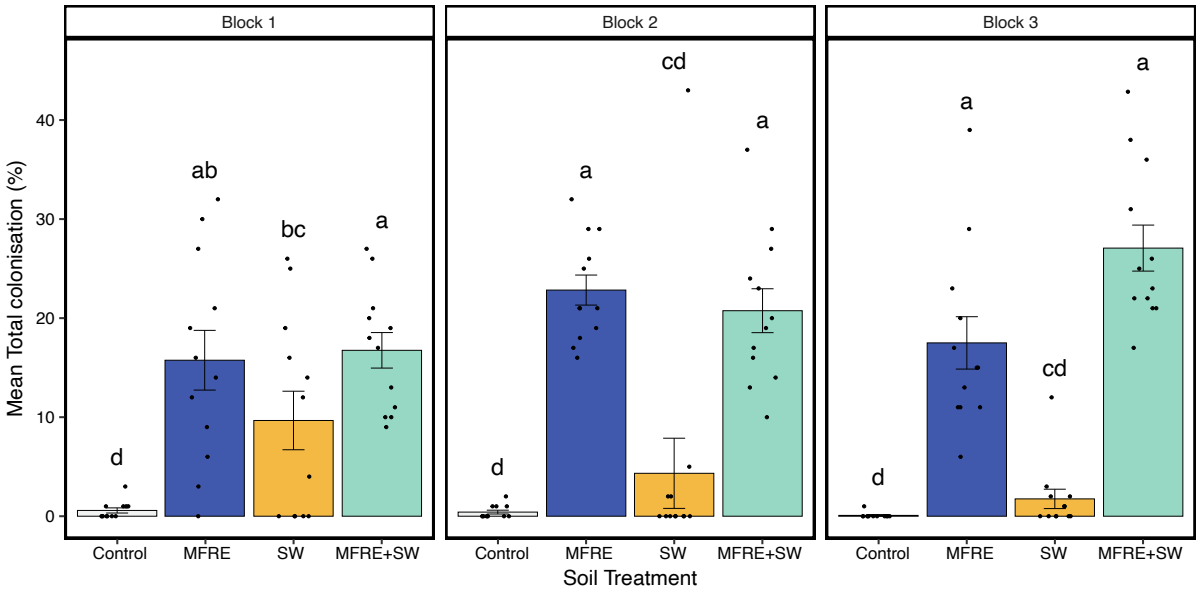

Figure 5.4 Total colonisation (%) compared between soil treatments for all three replicate blocks. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 12 per soil treatment.

5.4.3 Total N

Total nitrogen concentration (Figure 5.5) was quantified for all plant shoots by IRMS (see methods). There were no statistically significant differences between soil treatments (ANOVA: $F_{3,132}= 2.6556$, $p > 0.05$; Table S5.1). Similarly, no differences were observed between replicate blocks (ANOVA: $F_{2,132} = 3.0485$, $p > 0.05$; Table S5.1) and no interactions between these two variables were seen (Table S5.1). The only treatments that were statistically different from one another were the "MFRE+SW" treatment of replicate block 1, which contained significantly more N than the "MFRE" treatment of replicate block two (Tukey's HSD: $p < 0.05$).

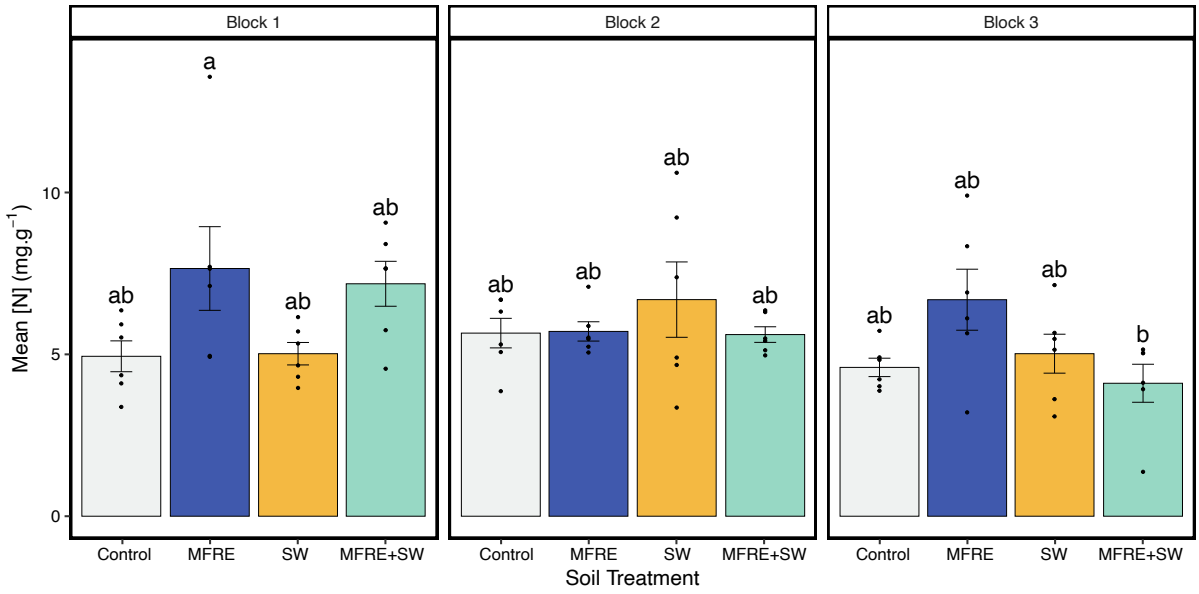

Figure 5.5 Mean total shoot [N] (mg.g^{-1}). Compared between soil treatments of three replicate blocks. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 6 per soil treatment. .

5.4.4 Total P

Contrary to total N concentration, the total P concentration of plant shoots was driven largely by replicate block (Figure 5.6; ANOVA: $F_{2,132} = 79.5797$, $p < 0.001$), with plants in the first replicate block containing significantly more total P than those in either of the other two for most soil treatments (Tukey's HSD: $p < 0.05$). the only treatment in replicate block one with statistically similar levels of total P was the "MFRE" treatment which was not statistically different from the "MFRE+SW" and "MFRE" treatments of replicate block two (Tukey's HSD: $p < 0.05$). In general, there were no real differences between plant total [P] within the same replicate block.

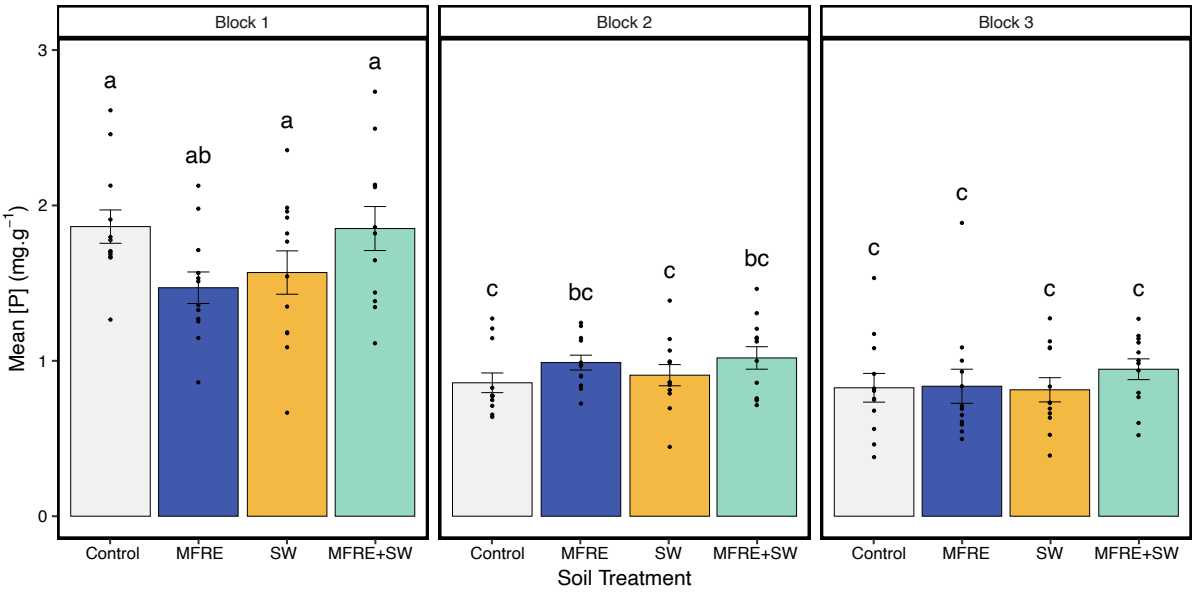

Figure 5.6 Total [P] (mg.g^{-1}) of *Plantago lanceolata* shoots. Compared between replicate blocks and soil amendments. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 12 per soil treatment.

5.4.5 MFRE ^{15}N transfer to plants

To determine how much microbial-acquired nitrogen is transferred to host plants, I applied one of three ^{15}N labelled N-containing compounds – ^{15}N-ammonium, ^{15}N-glycine, and ^{15}N-urea – into soil within a single soil core per mesocosm for a period of three weeks and quantified the subsequent ^{15}N concentration of plant shoots (ng.g^{-1}). To account for diffusion and bulk flow of ^{15}N tracers out of the meshed cores, half of the cores to which tracers were applied were rotated ~90º every 2-3 days to sever any hyphal continuity between the tracer and plant roots. The average ^{15}N concentration observed in plants of each treatment where cores were rotated was subtracted from the total ^{15}N concentration for individual unrotated mesocosms. There was no differen ce between treatments based on replicate block (ANOVA: $F_{2,36}$= 1.5315, $p > 0.05$; Table S5.1) therefore data were combined for presentation (Figure 5.7). There is a wide range of variation within the collected data, resulting in no statistically significant drivers of plant shoot ^{15}N concentration within this experiment (Table S5.1). Secondly, in plants labelled with ^{15}N-ammonium and ^{15}N-urea, there is the highest average concentration of ^{15}N tracer

in those only inoculated with MFRE ("MFRE") followed closely by those treated with both a soil wash and MFRE inoculum ("MFRE+SW").

Within the plants treated with ^{15}N-glycine, MFRE- plants had less ^{15}N than all other soil treatments which were all of a similar ^{15}N concentration. Across all ^{15}N treatments, there are comparable ^{15}N concentrations across all plants treated with no MFRE inoculum "SW" and "Control".

Overall, plants inoculated with MFRE contained more ^{15}N than those that weren't, aside from when treated with ^{15}N-glycine, which contained more ^{15}N in MFRE+SW treated plants, than MFRE- plants.

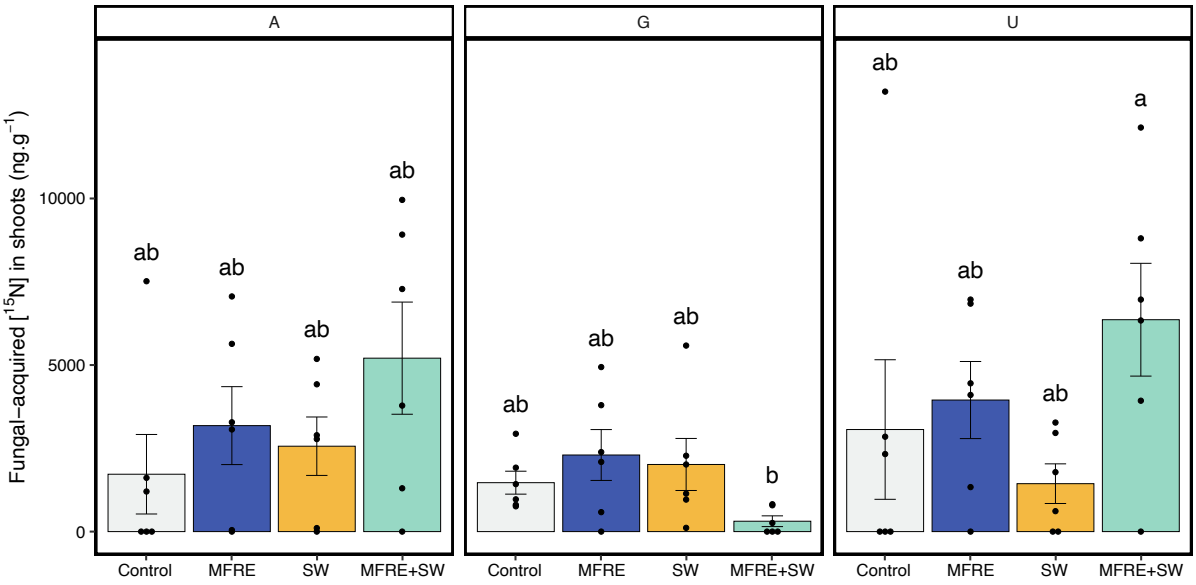

Figure 5.7 Mean fungal-derived [^{15}N] in plant shoots (ng.g^{-1}). Data are compared between ^{15}N source applied (A = ^{15}N-ammonium, G = ^{15}N-glycine, U = $^{15}N_2$-urea) and soil treatment. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 6 per soil treatment.

5.4.6 MFRE ^{33}P transfer to plants

The mean shoot ^{33}P concentration in plants where the core was rotated was subtracted from the ^{33}P concentration in individual plants where tracer was added to the static core.

There was no significant effect of the different soil treatments (ANOVA: $F_{3,60}$= 1.5175, p > 0.05) or replicate block (ANOVA: $F_{2,60}$= 1.2510, p > 0.05). Given these data are not significantly affected by replicate block, they were combined. This did not reveal any significant trends (table S5.1). However, there is a general trend of "SW" treatments containing less [33P] than the others. In plants of replicate blocks one and three the "SW" soil treatment contained no net fungal ^{33}P. Contrary to this, the same soil treatment in the second replicate block contains a comparable amount of ^{33}P to all other treatments and replicate blocks.

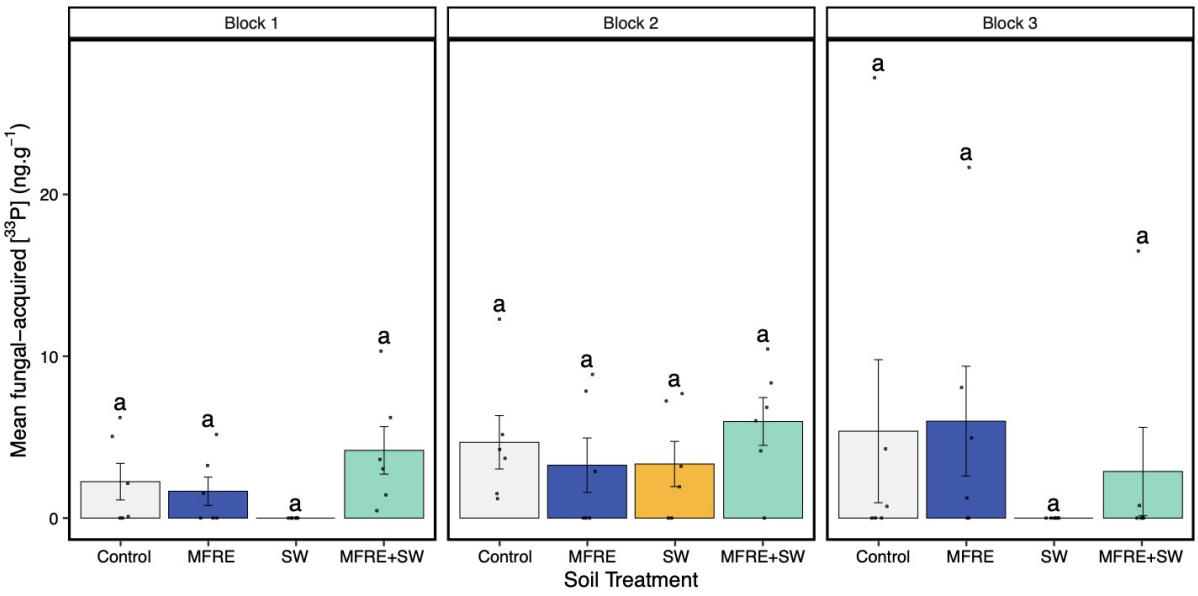

Figure 5.8 Mean fungal-acquired [^{33}P] in *Plantago lanceolata* shoots (ng.g^{-1}). Data are compared between replicate blocks and soil amendments. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: p < 0.05). n = 6 per soil treatment

5.4.7 C transfer to Fungi in soil

I used $^{14}CO_2$ to trace photosynthetically acquired carbon from the headspace of *Plantago lanceolata* mesocosms to the fungal biomass present in the soils of each. These data were then used to determine the total plant carbon transferred below ground (see methods for equations). The trends in these data are slight, with replicate block significantly impacting the amount of C transferred from plant to

fungus (Figure 5.9; ANOVA: $F_{2,132}$= 11.2273, p < 0.001). Additionally, differences were observed based on soil treatment (ANOVA: $F_{3,132}$= 2.8080, p < 0.05) but no interaction between these factors was observed (Table S5.1). Post-hoc testing revealed that, within replicate blocks, while there is some variation, there are no significant differences between treatments of the same replicate block (Tukey's HSD: p > 0.05). Where there are significant differences, between treatments, they occur across replicate blocks; as such, the "MFRE+SW" and "MFRE" treatments of block 3 contain significantly

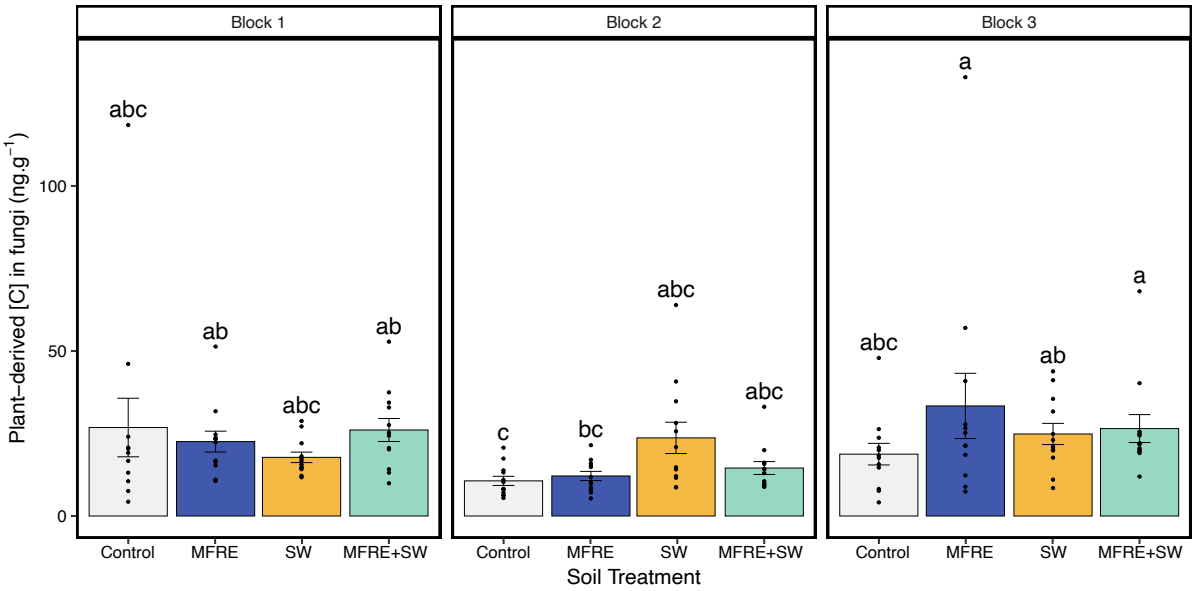

Figure 5.9 Plant-derived [C] in soil. Compared between replicate blocks and soil amendments. Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: p < 0.05). n = 12 per soil treatment.

more plant-derived carbon on average than the "MFRE" and "SW" treatments in replicate block 2 (Tukey's HSD: p < 0.05)

5.4.8 Bacterial and Fungal community abundance

I used qPCR to determine the approximate fungal and bacterial load in experimental pots at two separate time points during the experiment; one week post soil wash application, and at the end of the experiment after plants were

harvested and soil was freeze dried. These soil samples were taken from randomly selected pots within each treatment of the first replicate block (“Block 1”) and repeated at both time points.

Firstly, the overall number of copies of each gene per gram of soil differ greatly, with the number of 16S copies at the end of the experiment being greater than the copy number of the 18S gene by almost two orders of magnitude (or 100x). The number of copies of both the fungal (Figure 5.10a; ANOVA: $F_{1,32}= 76.6626$, $p < 0.001$) and bacterial (Figure 5.10b; ANOVA: $F_{1,32}= 22.3465$, $p < 0.001$) marker genes increase from the first time point to the second. However, post-hoc testing (Tukey’s HSD: $p < 0.05$) reveals that within treatments, 16S copy number does not significantly increase from one time point to the next.

Contrary to the trend seen based on time point, both the 18S (ANOVA: $F_{3,32}= 1.2267$, $p > 0.05$) and 16S (ANOVA: $F_{3,32}= 2.6387$, $p > 0.05$) load were unaffected by the soil treatment added to the soil. While overall all treatments have statistically similar means, there is a slightly greater number of 18S copies in the “SW” treatment at both time points (Figure 5.10a). This non-significant elevation in the “SW” treatment also appears in the 16S copy number at the second time point (Figure 5.10b, shaded bars), while at the earlier time point (open bars) both the “SW” and “Control” treatments contain slightly, however not significantly (Tukey’s HSD: $p > 0.05$), elevated mean copy numbers.

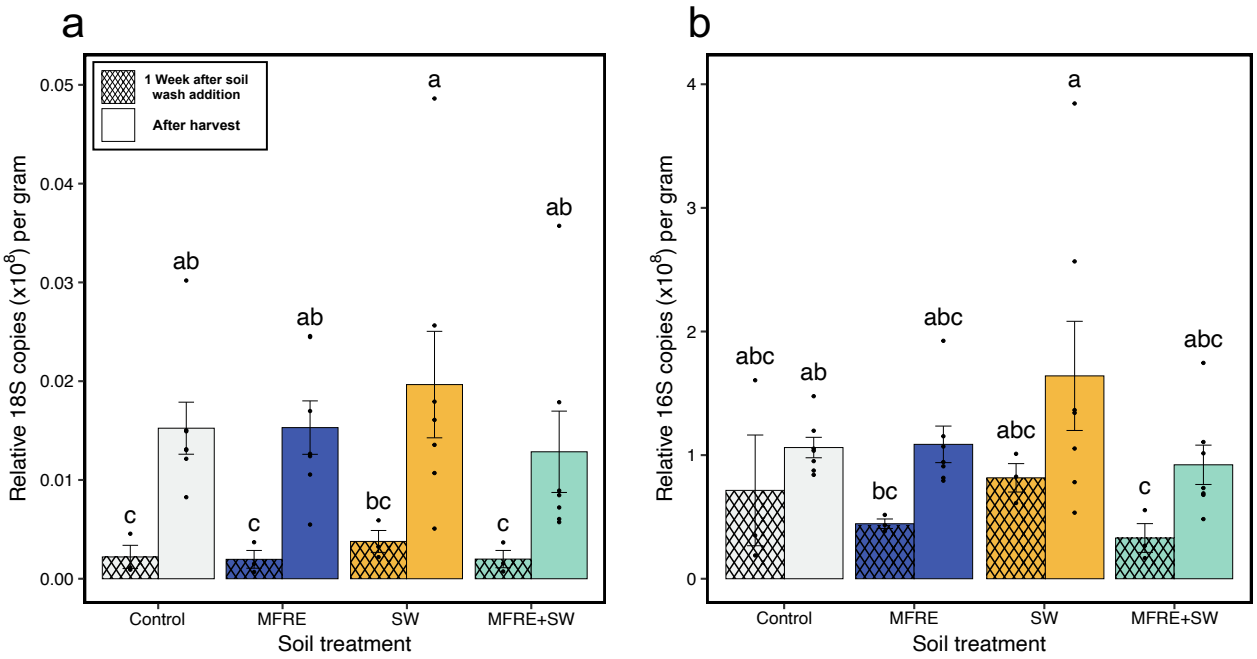

Figure 5.10 18S & 16S gene copies (x10^8). a) Mean 18S copy number compared between 1-week post inoculation (open bars) and after harvesting posts (shaded bars). **b)** Mean 16S copy number compared between 1-week post inoculation (hatched bars) and after harvesting posts (solid bars). Error bars represent ±SE, while different letters denote significantly different means (Tukey's HSD: $p < 0.05$). n = 3 per treatment in time point 1 (hatched bars), n = 7 per treatment in time point 2 (solid bars).

5.5 Discussion

The experiment conducted for this study was designed to elucidate any potential effects of soil bacterial communities on the colonisation and transfer of nutrients by MFRE fungi to *Plantago lanceolata* hosts. Owing to the complexity and need for a large number of replicates, the design included blocks of replicates established at different times (see table S5.2). Despite identical growth conditions and inoculation methods, each replicate block resulted in significantly different data trends across many of the measures taken.

The trends in colonisation based on soil inoculation treatment applied were, however, similar between replicate blocks. I observed significantly reduced

colonisation by fungi in the treatments where neither MFRE inoculum nor soil wash were directly applied (“Control”; Figure 5.4) compared to the two treatments where MFRE inoculum was included “MFRE+SW” and “MFRE” treatments. However, although not statistically significant, aside from the first replicate block, where the soil wash was added to pots not containing MFRE (“SW”) there was increased presence of fungi in *Plantago lanceolata* roots. It is hard to determine whether the fungus present in these roots was MFRE or AM fungi owing to significant morphological plasticity between both clades of mycorrhizal fungi.

In addition to observed colonisation in uninoculated treatments, there were similar numbers of copies of the fungal 18S gene in the soils of all mesocosms (Figure 5.10a) indicating that fungi were present in these systems in similar quantities in all treatments. This adds credence to the observations of hyphal colonisation in uninoculated *Plantago lanceolata* roots. However, the large differences in colonisation counts based on soil amendment may highlight the fact that, despite there being relatively similar amounts of fungi in each treatment as per the 18S data, there may be additional fungal to MFRE in mesocosms treated with a soil wash (“MFRE+SW” and “SW”) or those untreated and inoculated by uncontrolled means.

What can be determined from these is that the filtration method used to remove fungal propagules from the soil wash requires significant refinement to more effectively exclude fungi from future experiments. Molecular techniques to identify the taxa present in soil washes and verify the exclusion of AM fungi were not used here but are vital for future studies using this method.

The presence of fungal colonisation roots in supposedly uninoculated plants could also be due to the proximity of inoculated and uninoculated mesocosms within the growth chamber. There is a growing body of work investigating the dispersal of AM fungal spores which shows that spores are present in aerial samples even in relatively AM desolate urban centres (Chaudhary *et al.,* 2020).

Given the colocation and frequent rotation of mesocosms within replicate blocks to eliminate within-block effects throughout the growth period, spores from pots

inoculated with MFRE, or in the non-sterile atmosphere of the growth chamber may have entered uninoculated mesocosms, resulting in the non-zero colonisation of “Control” and “SW” treatments.

Concurrent with the potential cross contamination of MFRE to uninoculated mesocosms or unintentional inoculation with non-MFRE fungal spores, there are statistically similar numbers of bacterial 16S gene copies in the bulk soil of all treatments of the first replicate block at both sample time points (Figure 5.10b).

A noteworthy observation in the 16S data is that the average copy number of the “SW” and “Control” treatments is higher 1-week post inoculation than the two treatments where MFRE inoculum was added to pots (“MFRE+SW” and “MFRE” treatments). After the experiment was harvested, the elevated mean copy number of the 16S gene is only retained in the “SW” treatment (Figure 5.10b). Taken together, these could indicate that the presence of MFRE in the early stages of bacterial inoculation somewhat suppresses bacterial growth. However, at the end of the experiment, some 12 weeks after inoculation, the increased mean 16S copy number is only retained in the “SW” treatment. This could potentially be due to a greater diversity of bacteria introduced into these pots with the soil wash than could have potentially aerially cross-contaminated the “Control” mesocosms.

In terms of biomass, overall plants in replicate block 2 had greater shoot (Figure 5.2) and root biomasses (Figure 5.3) than any in replicate blocks 1 or 3. Within replicate blocks, no statistically significant differences were observed between mesocosms of different soil amendments. It is hard to draw any conclusions from the lack of growth promotion or inhibition observed in this experiment as fecundity, seed count or flower attractiveness to pollinators were not quantified. As such the long-term effects of these soil amendments cannot be determined.

Interestingly, this trend of greater mass in block 2 is not replicated by the total N concentration of *Plantago lanceolata* shoots. The only significant differences between treatments were observed between “MFRE+SW” treatment in block 1 and the “MFRE” treatment in block 3 which contained less N. Within replicate blocks no differences were observed between different treatments. From this, it is reasonable

to conclude that plant N concentration is not a function of biomass, and that in this experiment plants retain the same rate of N acquisition regardless of the presence of microorganisms.

Counter to this, the total P concentration in plant shoots (Figure 5.5) varies by replicate block with all treatments in replicate block 1 containing a significantly greater concentration of P than all treatments in both other replicate blocks, barring the “MFRE” treatment in block 1 which was not significantly different from the “MFRE” and “MFRE+SW” treatments in block 2. This pattern is not found in any of the other factors measured and cannot be explained. However, despite this difference being statistically significant, the range over which this difference occurs is very small, around 1mg. Therefore, this is unlikely to have a significant bearing on my interpretation of the data.

The important effects of mycorrhizal inoculation I established this experiment to investigate are the direct nutrient transfer between plants and their mycorrhizal fungal partners, and how this is complicated by the presence of soil bacteria.

I applied ^{33}P to soil cores within mesocosms to observe the effects of MFRE and bacteria have on plant ^{33}P acquisition (Figure 5.8). Net fungal transfer of ^{33}P to *Plantago lanceolata* was not significantly affected by any of the soil treatments applied to the mesocosms (Table S5.1). Despite this, the apparent lack of transfer of ^{33}P in the “SW” treatment of replicate block 1 does correspond with the slightly increased mean copy numbers of both the 18S and 16S genes. This could indicate that the presence of soil bacteria and unknown fungal species inhibit the mycorrhizal acquisition of ^{33}P by plants. It could be concluded, based on the fact that there is ^{33}P transfer in the “MFRE+SW” treatment, that symbiotic MFRE ameliorate the effects of non-mycorrhizal fungi and suppress the size of bacterial communities.

^{15}N was added to mesocosms in one of three forms, ^{15}N-ammonium chloride, ^{15}N-glycine, and ^{15}N-urea. These are common sources of N in soils (Hawkins *et al.,* 2000; Reay *et al.,* 2019) and have demonstrated transfer of ^{15}N from some of these sources in **Chapters 3** and **4**. These sources were injected in 3 separate

treatments into hyphosphere soil within meshed ingrowth cores. Within nitrogen source treatments, no differences were observed in ^{15}N assimilation based on the soil treatment. There were only two treatment combinations that were significantly different; the “MFRE” treatment treated with ^{15}N-glycine contained significantly less ^{15}N than mesocosms in the “MFRE” treated with ^{15}N-urea.

There is a similar pattern of ^{15}N assimilation based on soil treatment in mesocosms labelled with ^{15}N-ammonium and ^{15}N-urea. Within these two ^{15}N treatments, the greatest fungal-derived ^{15}N concentration is seen within the “MFRE” treatment followed by the “MFRE+SW” with the second greatest mean fungal-acquired ^{15}N concentration. After these, both “NF” treatments within the ^{15}N-ammonium and ^{15}N-urea had roughly similar mean fungal ^{15}N concentrations, albeit lower than the “MFRE+SW” treatments. What these very similar trends could show is that for these two sources of nitrogen, the presence of MFRE colonising plant roots enhances ^{15}N assimilation from the soil. The absence of a bacterial community (or a community originating in soil as discussed above) as in the “MFRE” treatments further increases this assimilation, showing that potentially, bacterial communities originating from environmental soil samples may compete with MFRE for available N in the system, leading to the observed reduction in fungal ^{15}N.

Urea, despite its toxic properties to some clades of bacteria (Motasim *et al.,* 2024) is also degraded in soils by some other bacterial groups such as *Sporosarcina pasteurii* and *Bacillus megaterium* via urease enzymes (Phang *et al.,* 2018; Mekonnen *et al.,* 2021). These enzymes hydrolyse the amino acid, releasing ammonium, a nitrogen source readily assimilated by MFRE for transfer to *Plantago lanceolata* (Fig. 3,4 and Fig. 4.6) and bicarbonate (Lasisi & Akinremi, 2021). This may explain the similar patterns in ^{15}N assimilation between mesocosms treated with ^{15}N-ammonium chloride and ^{15}N-urea.

The trends observed in mesocosms treated with ^{15}N-ammonium and ^{15}N-urea are not mirrored in the fungal assimilation of ^{15}N in mesocosms treated with ^{15}N-glycine. Within this ^{15}N treatment the “MFRE” soil treatment contained the lowest, mean fungal-derived [^{15}N] with all other treatments having roughly similar mean [^{15}N]. This is markedly different from the glycine-derived ^{15}N assimilation under

monoxenic conditions (Fig. 3,4 and Fig. 4.6) in which ^{15}N assimilation from glycine is assimilated by MFRE and transferred to *Plantago lanceolata* plants in greater quantities than the other ^{15}N sources applied. The reduced ^{15}N assimilation from glycine in these systems, is somewhat counterintuitive given the high colonisation rate in the "MFRE" treatment (Figure 5.4), and presence of MFRE and likely other fungal taxa along with bacterial contamination (Figure 5.10b). It is possible that the bacteria in the system presented competition with MFRE for the glycine, resulting in the elimination of any ^{15}N transfer to *Plantago lanceolata*. As a small amino acid, glycine represents a source of both carbon and nitrogen to all soil microorganisms, however, work has shown that bacteria are able to assimilate amino acids more rapidly than fungi (Bardgett *et al.,* 2003). While the mechanism of glycine assimilation suggested in **chapter 4** is untested, my data may suggest that these mechanisms are similar to other fungal groups in terms of amino acid assimilation.

The relatively even C acquisition by fungal species in the soil of mesocosms across all soil treatments, taken in conjunction with the 18S and 16S data indicates that the parameters of this experiment were not tightly controlled enough to prove or disprove the hypotheses of this experiment.

There are a few possible explanations for the variation between replicate blocks observed in this experiment. Firstly, the nature of the glasshouse conditions the experiment was conducted under. Despite supplementary light and heating, the growth space may have had some temperature and lighting variation according to when in the year mesocosms were established, resulting in the observed differences between replicate blocks. Secondly, there may be some effect of the storage of the soil sample taken to produce the soil wash. This was stored at 4ºC between blocks and used on the day of mesocosm establishment to prepare fresh soil wash. There was potentially some turnover or mineralisation of nutrients within the soil sample during this time, resulting in differing addition of nutrients to pots which had soil wash added.

This study was initially conducted with the aim of determining how the presence of a soil-derived bacterial community affects the nutritional symbiosis between MFRE and *Plantago lanceolata.* The data obtained have shown that the experimental

design requires refinement to more stringently control for several unexpected factors, namely the unintentional aerial inoculation of control mesocosms with off-target bacteria and fungi. Quantification of plant root colonisation and qPCR determination of fungal gene copies in the soil of mesocosms both indicate that control treatments ("Control") were not in fact devoid of fungi as intended. This could potentially be rectified through the generation of and inoculation with synthetic bacterial communities comprising the most abundant clades within a given soil type. Additionally, mesocosms could be established and maintained under sterile conditions ensuring the only vectors of microbiota are the MFRE inoculum the soil wash.

In addition, the relatively low level of root colonisation compared to the comparable 18S copy numbers seen in the bulk soil indicates that, while some endophytic fungi were present in these systems, the majority may not have been. Therefore, as the identity of these contaminants is unknown, their effects on MFRE-plant symbioses cannot be hypothesised. More in-depth sequencing to identify the key bacterial taxa present in these systems could resolve some of the functional discrepancies observed. However, these data would only be useful if the functional metabolism of these clades has previously studied. Identification of functional genes in soil samples such as nitrification, denitrification, or urea degradation indicator genes would circumvent any missing literature on the key bacterial species.

5.6 Conclusion

While the experimental aims were not met, the data presented here do contain some interesting trends, allowing more directed questions to be asked.

Counter to the observations made using monoxenic systems, I have shown that the assimilation of glycine-N by plants in the presence of MFRE is reduced in comparison to both ammonium-N and urea-N. This result requires replication in other soil-based systems as previous work conducted in monoxenic systems showed a clear preference in assimilation for glycine. The discrepancy between these two results requires resolution as they are both strong and clear however also conflicting.

There are a number of structural differences between urea and glycine, affecting their stability and recalcitrance to microorganisms in the soil, that may be the root cause of this. Alternatively, there may also be procedural causes compounding the chemical differences; in the previous experiments using monoxenic plates, the labelling period used was only 24 hours, whereas the present soil-based experiment employs a much longer period of exposure to the ^{15}N tracer compounds. Therefore, the amount of time for degradation of urea to occur is much greater.

I did not observe any differences in ^{33}P assimilation by plants or C acquisition by fungi between the different soil treatments. While concrete conclusions cannot be drawn from these data, they do demonstrate the need for greater research into MFRE in soil-based systems. Importantly, maintaining stricter control over cross contamination and aerial inoculation of bacteria and fungi throughout the growth period of the experiment. Greater control over the biotic environment of these mesocosms would necessitate a reduction in replicate numbers owing to the greater care more complex growth setups require. This sacrifice in repeat number would likely be compensated for by much lower variation and greater statistical strength of data.

These data do not show any differences in plant biomass or growth when *Plantago lanceolata* plants were inoculated with MFRE. This was expected given the lack of enhanced shoot growth observed in experiments presented **Chapters 3** and **4** (Fig. 3.2) and indeed growth inhibition under low N conditions (Fig. 4.2).

This experiment was also designed to observe any potential growth effects of MFRE on plants in longer-term, soil-based experiments. The 12-week period of growth utilised here was employed in order to allow the *Plantago lanceolata* seedlings to achieve maturity without flowering, precluding analysis of seed production, as a proxy for plant fecundity. As such conclusions cannot be drawn about the effect of MFRE on *Plantago lanceolata* reproductive fitness.

Chapter 6: General Discussion

The aim of this thesis was to determine how nitrogen is used by MFRE fungi and exchanged with *Plantago lanceolata* hosts and to investigate how the symbiosis formed between MFRE fungi and plants is affected by the saprotrophic capabilities of MFRE. I investigated this in several different nutritional contexts and in the presence of differing levels of additional microbiota. Using different isotopes (^{15}N, ^{33}P, and ^{14}C) I conducted nutrient tracing experiments to ascertain direct evidence of nutrient transfer.

6.1 Nitrogen preference of MFRE fungi

The functional responses of MFRE-plant symbioses to their environments were investigated based along two key lines of research. Firstly, I investigated the responses of MFRE fungi to different abiotic contexts. Using monoxenic systems I grew *Plantago lanceolata* seedlings colonised by MFRE to vary both the range and combination of different ^{15}N-labelled compounds (**Chapter 3**), as well as the N concentration in the growth media (**Chapter 4**) to determine how the nutritional symbiosis between MFRE and *P. lanceolata* responds to these different abiotic contexts.

To investigate the responses of MFRE to different N sources and concentrations, I conducted a series of experiments to determine the sources of N MFRE is able to utilise, how they are rewarded by plants in return for resource provision, and how nutritional context affects the transfer of N from MFRE to plant (**Chapter 3, 4**) in monoxenic systems.

I hypothesised that MFRE would, similarly to AM fungi, prefer inorganic sources of nitrogen for assimilation and transfer to host plants and they are less energetically costly to degrade (Johansen *et al*., 1996; Toussaint *et al.,* 2004; Marzluf, 1997), are generally more mobile within soils (Hagedorn *et al.,* 2000; Wang *et al*., 2015) and are therefore less restricted to distinct patches of N enrichment. To test this hypothesis, I established an experiment wherein microcosms containing *Plantago lanceolata* inoculated with MFRE were presented with one of four separate

compounds containing a ^{15}N tracer. These four sources – ammonium, nitrate, glycine, and urea – were chosen as they are all common N sources found in soils (Reay *et al*., 2019) and have been shown to be assimilated by AM fungi in plate-based assays and transferred to plants (Hwakins *et al.,* 2000).

I observed significantly greater transfer of ^{15}N from glycine and ammonium than from any other source (**Chapter 3**; Figure 3.4a), an unexpected result as ammonium and glycine are inorganic and organic respectively and were utilised at rates significantly greater than nitrate or urea which are also inorganic and organic respectively. This greater ^{15}N transfer from ammonium and glycine came at the cost of elevated C transferred from the *Plantago lanceolata* to the MFRE of microcosms supplied with ammonium or glycine. This apparent exchange of C for N is solely based on the type of N supplied to each microcosm.

When applied in combination with one another, the pattern of MFRE transfer of ^{15}N to host plants remained broadly the same (Figure 3.4b). This indicates that there is indeed a preference for glycine- and ammonium-N above other source of N. The preference for glycine in both experiments is consistent with the understanding that MFRE can exist without plant hosts and therefore possess the ability to assimilate organic nutrients which represent a source of C as well as N that can be traded with host plants for additional C.

The lack of N assimilation from urea is interesting as urea is often metabolised by soil bacteria to the extent that quantification of urease – the enzyme utilised by soil bacteria to hydrolyse urea – is often used as a metric for soil N mineralisation (Cordero *et al.,* 2019). Therefore, the observed lack of direct assimilation of urea by MFRE could be due to the lack of rhizosphere microbiome in the experimental systems used, leading to inability of MFRE to pick up urea-derived N from scavenging, or symbiosis with other soil microorganisms.

6.2 Organic N and C assimilation by MFRE across a Nitrogen concentration gradient

The finding in **Chapter 3** that MFRE preferentially assimilate N from glycine, an organic molecule is consistent with the current understanding of MFRE as a

facultatively symbiotic fungus. When not associated with plants, a source of C is required as plant photosynthates would not be available. I tested this hypothesis in **Chapter 4** by presenting MFRE-colonised *Plantago lanceolata* with two isotopically labelled forms of glycine; ^{13}C-glycine and ^{15}N-glycine in equal concentrations to track the fate of N- and C-based components of glycine. This experiment was conducted under different concentrations of N in the 1/2GB5 media to investigate the effects of different environmental nutrient availabilities on MFRE-plant N transfer. I also traced the movement of photosynthetically derived C from plants to MFRE hyphae using isotopically labelled $^{14}CO_2$.

I observed a significant increase in ^{15}N transfer from glycine to *Plantago lanceolata* when under the lowest N concentration treatment, and very little ^{15}N transfer under higher N conditions. At the same time, however, the pattern of ^{14}C transfer from plant to MFRE under the same N gradient was the inverted, with higher plant-derived C in MFRE hyphae under high N conditions (Figure 4.8). This striking result demonstrates the variable nature of MFRE symbiosis, with a greater 'carbon cost' to host plants under high N with little return from MFRE. Under low N conditions this is inverted, with plants gaining N in return for comparatively lower 'carbon cost'. These data are consistent with the literature around AM fungal symbiosis and N transfer, which shows that transfer of N to plants is inhibited under N fertilisation (Jach-Smith & Jackson, 2020). However, my data challenge the applicability of the theory of biological markets to diverse mycorrhizal symbioses (Kiers *et al.,* 2011). If MFRE symbiosis follows this model of nutrient exchange dynamics, the patterns of ^{14}C transfer to MFRE would mirror plant ^{15}N assimilation. This is not the case, however, and under abundant N, MFRE cease N transfer to host plants while still gaining plant C, shifting to an apparently more parasitic mode of C acquisition. The long-term effects of this parasitic nutritional relationship on host plants are currently open to speculation, however there may be alternative benefits of symbiosis with MFRE to plants other than simple nutrition. While metrics of non-nutritional benefit were not measured in the studies presented here, this is a promising avenue for future research.

I also observed a significant reduction in plant C assimilation from ^{13}C labelled glycine in inoculated microcosms compared with uninoculated ones under low N

conditions (Figure 4.9). This, coupled with the increased growth of MFRE hyphae beyond plant roots under low N could imply that MFRE assimilate prevent plants from assimilating small amino acids, scavenging the glycine C skeleton to fuel hyphal growth, while simultaneously exchanging glycine-derived N with plants for additional C.

Additionally, I observed significantly reduced root colonisation under low N, which, in conjunction with observed increases in N transfer to plants, challenges the understanding that mycorrhizal colonisation and mycorrhizal benefits to plants are correlated.

6.3 The impact of secondary biotic interactions on MFRE-*Plantago* symbiosis

I attempted to translate my findings using sterile, monoxenic microcosms, to non-sterile soil-based mesocosms using blended ½ GB5-grown MFRE mycelium as MFRE inoculum (**Chapter 5**). This experiment was conducted to investigate how the presence of a bacterial community derived from environmental soil samples impacted the assimilation and transfer of various organic and inorganic soil N and P sources to from MFRE to host plants. I also established this experiment with the wider purpose of bringing MFRE research into greater ecological relevance; as informative of the fundamental biology of MFRE these monoxenic systems are, they cannot accurately simulate conditions of MFRE natural habitats. Thus, I designed this experiment to test the efficacy of using colonised axenic ½ GB5 plates as inoculum in soil-based systems.

Briefly, I inoculated soils with a fully factorial combination of live and mock MFRE inoculum, and a live and mock soil wash to introduce MFRE and soil bacteria to pot-based mesocosms respectively in different combinations. After a 12-week growth period, I then presented meshed ingrowth cores in each mesocosm with ^{33}P-orthophosphate and one of three ^{15}N-labelled compounds – ammonium, glycine, and urea – for a period of up to 3 weeks. During this time, half of the cores were rotated daily to sever direct hyphal connection between the

isotope tracers and the plant roots. After the assimilation period, the headspace of each mesocosm was sealed in a gas tight chamber and a pulse of $^{14}CO_2$ was released containing 0.25 MBq ^{14}C and plants were allowed to photosynthesise for a 24-hour period prior to harvest.

After harvest, the fungal and bacterial DNA was extracted from the soil of a subset of mesocosms and the number of copies of the fungal 18S and bacterial 16S ribosomal subunits was determined by qPCR as a proxy for the abundance of each clade present per gram of soil. This revealed the flaws in the inoculation control of this experiment as there were roughly even copy numbers of both 16S and 18S genes were observed across all treatments. This severely limits the reliability of the dataset obtained from the experiment as in three of the treatments applied the intention was that there would be no MFRE, no bacteria, or neither bacteria nor MFRE present. This trend was not mirrored in the root colonisation observed after staining with ink vinegar solution. In this data there is a clear trend of high colonisation in roots of MFRE inoculated plants, and low colonisation in those inoculated with mock MFRE inoculum. These two datasets together indicate that there were indeed fungi present in the soil of uninoculated pots but that the fungi present were not necessarily endophytic in nature. Regardless, the presence of these unidentified fungi, especially in mesocosms that were intended to contain no fungi is a severe limitation in the experimental setup.

The presence of contaminant fungi and bacteria in could have originated from several sources, the most obvious being the inocula used. I prepared the bacterial inoculum by mixing MES buffer and soil from the Bradfield Environment Laboratory research station high (Peak District, UK) before several filtration steps, progressively decreasing the mesh size used down to 45µm supposedly fine enough to exclude fungal propagules according to the literature (Walker, 2013; Boyno *et al.,* 2023). This method was most likely the source of contamination, however there may also have been aerial introduction of fungal spores and bacteria to mesocosms as they were grown in a non-sterile environment, with individuals of all four treatments in close proximity to one another. This has reduced the statistical power of the results, however there were some interesting trends worthy of discussion.

The most striking result I observed in this experiment is that there was significantly greater transfer of ^{15}N from urea in mesocosms with only MFRE applied (the "MFRE" treatment) than transfer of ^{15}N from glycine in mesocosms of the same "MFRE" treatment (Fig. 5.6). This is a radical departure from what observations made in **Chapter 3**. In these systems there was little to no transfer of ^{15}N from urea and significantly greater transfer of ^{15}N from glycine (Fig. 3.4). There are several potential causes of this altered pattern of N transfer by MFRE. Urea is degraded in soils to ammonium and bicarbonate via the ubiquitous urease enzymes produced by a number of soil-dwelling bacteria (Phang *et al.,* 2018; Mekonnen *et al.,* 2021; Lasisi & Akinremi, 2021). Similar patterns of ^{15}N assimilation were seen in mesocosms treated with ^{15}N-urea and ^{15}N-ammonium (Fig. 5.6), indicating that contaminant bacteria potentially degraded the labelled ^{15}N-urea into ^{15}N-ammonium that was then assimilated by MFRE and transferred to *Plantago lanceolata* hosts. The disparity between urea ^{15}N and glycine ^{15}N transfer to host plants was only observed in mesocosms inoculated solely with MFRE ("MFRE" treatments) indicating that, even if contaminant bacteria and fungi were present, the interaction between the MFRE and contamination is a unique one.

The limited transfer of ^{15}N to plants is of note. In the monoxenic experiments I observed the highest transfer of ^{15}N to host plants from glycine. While no definite conclusions can be drawn from the data, it is possible to generate hypotheses for why this may have occurred. There may be some level of competition between MFRE and soil bacteria, resulting in limited mycorrhizal transfer of ^{15}N to plants. The assimilation of small free amino acids in soils by bacteria and subsequent conversion to ammonium and subsequently nitrate (Moe, 2013) could explain this reduction in glycine-^{15}N in plant hosts. Further dedicated investigation using more rigorously controlled systems is required to determine the true causes of this interesting phenomenon.

The method I used to inoculate plants with MFRE in soil-based systems requires either refinement or replacement with a more effective methodology to obtain reliable data. While conceptually simple and requiring minimal effort, the use of blended agar plate inoculum does not result in a comparable total colonisation percentage to that seen in monoxenic agar-based systems. This method of

inoculation was used as the pot culture inoculum detailed in **Chapter 2** had not yet been developed. It is, however, the same method used to inoculate pot cultures with MFRE. The reduced level of colonisation seen in this experiment compared to colonisation seen in **Chapter 2** may be due to the length of time allowed for colonisation to occur. In **Chapter 4** I present colonisation data from the 'N concentration' experiment which was conducted using plants grown on agar in monoxenic systems (Fig. 4.3a). The average total colonisation (%) observed seven weeks after inoculation is between ~20% and ~40%. In contrast, soil-based systems inoculated with blended MFRE agar (Fig. 5.3) have an average total colonisation between ~15% and ~25%. This is a notable reduction when the homogenous distribution of MFRE inoculum throughout the soil and larger *Plantago lanceolata* root network is considered. Blended MFRE inoculum was mixed through the soil used in this experiment until an even distribution was achieved. This was done to ensure that plant roots would encounter MFRE propagules such as hyphal fragments and spore-like spherical swellings throughout the growth of the roots, theoretically infecting all new root tissue as early as possible. The procedure was adapted from the anecdotal observation that monoxenic systems where inoculating agar containing MFRE was applied directly to plant roots, colonisation appeared to occur earlier than if MFRE was applied to the systems further away from the roots. An example of this can be seen in figure 5e in appendix 2 (Howard *et al.,* 2024), the section of MFRE agar directly on the plant's root system appears to have more hyphae growing from it than either of the other two agar sections applied to the plate.

The comparatively reduced colonisation seen in the soil-based systems could be caused by a number of factors; firstly, there may be an effect of physical disruption to the MFRE structures caused by the blending process of the agar.

Physical damage to MFRE may reduce the viability of propagules plant roots encounter, causing the observed reduction in colonisation. There may also be an effect of the periodic drying of soil on the viability of the MFRE within each system. The MFRE isolate used, *Lyc-1*, was isolated from *Lycopodiella inundata* (Hoysted *et al.,* 2023) a clubmoss with a preference for wet habitats such as bogs and ponds, therefore this strain of MFRE may be susceptible to periodically dry conditions,

such as between watering, resulting in the observed reduced colonisation. There may be an impact of fungal mode of growth on the colonisation we observe; in blended plate inoculum, MFRE are not grown in the presence of or colonising plants. This likely causes the fungi to revert to a saprotrophic growth mode and could potentially delay colonisation of new plant roots by expending the energy needed to switch to a symbiotic mode of growth. Finally, there may be an effect of time on the colonisation efficacy of the method used. This experiment was conducted over the period of twelve weeks, nearly twice the duration of the monoxenic experiments. This comparatively longer time period may expose the small fragments of MFRE hyphae and propagules to nutrient, temperature, and drought stress prior to physical contact with live roots reducing the likelihood of vigorous colonisation.

The efficacy of MFRE inoculum could be improved by simply increasing the amount of blended agar inoculum added to each pot, this could potentially increase the total colonisation, however, the addition of a nutrient-rich agar medium to pots may not be controllable in experiments relying on nutrient manipulation. The isolation of MFRE spores and hyphae from agar medium may be a solution to this issue, however, the protocol for the extraction of hyphae from agar is time consuming, resource intensive, and requires large amounts of MFRE agar for limited extraction.

An alternative to generating inoculants from axenic agar plates altogether could be the use of root fragments from pre-colonised plants as described in **Chapter 2**. The use of recently harvested MFRE-colonised roots as inoculum could remedy many of the issues presented by the use of blended plate inoculum. Firstly, the production of this inoculum can involve the removal of excess soil medium from the inoculant roots, negating any potential impacts of nutrient content in the inoculant medium on experimental mesocosms. Secondly, MFRE propagules originating from symbiotic plants may be more primed to colonise new plants than propagules derived from axenic plates where MFRE is not in symbiosis with plants. In addition, there is a benefit to not blending the MFRE biomass as the production of AM fungal inoculum using pre-colonised plants requires cutting the colonised roots into 1-2 cm sections (See **Chapter 2**). This results in significantly more intact fungal biomass in the inoculum than blended agar inoculum as hyphae and other

structures are protected within the inoculum roots. The presence of the plant roots may also present as refuges for MFRE, physically sheltering the majority of MFRE fungal biomass from fluctuations in moisture, temperature, and light levels. They may also act as nutrient reservoirs, with whole fungal storage organs containing plant-derived C. This would buffer the MFRE from the stress of inoculant root harvest and fragmentation, allowing more time for MFRE to grow out from dead inoculant roots into new living plants. This inoculation conundrum is just one of the challenges to be addressed for MFRE research to advance and meet parity with AM fungal research.

6.4 The state of the art in MFRE research

The work I have presented in this thesis adds to the accepted literature in several ways:

Firstly, I have presented evidence that MFRE can degrade and assimilate organic compounds and utilise the breakdown products independently of one another (**Chapter 3**). Crucially, I demonstrate that this relationship is not obligate, and that under a decreasing N gradient, MFRE transfer more organically derived N to their hosts. This concurs previous work that suggests MFRE are not obligate biotrophs of plants as AM fungi are (Smith & Read, 2008; Field *et al.,* 2015a) and that MFRE use their saprotrophic capacity to occupy a niche when in symbiose with plants that AM fungi largely do not.

While they can live without symbiosis with plants, MFRE frequently do form associations with plants and have been shown to form nutritional mutualisms with several plant species including *Trifolium repens* (Hoysted *et al.,* 2023), several including *Allisonia* and *Neohodgsonia* (Field *et al.,* 2015a). Building on this, I have demonstrated throughout this thesis that *Plantago lanceolata* can be added to this roster of known MFRE partners (**Chapter 3; 4; 5**).

The facultatively saprotrophic capabilities of MFRE allows these fungi to be cultured *in vitro* in the absence of a host plant (**Chapter 2;** Field *et al.,* 2015a), allowing inoculation with single MFRE isolates to be carried out in experiments,

such as used by Hoysted *et al*. (2023) and in work presented in **Chapters 3** and **4** of this thesis. These methods of establishing experimental microcosms on solid agar media differ greatly from those employed by Hawkins et al. (2000) which were used to investigate nitrogen assimilation in AM fungal symbioses. The methods used by Hawkins *et al.* are complex, requiring multiple compartments with a 4–6-week initial setup time to allow for the slow growth of transformed carrot roots. While Hawkins et al. (2000) could demonstrate organic N assimilation by these AM fungi, the reliance on non-photosynthetic plant material precludes the ability to investigate the systemic effects of AM symbiosis on green plants.

The combination of factors which control the shift in MFRE lifestyle from saprotroph to mutualist are unknown. Using nutrient tracing data from destructively harvested samples (**Chapter 4**) I have shown that inorganic N availability is one of the key factors controlling the state of MFRE-plant symbiosis.

Work conducted by Field *et al.* (2016) demonstrated that MFRE and AM fungi can simultaneously colonise the same plant host and engage in reciprocal nutrient exchange. This work also showed that, in terms of nutritional function, MFRE and AM fungi may be complementary, with AM fungi transferring greater amounts of phosphorus (P) and MFRE concurrently transferring more N to plant hosts. This division of labour between the two fungal symbionts renders much of what is thought about the functioning of AM fungi questionable. Many laboratory investigations into the symbiotic transfer of nutrients between AM fungi and plants do not ensure fully sterile conditions, rely solely on root staining and microscopy for assessment of colonisation, and do not routinely identify fungal symbionts to the species level post-harvest. Similarly, field investigations investigating the functions of AM fungi seldom utilise molecular primers that are incapable of identifying MFRE in environmental samples (Bidartondo *et al.,* 2011). There is an assumption that the only endomycorrhizal fungi to colonise plants are AM fungi of the Glomeromycota. There is also a general lack of awareness of the existence of MFRE in the wilder mycorrhizal research context, stemming from the long history of MFRE being taxonomically grouped with AM fungi. The two misconceptions have led to the severe underestimation the contributions of MFRE to plant nutrition globally. Much of what was previously believed about how AM fungi function

specifically regarding nitrogen transfer to plants, especially the results of environmental studies that fail to rule out the presence of MFRE in plant roots, now require re-evaluation in the context of MFRE nitrogen assimilation.

There is a pressing need to understand the functions of MFRE and their interactions with AM fungi and other soil microbes for two main reasons:

Firstly, in agricultural production, plant N fertilisation is one of the limiting factors of productivity with significant expenditure of non-renewable resources in producing nitrogen fertiliser to meet global demand (Anas *et al.,* 2020). AM fungi have been suggested as an alternative to synthetic N fertilisation (Watts *et al.,* 2023) and a number of products have been produced on the assumption that AM fungi alone can enhance plant nitrogen nutrition (Tarbell & Koske, 2007). If indeed, MFRE do play a larger role in global plant N acquisition, the production and use of solely AM fungal inoculants and the management of agroecosystems to promote AM fungi, and use of organic fertilisers may prove less effective than expected. Therefore, it is imperative to establish the basic biology of MFRE, namely the sources of N they assimilate and how they exchange these with plants for C.

Secondly, as a globally distributed group of fungi with a broadly distributed range of host plant species (Rimington *et al.,* 2015; Kowal *et al.,* 2020, Albornoz *et al.,* 2021), if MFRE play a significant role in plant and soil N cycling, as data presented in this thesis suggest, they necessarily play a significant role in global N cycling. If this is the case, the presence and normal functioning of MFRE in natural settings then requires close monitoring as MFRE would be a key indication of healthy soil functioning. As stated above, many accepted and widely used protocols for detecting mycorrhizal fungi in environmental soil samples do not utilise primers capable of identifying MFRE (Bidartondo *et al.,* 2011), and this would need rectification if MFRE were found to be of global significance. Additionally, recent work has begun to investigate the interactions between AM fungi and soil bacteria (Zhang *et al.,* 2016; Zhang *et al.,* 2018a; Zhang *et al.,* 2021; Jiang *et al.,* 2021). These studies were also designed and conducted with a baseline ignorance of the presence and functions MFRE. If, as the work presented in this thesis indicates, MFRE have significant impacts on soil N utilisation and plant nutrient acquisition

and interact with soil bacteria to achieve this, the clades of bacteria fostered by MFRE also by default become worthy of investigation. Therefore, the study and characterisation of the functions of MFRE fungi in relation to soil and plant nitrogen cycling are imperative for resolving the current uncertainty surrounding these enigmatic endophytes.

6.5 Conclusions

I have demonstrated carbon-for-nitrogen symbiosis between MFRE and *Plantago lanceolata*, preferential assimilation and transfer of glycine-N, and variation in symbiotic reciprocity based on nutritional context (**Chapter 4**). The data presented in this chapter demonstrates that MFRE fungi occupy their own nutritional niche, engaging in mutualistic symbiosis with plants under low N conditions and parasitising plants for photosynthetically derived C while assimilating organic C under high N.

The work I have presented here has developed on the systems used to investigate MFRE and has established a firm grounding from which future research can be based. Currently, without the extensive molecular toolkit available to the field of AM fungal research, the mechanisms of N assimilation and transfer to host plants remain under question. However, the data presented in this thesis allows us to propose some hypothetical mechanisms (Figure 6.1) to be tested as the necessary resources, such as an annotated genome, become available. The assimilation of C and N from glycine, as shown in **Chapter 3**, necessitates the degradation and separation of the N-containing amine group from the carbon skeleton of the full molecule. Whether organic compounds in the hyphosphere soil are hydrolysed by MFRE in-situ, by the secretion of degradative enzymes, or translocated into MFRE hyphae via amino acid permeases (AAPs) prior to hydrolysis, remains to be determined. Figure 6.1 also proposes that by bacterial-mediated processed, urea and nitrate are converted to ammonium which is then available to MFRE for direct assimilation. Both denitrification and urease hydrolysis are ubiquitous in soils (Berg and Bothe, 1992; Guettes *et al.,* 2002), therefore it is logical to hypothesise that MFRE evolved to use the common byproducts of bacterial metabolism instead of investing energy in producing the necessary enzymes to directly catalyse the

breakdown of these N sources. The data presented here suggest several mechanisms of MFRE-mediated N assimilation by plants summarised in Figure 6.1.

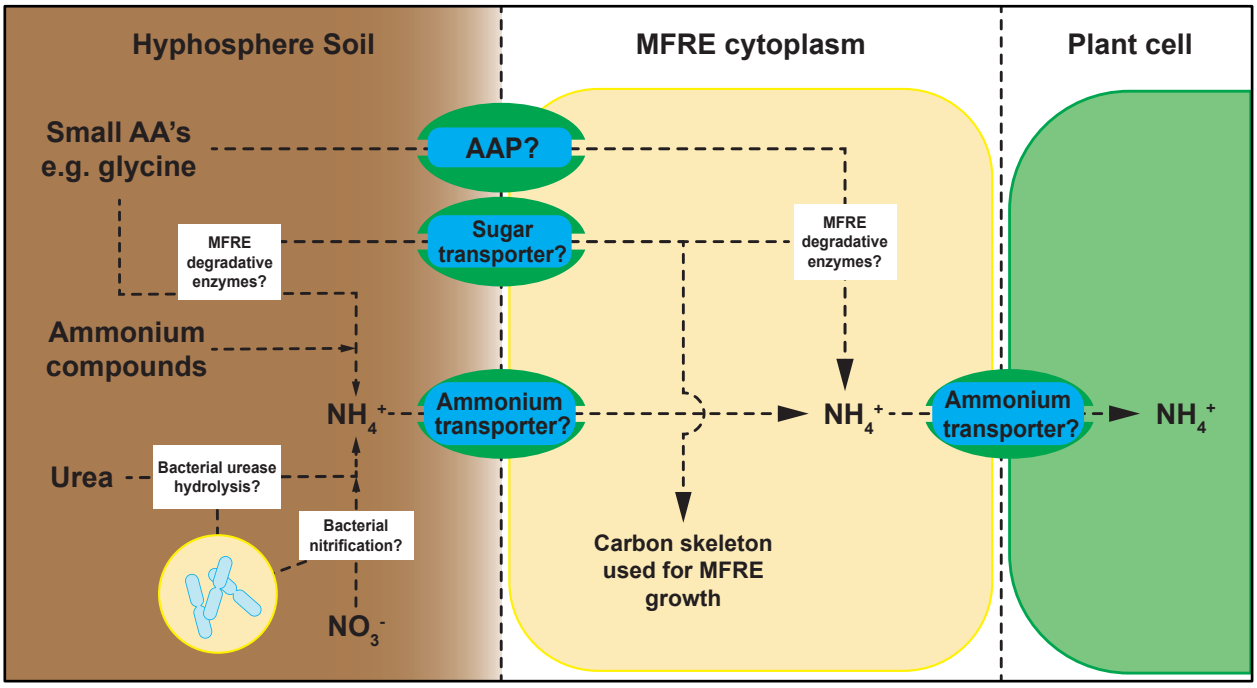

Figure 6.1 Diagram of proposed mechanisms of the assimilation and transport of organic and inorganic N sources from soils to plants via MFRE.

6.6 Future research directions

The work presented here has attempted to answer some of the basic questions surrounding the N use of MFRE. This data demonstrates the importance of MFRE in the nitrogen nutrition of their host plants. It also brings to light the assimilation of organic matter by MFRE and suggests that the fungi degrade organic compounds to utilise their C for growth while exchanging the N gained with plants for additional C. Additionally, the data presented in **Chapter 5** and the challenges faced in the reliable inoculation of plants by MFRE highlight how fundamental the state of research of MFRE currently is. It also highlights the need for dedicated and concerted collaboration between research groups to standardise inoculation protocols, experimental systems, and analysis pipelines to bring make datasets more comparable with one another.

The data I have presented raise some important questions to do with the specific nature of the biochemistry, physiology, and biotic interactions of MFRE, their hosts, and other soil microbes. These are as follows:

- By what mechanism do MFRE assimilate and degrade organic molecules?
- Are organic compounds broken down before or after assimilation by the MFRE fungi?
- Is the range of organic compounds MFRE can utilise limited to small amino acids?
- What are the longer-term effects of MFRE inoculation on plant growth and fecundity?
- Is N availability the only control over the position in the spectrum of mutualism that MFRE occupy when colonising plants?
- Which combination of factors influence the colonisation of plants by MFRE?
- Do MFRE assimilate the products of bacteria urea degradation for transfer to host plants?
- Is there competition between MFRE and soil bacteria for glycine?

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Table S2.1 Statistical tests, results, and transformations performed on data in chapter 2

Figure	Statistical test	Variable(s) tested	Transformation	P value	Post hoc test
2.2 Monoxenic time series colonisation	ANOVA; d.f = 6,14, F = 2.4512	**Time since inoculation**	**N/A**	p > 0.05	**Tukey's HSD**
2.6 Grouped wheat colonisation	ANOVA; d.f = 2,15, F = 24.076	**Time since inoculation**	**N/A**	p < 0.001	**Tukey's HSD**
S2.1 Wheat colonisation	ANOVA; d.f.= 2,9, F= 46.2180	**Time point**	**log transformed**	p < 0.001	**Tukey's HSD**
	ANOVA; d.f.= 2,9, F= 6.3304	**Cultivar**		p < 0.05	
	ANOVA; d.f.= 4,9, F= 2.0764	**Interaction**		p > 0.05	

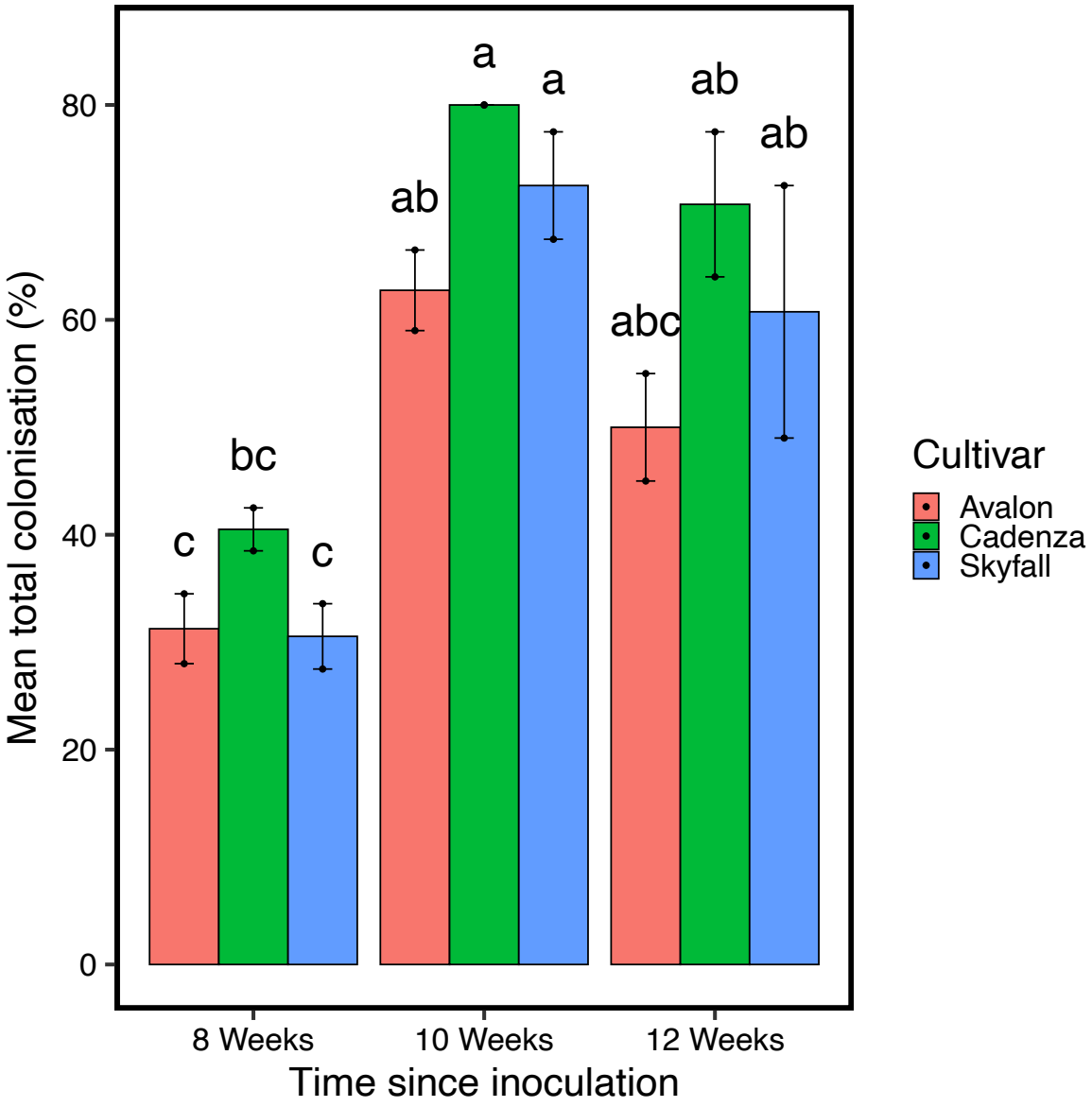

Figure S2.1 Mean colonisation of wheat plants compared over three time points and between three cultivars. n = 2 per treatment combination.

Figure S2.2 Protocol for establishing non-sterile MFRE inoculum from axenic plates.

Preparation of non-sterile MFRE inoculum

This protocol is for the propagation of non-sterile mycorrhizal inoculum. This is ideal if experiments require large volumes of mycorrhizal inoculum and/or require greater colonisation, as this method results in more successful colonisation of host plants. The inoculum is made by growing MFRE associated with the roots of a mixture of perennial plant species (first established with *Plantago lanceolata*, *Trifolium pratense* and *Holcus lanatus* (ribwort plantain, red clover and Yorkshire fog)).

Stocks are grown on 50:50 mix of pre-sterilised sand and perlite and are maintained in the greenhouse/growth chamber. 50% Gamborg nutrient feed should be given as required, good results have been obtained with regular weekly feedings of ~10ml.L.

Keeping the growth substrate well-watered is essential, using deionised water if possible, to avoid contamination.

Inoculum should be suitable to use when pots are between 6 and 18 months old. Make sure you use separate clean trays and wear clean new gloves to prevent cross contamination if there are other fungal species in the vicinity

Protocol

Surface sterilise seeds of *Plantago lanceolata, Trifolium repens,* and *Holcus lanatus* in a 4.5% solution of sodium hypochlorite. Pre-germinate on damp filter paper 7 days before sowing.

Homogenise 2 agar plates (~200ml) of ½ gamborg B5 well colonised with MFRE (lyc-1) with 50ml dH_2O to a loose consistency. Do this in a sterilised blender

Sow pregerminated seeds in the sand/perlite mix. Usually ,10-20 seedlings per species per 5L pot is good, some will die and having extra allows for this.

Add the blended plate inoculum on top of the seedlings and water well with dH_2O to draw the inoculum down into the soil medium.

Cover pots with cling film for the first 1-2 weeks until plants have become well established.

Keep cultures under glasshouse conditions (16hr day, 18 ºC) and water twice weekly with 200-300ml dH_2O while feeding once a week with 50ml ½ Gamborg B5 solution

Notes

If there is a low survival rate of seedlings initially sown, new seeds can be sterilised and directly sown into the soil.

When pot cultures are well established, make sure to pinch off flowers to prevent senescence

If pots become too overgrown, use sterilised shears to defoliate to the substrate surface, this allows for new growth, and encourages sporulation of the fungus by starving it of plant carbon.

First done after 3 months of growth

Pots can be kept for 6 months - 1yr. Make sure not to let them get too old as they can become root bound and some roots may start to decay.

Establishing new pots from initial pot culture

To establish new cultures from existing ones, defoliate starting pots up to 1 week before they are to be subcultured.

Remove roots from pots and break them into 1-2cm pieces by tearing/cutting with sterilised shears.

Mix existing sand/perlite/root fragment with an equal volume of fresh sterile substrate of the same composition (50:50 sand:perlite by vol) and plant pre-germinated seedlings.

Maintain as above.

Table S3.1 Statistical tests, results, and transformations performed on data in chapter 3.

Fig.	Statistical test	Variable(s) tested	Transformation	P value	Post hoc test & p value
3.2a	ANOVA; d.f.=1,110, F=0.5905	Fungal inoculation	N/A	p > 0.05	N/A
	ANOVA; d.f.=3,110, F=2.2187	^{15}N treatment		p > 0.05	
	ANOVA; d.f.=3,110, F=1.2649	Interaction		p > 0.05	
3.2b	ANOVA; d.f.=1,110, F=7.0530	Fungal inoculation	Log transformed	p < 0.01	Tukey's HSD; p<0.05
	ANOVA; d.f.=3,110, F=3.2880	^{15}N treatment		p < 0.05	
	ANOVA; d.f.=3,110, F=1.7085	Interaction		p > 0.05	
3.2c	ANOVA; d.f.=1,99, F=0.0009	Fungal inoculation	N/A	p > 0.05	N/A
	ANOVA; d.f.=3,99, F=1.4947	^{15}N treatment		p > 0.05	
	ANOVA; d.f.=3,99, F=0.9285	Interaction		p > 0.05	
3.2d	ANOVA; d.f.=1,99, F=2.2155	Fungal inoculation	N/A	p > 0.05	N/A
	ANOVA; d.f.=3,99, F=0.0001	^{15}N treatment		p > 0.05	
	ANOVA; d.f.=3,99, F=1.0878	Interaction		p > 0.05	
3.3a	ANOVA; d.f.=1,109, F=4.80	Fungal inoculation	Log transformed	p < 0.05	Tukey's HSD: p<0.05
	ANOVA; d.f.=3,109, F=3.91	^{15}N treatment		p < 0.01	
	ANOVA; d.f.=3,109, F=1.52	Interaction		p > 0.05	
3.3b	ANOVA; d.f.=1,99, F= 7.26	Fungal inoculation	Log transformed	p < 0.01	Tukey's HSD: p<0.05
	ANOVA; d.f.=3,99, F= 4.25	^{15}N treatment		p < 0.01	
	ANOVA; d.f.=3,99, F= 1.54	Interaction		p > 0.05	
3.4a	ANOVA: d.f.=3,74, F=3.7022	Shoot [^{15}N] ($\mu g.g^{-1}$)	Square root transformed	p < 0.05	Tukey's HSD; p<0.05
3.4b	ANOVA: d.f.=3,35, F= 4.3933	Shoot [^{15}N] ($\mu g.g^{-1}$)	Square root transformed	p < 0.01	Tukey's HSD; p<0.05
3.5	Kruskal-Wallis; X^2=20.256, d.f.=3	Plant-derived ^{14}C in MFRE containing media	N/A	p < 0.001	Dunn's post-hoc: p<0.05

Table S4.1 Statistical tests, results, and transformations performed on data in chapter 4.

Fig.	Statistical test	Variable(s) tested	Transformation	p value	Post hoc test & p value
4.2	ANOVA; d.f.=1,51, F=37.6797 ANOVA; d.f.=2,51, F=0.7200 ANOVA; d.f.=2,51, F=2.9633	Fungal inoculation N concentration Interaction	Log transformation	p < 0.001 p > 0.05 p > 0.05	Tukey's HSD: p<0.05
4.3a	ANOVA; d.f.=2,39, F=6.5694	Total Colonisation	N/A	p < 0.01	Tukey's HSD: p<0.05
4.3b	ANOVA; d.f.=2,39, F=10.561	Vesicular Colonisation	N/A	p < 0.001	Tukey's HSD: p<0.05
4.4	ANOVA; d.f.=2,39, F= 20.186 ANOVA; d.f.=1.91,74.37, F= 350.927 ANOVA; d.f.=3.81,74.37, F= 14.856	N Concentration Time Point Interaction	N/A	p < 0.001 p < 0.001 p < 0.001	Pairwise t-tests (Table S2)
4.5	ANOVA; d.f.=1,51, F=15.5000 ANOVA; d.f.=2,51, F=15.7688 ANOVA; d.f.=2,51, F=0.3617	Fungal inoculation N concentration Interaction	N/A	p < 0.001 p < 0.001 p > 0.05	Tukey's HSD: p<0.05
4.6	Kruskal-Wallis: d.f. = 2, X^2= 9.9668	Shoot [^{15}N] ($\mu g.g^{-1}$)	N/A	p < 0.01	Dunn's post-hoc: p<0.05
4.7	ANOVA: d.f = 2,39, F= 1.8301	[C] in MFRE hyphae ($ng.g^{-1}$)	N/A	p > 0.05	Tukey's post-hoc: p > 0.05
4.8	ANOVA; d.f.=1,50, F= 20.6347 ANOVA; d.f.=2,50, F=5.0497 ANOVA; d.f.=2,50, F=1.2897	Fungal inoculation N concentration Interaction	N/A	p < 0.001 p < 0.01 p > 0.05	Tukey's HSD: p<0.05

Table S4.2 Pairwise T-test statistics for hyphal growth at each time point (1-7)

Time point	Treatment 1	Treatment 2	T_{13}	p value	Significance code
1	187.4 $\mu g.gN^{-1}$	93.7 $\mu g.gN^{-1}$	0.2919211	p > 0.05	ns
	187.4 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-5.4530972	p < 0.001	***
	93.7 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-4.7877685	p < 0.01	**
2	187.4 $\mu g.gN^{-1}$	93.7 $\mu g.gN^{-1}$	-1.9652583	p > 0.05	ns
	187.4 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-5.0535088	p < 0.001	***
	93.7 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-4.8184044	p < 0.01	**
3	187.4 $\mu g.gN^{-1}$	93.7 $\mu g.gN^{-1}$	-2.164201	p > 0.05	ns
	187.4 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-5.4294239	p < 0.001	***
	93.7 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-4.3429743	p < 0.01	**
4	187.4 $\mu g.gN^{-1}$	93.7 $\mu g.gN^{-1}$	-1.4703968	p > 0.05	ns
	187.4 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-4.2385481	p < 0.01	**
	93.7 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-3.1249716	p < 0.05	*
5	187.4 $\mu g.gN^{-1}$	93.7 $\mu g.gN^{-1}$	-3.4933944	p < 0.05	*
	187.4 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-4.6848381	p < 0.01	**
	93.7 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-1.543355	p > 0.05	ns
6	187.4 $\mu g.gN^{-1}$	93.7 $\mu g.gN^{-1}$	-4.8014318	p < 0.01	**
	187.4 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-7.8208239	p < 0.001	***
	93.7 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-2.366826	p > 0.05	ns
7	187.4 $\mu g.gN^{-1}$	93.7 $\mu g.gN^{-1}$	-4.6667448	p < 0.01	**
	187.4 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-6.173636	p < 0.001	***
	93.7 $\mu g.gN^{-1}$	25 $\mu g.gN^{-1}$	-1.2448251	p > 0.05	ns

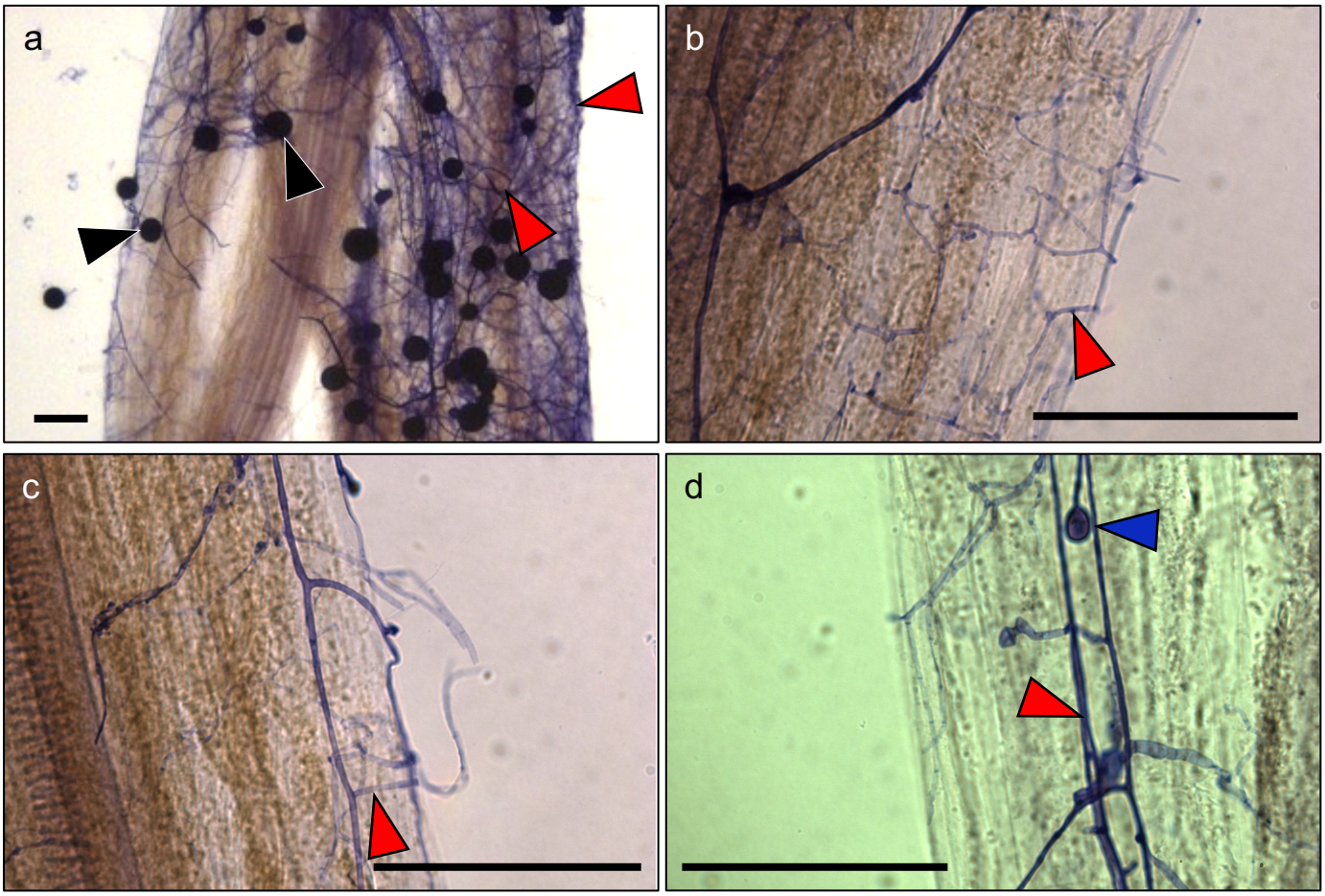

Figure S4.1 Micrographs of MFRE in *Plantago lanceolata* roots. **a)** Fine hyphae (Red arrow) and spherical structures (Black arrows) on root surface (20x magnification). **b-d)** Fine branching hyphae within *Plantago* root epidermal cells (red arrows; 100x magnification). **d)** Branching hyphae with terminal swellings (Blue arrows; 100x magnification). Scale bars = 50µm.

Table S5.1 Statistical tests, results, and transformations performed on data in chapter 5.

Figure	Statistical test	Variable(s) tested	Transformation	P value	Post hoc test
5.2 Shoot mass	ANOVA; d.f.= 3,132, F= 0.0289	Soil treatment	N/A	p > 0.05	Tukey's HSD
	ANOVA; d.f.= 2,132, F= 153.5916	Replicate block		p < 0.001	
	ANOVA; d.f.= 6,132, F= 1.2371	Interaction		p > 0.05	
5.3 Root mass	ANOVA; d.f.= 3,132, F= 0.2230	Soil treatment	N/A	p > 0.05	Tukey's HSD
	ANOVA; d.f.= 2,132, F= 67.5191	Replicate block		p < 0.001	
	ANOVA; d.f.= 6,132, F= 2.0764	Interaction		p > 0.05	
5.4 Colonisation	ANOVA; d.f.= 3,132, F= 114.5991	Soil treatment	Square root transformed	p < 0.001	Tukey's HSD
	ANOVA; d.f.= 2,132, F= 0.1108	Replicate block		p > 0.05	
	ANOVA; d.f.= 6,132, F= 3.8985	Interaction		p < 0.01	
5.5 Total [N]	ANOVA; d.f.= 3,132, F= 2.6556	Soil treatment	Square root transformed	p > 0.05	Tukey's HSD
	ANOVA; d.f.= 2,132, F= 3.0485	Replicate block		p > 0.05	
	ANOVA; d.f.= 6,132, F= 2.1110	Interaction		p > 0.05	
5.6 Total [P]	ANOVA; d.f.= 3,132, F= 2.0986	Soil treatment	Log transformed	p > 0.05	Tukey's HSD
	ANOVA; d.f.= 2,132, F= 79.5797	Replicate block		p < 0.001	
	ANOVA; d.f.= 6,132, F= 1.0760	Interaction		p > 0.05	
5.7 Plant shoot [^{15}N]	ANOVA; d.f.=3,36, F= 1.3066	Soil treatment	Square root transformed	p > 0.05	Tukey's HSD
	ANOVA; d.f.=2,36, F= 1.9269	N source applied		p > 0.05	
	ANOVA;2,36, F= 1.5315	Replicate block		p > 0.05	
	ANOVA; 6,36, F= 2.2263	Soil treatment/replicate block interaction		p > 0.05	
	ANOVA; d.f.= 6,36, F= 0.7299	Treatment/Replicate block interaction		p > 0.05	
	ANOVA; d.f.=4,36, F= 2.5750	N source/Replicate block Interaction		p > 0.05	
	ANOVA; d.f.=12,36, F= 1.4791	N source/Treatment/Replicate block interaction		p > 0.05	
5.8 Plant shoot [^{33}P]	ANOVA; d.f.= 3,60, F= 1.5175	Soil treatment	N/A	p > 0.05	Tukey's HSD
	ANOVA; d.f.= 2,60, F= 1.2510	Replicate block		p > 0.05	
	ANOVA; d.f.= 6,60, F= 0.6223	Interaction		p > 0.05	
5.8 Plant shoot [^{33}P]	ANOVA; d.f.=3,68, F = 1.5579	Soil treatment	N/A	p > 0.05	Tukey's HSD
5.9 Plant-derived [C] in soil	ANOVA; d.f.= 3,132, F= 2.8080	Soil treatment	Log transformed	p < 0.05	Tukey's HSD
	ANOVA; d.f.= 2,132, F= 11.2273	Replicate block		p < 0.001	
	ANOVA; d.f.= 6,132, F= 1.5982	Interaction		p > 0.05	
5.10a	ANOVA; d.f.=3,32, F= 1.2267	Soil treatment	Log transformed	p > 0.05	Tukey's HSD
	ANOVA; d.f.=1,32, F= 76.6626	Time post inoculation		p < 0.001	

Fungal community size	ANOVA; d.f.=3,32, F= 0.4645	**Interaction**		p > 0.05	
5.10b Bacterial community size	ANOVA; d.f.=3,32, F= 2.6387	**Soil treatment**	**Log transformed**	p > 0.05	**Tukey's HSD**
	ANOVA; d.f.=1,32, F= 22.3465	**Time post inoculation**		p < 0.001	
	ANOVA; d.f.=3,32, F= 0.4423	**Interaction**		p > 0.05	

Table S5.2 Experimental timeframe

Replicate block	Date established	Date of soil labelling (days since establishment)	Date of $^{14}CO_2$ fumigation (days since soil labelling)
1.1	10/05/22	02/08/22 (84)	**23/08/22 (21)**
1.2	10/05/22	02/08/22 (84)	**25/08/22 (23)**
2.1	24/05/22	16/08/22 (84)	**15/09/22 (30)**
2.2	24/05/22	16/08/22 (84)	**21/09/22 (36)**
3.1	07/06/22	06/09/22 (91)	**04/10/22 (28)**
3.2	07/06/22	06/09/22 (91)	**06/10/22 (30)**

Appendices

Appendix 1 Howard *et al.*, (2022)

Received: 10 December 2021 | Revised: 19 April 2022 | Accepted: 10 May 2022

DOI: 10.1111/ppl.13715

SPECIAL ISSUE ARTICLE

Physiologia Plantarum

The potential role of Mucoromycotina 'fine root endophytes' in plant nitrogen nutrition

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Funding information
H2020 European Research Council, Grant/Award Number: 865225; Natural Environment Research Council, Grant/Award Number: NE/S009663/1

Edited by A. Sandanandom

Abstract

Mycorrhizal associations between fungi and plant roots have globally significant impacts on nutrient cycling. Mucoromycotina 'fine root endophytes' (MFRE) are a distinct and recently characterised group of mycorrhiza-forming fungi that associate with the roots of a range of host plant species. Given their previous misidentification and assignment as arbuscular mycorrhizal fungi (AMF) of the Glomeromycotina, it is now important to untangle the specific form and function of MFRE symbioses. In particular, relatively little is known about the nature of MFRE colonisation and its role in N uptake and transfer to host plants. Even less is known about the mechanisms by which MFRE access and assimilate N, and how this N is processed and subsequently exchanged with host plants for photosynthates. Here, we summarise and contrast the structures formed by MFRE and arbuscular mycorrhizal fungi in host plants as well as compare the N source preference of each mycorrhizal fungal group with what is currently known for MFRE N uptake. We compare the mechanisms of N assimilation and transfer to host plants utilised by the main groups of mycorrhizal fungi and hypothesise potential mechanisms for MFRE N assimilation and transfer, outlining directions for future research.

1 | INTRODUCTION

Nitrogen (N) is a key macronutrient in plant nutrition (Evans, 1989) and, as a result of global N limitation (Vitousek & Howarth, 1991), is a key driving force in plant competition and evolution (Kang et al., 2015; Pankoke et al., 2015). N occurs within soils in both inorganic mineral (e.g. ammonium and nitrate salts; Matsumoto et al., 2000) and organic forms (derived from plant, animal and microbial decay; Greenfield, 2001); organic N can be as high as 90% of total soil N in some habitats (e.g. moorland soil in the vicinity of *Calluna vulgaris*; Abuarghub & Read, 1988). Major classes of organic N-containing compounds found within soils include free amino acids, polypeptides and proteins, purines, pyrimidines and vitamins. There is evidence that organic N is important for many plant N budgets across a variety of ecosystems as plant-available inorganic N pools are often limiting (Talbot & Treseder, 2010).

>500 My of plant evolution has driven a huge array of plant adaptations and strategies to enhance N access and assimilation in N-limited environments, including in extreme cases carnivory to directly access different N pools (Bott et al., 2008; Roberts & Oosting, 1958). In less extreme and more widespread cases, symbiotic associations with soil microbes provide indirect access to otherwise unavailable soil N pools (Phillips et al., 2011; Smith & Read, 2008). A diverse array of microorganisms live within the rhizosphere and surrounding soil, and play an important role in plant N nutrition through cycling and degradation of mineral and organic N (Phillips et al., 2011; Truu et al., 2020) and some by forming symbioses with plants. For example, rhizobia colonise the roots of legumes, inducing nodule

formation, which facilitates atmospheric N_2 fixation by the bacteria and transfer of N_2 to the host plant in return for plant-fixed carbon (C) (Andrews & Andrews, 2017). A different nutritional symbiosis is formed between the vast majority of plants and certain groups of soil fungi; these partnerships are known as mycorrhizas (Brundrett, 2009; Brundrett & Tedersoo, 2018). Mycorrhizal fungi have until recently been classified into four main groups based on colonisation structures, morphology, and host range (Brundrett, 2009; Brundrett & Tedersoo, 2018; Table 1), all of which show evidence of nitrogen transfer from the fungi to the host plants (Field et al., 2019; Fochi et al., 2017; Makarov, 2019; Stuart & Plett, 2020). The most commonly occurring groups of mycorrhizal fungi are spread across three fungal phyla: Mucoromycota, Ascomycota, and Basidiomycota (Spatafora et al., 2016; Stuart & Plett, 2020). Within Mucoromycota, arbuscular mycorrhizal fungi—the most researched group of mycorrhiza-forming fungi—are found within the subphylum Glomeromycotina (syn. Glomeromycota) (Schüßler & Walker, 2011). Glomeromycotina arbuscular mycorrhizal fungi (AMF) are estimated to form associations with ~72% of vascular plants (Brundrett & Tedersoo, 2018). Recent molecular, cytological and physiological evidence suggests another group of widely occurring (Orchard, Standish, et al., 2017), mycorrhiza-forming (Hoysted et al., 2019) fungi should now be considered alongside these; 'fine root endophytes' of the subphylum Mucoromycotina, within the Mucoromycota (Spatafora et al., 2016; Orchard, Hilton, et al., 2017; Walker et al., 2018; Table 1).

Despite their distinctive fine hyphae and colonisation morphology, the production of arbuscule-like structures in planta by Mucoromycotina 'fine root endophytes' (MFRE) led to their misclassification as AMF within the subphylum Glomeromycotina (Orchard, Standish, et al., 2017). Following application of novel molecular detection and identification methods across a range of host plants (Bidartondo et al., 2011; Orchard, Hilton, et al., 2017) and subsequent reclassification (Spatafora et al., 2016), MFRE were recognised as a distinct symbiotic fungal type (differences detailed in Table 1). Experimental evidence suggests that, like AMF, MFRE play an important role in facilitating plant nutrient uptake in return for plant-fixed carbon from their host (Hoysted et al., 2019). Unlike obligately biotrophic AMF (Lin et al., 2014; Smith & Smith, 2011), MFRE are thought to be facultatively saprotrophic in nature (Field et al., 2015; Field et al., 2016; Lin et al., 2014), as evidenced by isolation and in vitro culture experiments (Field et al., 2015) whereby MFRE proliferate on synthetic media without a host plant. These apparently saprotrophic qualities, together with their frequent co-colonisation of host plants with AMF, open the possibility that MFRE may play an important role in plant nutrition that is distinct from AMF.

Recent experimental evidence in both non-vascular liverworts (Field et al., 2019), and in a vascular plant (Hoysted et al., 2019, 2021), suggests that there is a degree of complementarity between MFRE and AMF function, with MFRE playing a more prominent role in facilitating plant N nutrition alongside AMF-acquired P (Field et al., 2019). This hitherto unappreciated complementarity may have caused a potential source of confusion so far, whereby fungal-mediated transfer of N to host plants has been misattributed as being wholly due to AMF. Thanks to the use of specific MFRE primers (Bidartondo

TABLE 1 Summary of key points to compare and contrast the different mycorrhizal types using the extensive literature on non-MFRE mycorrhizal types, and comparatively sparse literature on MFRE

Distinguishing features	Mycorrhizal group				
	Arbuscular mycorrhizas	Ectomycorrhizas	Ericoid mycorrhizas	Orchid mycorrhizas	Mucoromycotina fine root endophytes (MFRE)
Host range	Angiosperms, gymnosperms, ferns, liverworts, lycopods, hornworts	2 gymnosperm lineages, 28 angiosperm lineages	Ericaceae, Diapensiaceae (Ericales)	Orchidaceae	Angiosperms, liverworts, hornworts, lycopods (Lycopodiella *inundata*)
Lifestyle	Obligate biotroph	Partial saprotroph	Partial saprotroph	Partial saprotroph	Partial Saprotroph
Known forms of N assimilated	NH_4^+, NO_3^-, soluble proteins	NH_4^+, NO_3^-	NH_4^+, NO_3^-	NH_4^+, amino acids	NH_4^+
N forms transferred to plant host	NH_4^+, potentially NO_3^-	NH_4^+, potentially amino acids	NH_4^+	Amino acids?	NH_4^+?
Notes on colonisation structures and fungal morphology	Intracellular formation of arbuscules, coils, vesicles, formation of 'coarse' (>3 μm diameter) hyphae, large (91 to >300 μm diameter) spores	Intercellular hyphal proliferation (Hartig net)	Intracellular hyphal complex within external layer of root epidermal cells	Intracellular formation of pelotons	Intracellular formation of arbuscules, coils, vesicles, 'fine' (<2 μm diameter) hyphae, 'fan-like' hyphal branching, small (10–12 μm diameter) spores

Note: Columns in green indicate the four traditional groupings of mycorrhizal fungi. The blue column shows Mucoromycotina 'fine root endophyte' (MFRE) as a fifth mycorrhizal group to be considered along with the four existing groups (Brundrett, 2009; Brundrett & Tedersoo, 2018; Chalot & Brun, 1998; Fochi et al., 2017; Hoysted et al., 2019; Rimington et al., 2020; Smith & Read, 2010; Sinanaj et al., 2021).

et al., 2011; Desirò et al., 2017), consideration of a more cosmopolitan fungal endophyte community is now possible.

Owing to the dearth of research into the functions and mechanisms of MFRE compared to AMF, in this review we draw on existing bodies of research into MFRE, AMF, and other groups of mycorrhizal fungi in order to compare and contrast the function and potential role of MFRE in plant N nutrition with those of other symbiotic fungi. We review the current literature regarding molecular mechanisms of nutrient exchange and how soil environment may affect mycorrhizal nutrient exchange. Against this background, we pose important outstanding research questions (Box 1) and put forward hypotheses for the nutritional functions of MFRE in relation to host plants.

2 | FUNGAL ORGANS OF NUTRIENT EXCHANGE

The colonisation morphology of different mycorrhizal fungal groups is highly characteristic and is widely used as the basis by which they are distinguished from one another without molecular characterisation and vice versa (Brundrett, 2009; Brundrett & Tedersoo, 2018; Table 1). It is important to note that the extent and types of the fungal structures that form within plant roots during mycorrhizal symbioses are highly dependent on the species of both plant and fungus (Dickson, 2004). AMF form characteristic structures within the root cells of their host plants. These include arbuscules that are terminally differentiated, highly branched structures that provide a large surface area for nutrient transfer between symbiotic partners, vesicles for lipid storage, and spores for lipid storage and propagation (Luginbuehl & Oldroyd, 2017; Figure 1). Ectomycorrhizal fungi (EcM) colonise the lateral roots of their host plants by forming an interlacing mycelial structure, known as the Hartig net, which penetrates between and surrounds the epidermal cells (Stuart & Plett, 2020) and, like the arbuscule, provides a large surface area for nutrient exchange. However, unlike AMF arbuscules, plant cell walls are not penetrated during the formation of the Hartig net. This is compensated for by the development of lateral root clusters and tubercles that increase the interface surface area (Smith & Read, 2008). Roots of ericoid mycorrhizal fungi (ErM)-colonised plants are highly specialised; they lack root hairs and have very narrow diameters (100–<50 μm in distal regions). The roots have a single layer of epidermal cells that are colonised by ErM; typically, the cell wall is penetrated once and a hyphal complex occupies the whole cell (Smith & Read, 2008). All orchid species rely on orchid mycorrhizal fungi (OrM) at some stage during their life cycle. Owing to their small seed size and lack of sufficient nutrition, OrM are required for growth of orchids beyond the protocorm stage of development into adulthood (Smith & Read, 2008). In both the protocorm and adult orchid roots, the fungus forms pelotons through the growth and anastomosis of intracellular hyphae after cell wall penetration, increasing the interfacial area between fungus and plant (Smith & Read, 2008). Individual pelotons have a duration of less than 2 weeks (Mollison, 1943) at the end of which they collapse and are digested, which is traditionally proposed as the main mechanism for nutrient transfer to the host plant (Fochi et al., 2017; Smith & Read, 2008). However, although fungal lysis may contribute to some plant nutrient gain, there is now convincing evidence of transfer across intact membranes as in other mycorrhizal symbioses (Fochi et al., 2017; Kuga et al., 2014).

MFRE in angiosperms typically produce fine arbuscules together with fan-like hyphal structures and hyphal ropes (Albornoz et al., 2020; Hoysted et al., 2019; Orchard, Standish, et al., 2017) and are distinguished morphologically from the 'coarser' AMF (Figure 1A, B) by their characteristic finer hyphae (<2 μm in diameter vs. >3 μm in AMF) with small intercalary and terminal vesicles or swellings (Orchard, Standish, et al., 2017; Figure 1C). Fine arbuscules (Figure 1D) alongside coarser ones have also been observed in liverworts co-colonised by MFRE and AMF (Field et al., 2016, 2019) but, in this group and in other early-divergent spore-producing lineages, the morphology of colonisation by MFRE appears highly plastic (Field & Pressel, 2018; Pressel et al., 2021). Both intra- and intercellular phases of colonisation are present in the earliest divergent Haplomitriopsida liverworts and in the gametophyte and early sporophytic stage (protocorm) of several lycophyte species (Schmid & Oberwinkler, 1993; Duckett & Ligrone, 1992; Hoysted et al., 2019; Hoysted et al., 2020). Intracellular colonisation results in a variety of structures, including tightly wound hyphal coils with terminal swelling

BOX 1 Key outstanding research questions

- What are the key intracellular symbiotic structures formed by MFRE for nutrient exchange, what determines their development and
- phenology?
- What is the range of nitrogen sources MFRE can access and assimilate, how does this contribute to host plant nitrogen nutrition and how does it compare to other symbiotic fungi?
- By what mechanisms do MFRE take up and assimilate nitrogen from soils?
  - Does the uptake and assimilation mechanism differ when MFRE are presented with organic versus inorganic nitrogen sources?
  - How and in what form/forms is nitrogen translocated along MFRE hyphae and into host plants?
- Does the form of nitrogen assimilated by MFRE affect fungal acquisition of plant-assimilated carbon from host plants?
- How do the absolute concentrations of soil nitrogen and phosphorus, as well as N:P ratio affect MFRE-mediated plant nutrient acquisition?
- What is the role of soil microbes in MFRE-mediated nitrogen uptake by host plants?

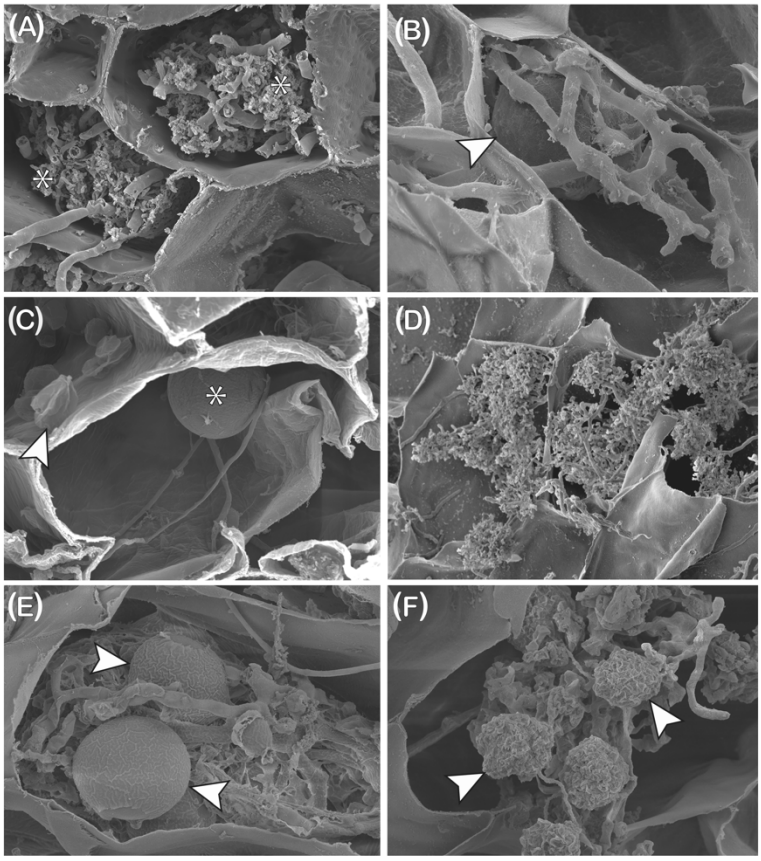

FIGURE 1 Scanning electron micrographs of structures produced by AMF (A,B) and MFRE (C–F) during colonisation of host plant tissues. (A,B) Typical arbuscular mycorrhizal fungi (AMF) intracellular 'coarse' arbuscules (A, arrowed) and large vesicle (B, arrowed) with coarse hyphae, shown here in the thallus of the liverwort *Neohodgsonia mirabilis*. (C) Mucoromycotina 'fine root endophyte' (MFRE) intracellular intercalary and terminal small vesicles/swellings, shown here at different stages of development (young*; collapsed, arrowed) with fine hyphae in a root of the vascular plant *Lycopodiella inundata*. (D) Intracellular fine arbuscules (arrowed) in the liverwort *N. mirabilis*. (E,F) Intracellular tightly wound hyphal coils with young (E, arrowed) and collapsed (F, arrowed) 'lumps' in the thallus of the Haplomitriopsida liverwort *Treubia lacunosa*. Scale bars: (A, B, D) 20 μm; (C, E, F) 10 μm

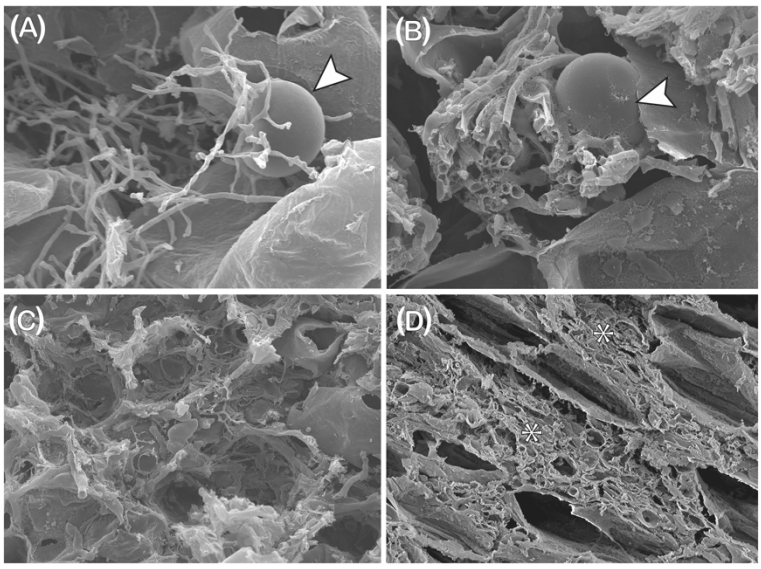

FIGURE 2 Scanning electron micrographs of structures produced by MFRE during colonisation of host plant tissues. (A) Intracellular small vesicle with fine, branching hyphae in the sporophytic protocorm of *Lycopodiella inundata*. (B) Intracellular tightly wound hyphal coil with larger vesicle in the outermost cortical layers of a gametophyte of *L. inundata*. (C,D) *L. inundata* sporophytic protocorm. During intercellular colonisation, the fine hyphae enlarge (C) until the intercellular spaces are filled with masses of collapsed pseudoparenchymatous hyphae (D*). Scale bars: (C) 50 μm; (B,D) 20 μm; (A) 10 μm

or "lumps" (Haplomitriopsida and outermost cortical layers of lycophyte gametophyte) (Figures 1E and 2B) and branched fine hyphae with intercalary and terminal vesicles (lycophyte gametophyte and protocorm) (Figure 2A) but, consistently, no arbuscule-like structures (Carafa et al., 2003; Duckett et al., 2006; Hoysted et al., 2019). During intercellular colonisation, the fine hyphae enlarge (Figure 2C), eventually forming masses of swollen pseudoparenchymatous structures lacking vesicles and which soon collapse and degenerate (Figure 2D). This short life-span mirrors that of the Haplomitriopsida intracellular fungal 'lumps'. It has been suggested that the collapse

and lysis of these structures (Figure 1F) may provide a source of nutrients, including N, passed from MFRE to their host liverworts, comparable to the previously discussed mechanism employed by OrMs (Duckett et al., 2006; Hoysted et al., 2020).

MFRE colonisation in lycophytes also varies depending on the plant's life stage. In the adult sporophytes of *Lycopodiella inundata*, colonisation is strictly intracellular and consists solely of fine branching hyphae with intercalary and terminal swellings (Figure 1C); as in earlier developmental stages, arbuscule-like structures have never been observed in MFRE-colonised roots of *L. inundata* (Hoysted et al., 2019; Hoysted et al., 2020). Recent demonstrations of nutrients-for-carbon exchange in Haplomitriopsida liverworts (Field et al., 2015) and adult sporophytes of *L. inundata* (Hoysted et al., 2019; Hoysted et al., 2020) by isotope tracer experiments indicate that arbuscules are not required for this exchange to occur and that other MFRE structures must therefore be involved in active metabolic interactions with the host cells. Several questions remain regarding the development, phenology, and functioning of MFRE structures in symbiosis with host plants (Box 1).

There is increasing evidence of a tightly controlled interplay between soil nitrogen and phosphorus dynamics and the characteristics of mycorrhizal colonisation and nutrient exchange with host plants. Low soil N and P, both individually and in combination, result in increased colonisation by the AMF *Rhizophagus irregularis* in the model legume *Medicago truncatula* (Chen et al., 2018). Starvation of these nutrients results in greater colonisation by mycorrhizal symbionts, likely due to the stress response induced by lack of these key nutrients (Bonneau et al., 2013). N and P interplay may also influence fungus to plant transfer of nutrients. The *M. truncatula* phosphate transporter mutant knockout line *Mtpt4* displays a premature arbuscule degeneration (PAD) phenotype; in plants supplied with low levels of N, PAD is suppressed (Javot et al., 2011). Suppression of PAD is dependent on the expression of the ammonium transporter AMT2;3 (Breuillin-Sessoms et al., 2015), suggesting that the amount of N and P passed from the fungus to the plant is linked to soil N and P status. Despite the increasing evidence of the interaction between N and P nutrition in the dynamics of the symbiosis between mycorrhizal fungi and plants, the extent of this and the mechanisms underpinning it remain elusive. This is potentially further complicated by the apparent functional complementarity between MFRE and AMF, wherein MFRE may play a more significant role in host plant N nutrition with AMF playing a more significant role in plant P nutrition (Field et al., 2019). This could suggest that there is cross-talk between the two symbionts or that the levels of each nutrient transferred by each symbiont are controlled by either the plant or nutrient source-sink dynamics. Further research is urgently needed to explore this.

3 | N SOURCE PREFERENCE

AMF are able to assimilate various forms of N, including urea, amino acids (AA), ammonium (NH_4^+), nitrate (NO_3^-), and various soluble proteins (Jin et al., 2012). There is some evidence that inorganic N is favoured over organic N when both are available (Whiteside et al., 2012), with NH_4^+ preferred to NO_3^- (Johansen et al., 1996; Toussaint et al., 2004). This preference for NH_4^+ may decrease the energetically costly reduction of NO_3^- by the fungus prior to assimilation (Marzluf, 1997). However, in some cases, it appears that the opposite preference is true (Ngwene et al., 2013; Thirkell et al., 2019), suggesting a degree of plasticity in N source preference in AMF.

Experiments have shown that both AMF and MFRE transfer N to their host plants (Fellbaum et al., 2012; Hawkins et al., 2000; Hoysted et al., 2019). Unlike the obligately biotrophic AMF, it is possible to culture MFRE axenically in the absence of a host plant, suggesting that these fungi have at least some facultative saprotrophic capabilities (Field et al., 2015). MFRE appear able to assimilate and transfer N from both organic (Field et al., 2019) and inorganic sources to their host plants (Hoysted et al., 2019), although the degree to which MFRE rely on organic N acquisition to maintain symbioses remains to be investigated (Box 1). Other mycorrhiza-forming fungi also have saprotrophic capabilities; EcM fungi (Basidiomycota and Ascomycota) acquire both organic and inorganic forms of N, although they are likely to access organic sources more frequently in nature given how abundant (over 95% in some woodlands) organic material is in the woodland ecosystems where EcM are most common (Chalot & Brun, 1998; Nicolás et al., 2019). ErM are also capable of utilising nitrogen from both organic and inorganic sources; experiments using labelled tannins (polyphenol–protein complexes) showed N transfer to *Rhododendron maximum* (Makarov, 2019), while colonisation by the ErM *Rhizoscyphus ericae* increased the capacity of cranberry to absorb nitrate (Wei et al., 2016). The N source preference of *R. ericae* has been shown to depend on both the strain of the fungus and the availability of carbon (Grelet et al., 2005). Orchid mycorrhizas such as *Tulasnella calospora* have also been shown to assimilate both inorganic (ammonium nitrate) and organic (glycine) sources of N and transfer N to their hosts (Fochi et al., 2017). Based upon the nitrogen source preferences of other mycorrhizal fungi, it seems reasonable to assume that the preferred inorganic nitrogen source for MFRE would be ammonium, eliminating the energetic cost of reducing nitrate, although, like ErM, this may be dependent upon the availability of a carbon source to utilise for energy among other factors such as soil pH. While the direct mechanisms of N uptake in MFRE remain unknown, it is possible that MFRE may break down or assimilate organic N as well as assimilate inorganic N, thus resulting in the transport of substantially more N to host plants, per unit biomass, than AMF (Hoysted et al., 2019).

4 | MECHANISMS OF MYCORRHIZAL-MEDIATED N ASSIMILATION

Ammonium (NH_4^+) uptake by AMF is achieved via transport proteins such as the high-affinity ammonium transporter GintAMT1 expressed in the extraradical mycelium of *Glomus intraradices* in the presence of low concentrations of NH_4^+ (López-Pedrosa et al., 2006; Calabrese et al., 2016; Figure 3). Similar NH_4^+ importers have been either

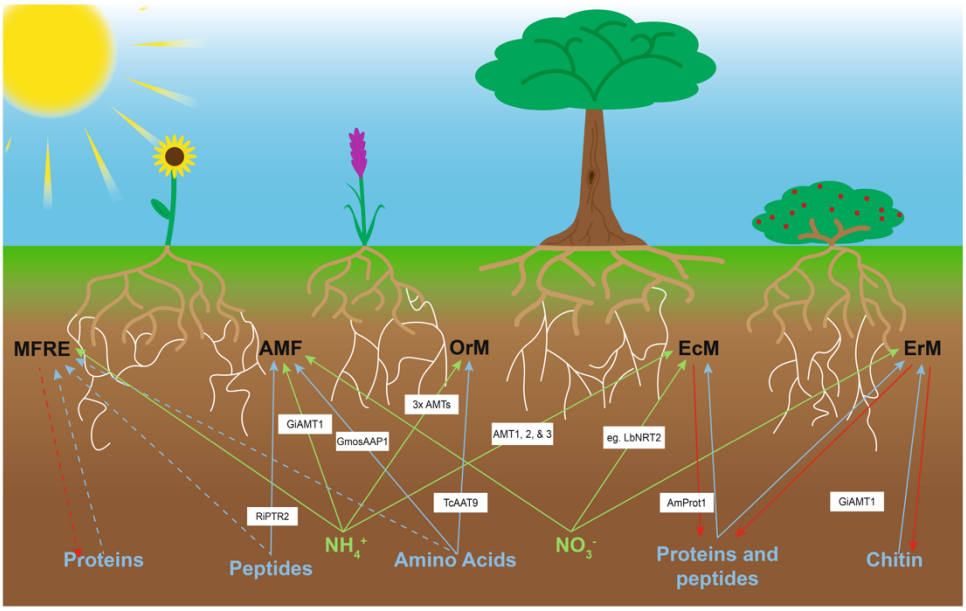

FIGURE 3 Forms of nitrogen present in soil and capabilities of mycorrhiza-forming fungi to access and assimilate them with known key transporters and enzymes involved shown. Solid arrows represent known assimilation, whereas dashed arrows represent possible assimilation pathways. Red arrows indicate secretion of enzymes by fungi to degrade organic nutrient sources, green and blue arrows indicate inorganic and organic nitrogen sources, respectively (Belmondo et al., 2014; Cappellazzo et al., 2008; Fochi et al., 2017; López-Pedrosa et al., 2006; Nehls et al., 2001; Stuart & Plett, 2020)

functionally characterised or identified in a variety of mycorrhizal fungi, such as in the EcM species *Hebeloma cylindrosporum* and *Amanita muscaria* (Stuart & Plett, 2020) as well as the OrM species *Tulasnella calospora* (Fochi et al., 2017; Figure 3). MFRE are also capable of using NH_4^+ as a nitrogen source (Hoysted et al., 2019; Field et al., 2015; Figure 3) and although there are no genomic or transcriptomic data yet available to confirm this, it seems likely that, due to the commonality of NH_4^+ uptake mechanisms across the other groups of mycorrhizal fungi, MFRE also possess AMTs or similar NH_4^+ transporters. Nitrate (NO_3^-) uptake by AMF has been shown to occur via a H^+ mediated symporter, GiNT, that is expressed in the extraradical mycelium (Bago et al., 1996; Tian et al., 2010). Similar nitrate transporters have also been found in the genomes of the EcM *H. cylindrosporum* and *Laccaria bicolor* (Stuart & Plett, 2020), suggesting that MFRE may also possess similar NO_3^- transporters. However, an isolate of the OrM *Tulasnella calospora* lacks a nitrate uptake system (Fochi et al., 2017), perhaps indicative of a preference for organic N. Currently, N assimilation for MFRE has only been shown for NH_4^+ sources with the capacity for NO_3^- assimilation and transfer representing a significant unknown (Box 1).

Although the capacity of AMF to assimilate organic forms of N remains ambiguous (Smith & Read, 2008), several potential mechanisms for organic N assimilation have been identified. The amino acid permease GmosAAP1, expressed by *Glomus mosseae*, is capable of actively importing proline via a H^+ and pH-mediated mechanism (Cappellazzo et al., 2008). It is expressed in both the intraradical and extraradical mycelium and transports multiple amino acids, including arginine, asparagine, and glutamine (Jin et al., 2005). In addition, the dipeptide transporter RiPTR2 has been shown to be expressed by *R. irregularis* in both the intra- and extraradical mycelium (Belmondo et al., 2014); however, the significance of this contribution to overall host plant N nutrition remains to be elucidated. A key deficiency in the ability of AMF to utilise complex organic nitrogen forms is that they do not appear capable of polymeric N degradation, relying instead on the breakdown of large organic compounds by other microbes in the rhizosphere (Chowdhury et al., 2022; Talbot & Treseder, 2010). It is possible that greater access to soil organic N pools derived from the saprotrophic capabilities of MFRE may result in the observed transport of substantially more N to host plants than AMF. Other mycorrhizal fungal groups with saprotrophic capabilities have complex mechanisms for accessing organic N; EcMs utilise a wide range of amino acids as both sources of N and C, and many of these fungi also often possess the enzymes necessary for the degradation of chitin (Nygren et al., 2007; Figure 3). EcM fungi secrete a diverse range of peptidases and have a number of amino acids, oligopeptides and dipeptide transporters that take up the resulting products, suggesting that protein is an important N source for these fungi (Nygren et al., 2007; Stuart & Plett, 2020). EcMs produce a range of different proteases, including aspartic proteases, serine proteases, metalloproteases, and cysteine proteases, such as the aspartic protease AmProt1 found in the EcM *Amanita muscaria* (Nehls et al., 2001). ErMs also produce enzymes capable of decomposing other complex organic molecules allowing, for example, mobilisation of N from chitin and polyphenol–protein complexes (Makarov, 2019). An amino acid transporter/permease (TcAAT9)

has been found in the OrM *T. calospora* that is upregulated when provided with glutamine as the N source as opposed to ammonium, suggesting that OrMs are also capable of utilising amino acids as a N source (Fochi et al., 2017). Given their saprotrophic capabilities, it is plausible that MFRE may also possess a suite of degradative enzymes such as proteases that, in a manner similar to EcMs and ErMs, are secreted into the soil by transporters, allowing MFRE to assimilate the degradation products. Investigating into potential mechanisms of organic matter degradation using established methods (Shah et al., 2013) is now needed (Box 1). Once a genome for MFRE is available, comparisons between the suites of enzymes possessed by saprotrophic mycorrhiza-forming fungi and MFRE will allow to predict the degradative capabilities of MFRE. This work can then inform further targeted study of putative degradation mechanisms using established techniques (Shah et al., 2013).

5 | FUNGUS-TO-PLANT TRANSFER OF N

After N is assimilated into AMF hyphae, it is converted to arginine for transport to the arbuscule; however, how N is processed into this form depends upon the original form of nitrogen imported (Jin et al., 2012). Nitrate is reduced to nitrite by nitrate reductase before being further reduced to ammonium by nitrite reductase (Jin et al., 2012; Figure 4a), which is then incorporated into arginine via the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle (Govindarajulu et al., 2005). In *Rhizophagus irregularis*, GiGS1 and GiGS2 incorporate inorganic nitrogen into glutamate to produce glutamine, which is then converted into arginine (Tian et al., 2010; Figure 4a). Organic sources of N also require conversion into arginine for transport to the intraradical mycelium unless direct assimilation of arginine has occurred (Figure 4A). However, not all common amino acids are utilised by AMF. Cyclic amino acids and amino acids with high bond strengths resist hydrolysis and are consequently not efficient for use by AMF, regardless of size, and are thus assimilated less often (Talbot & Treseder, 2010).

Less is known about the processing of nitrogen once inside the extraradical mycelium of the other mycorrhizal types; in EcM neither the form of N transported to the intraradical mycelium nor the mechanisms behind this are fully known (Stuart & Plett, 2020). Wei et al. (2016) suggest that in the ErM *Oidiodendron maius*, nitrate is converted to arginine in a similar manner to AMF. Based on transcriptional evidence, it seems unlikely that the OrM *T. calospora* uses the arginine-based transport pathway from extraradical mycelium to the

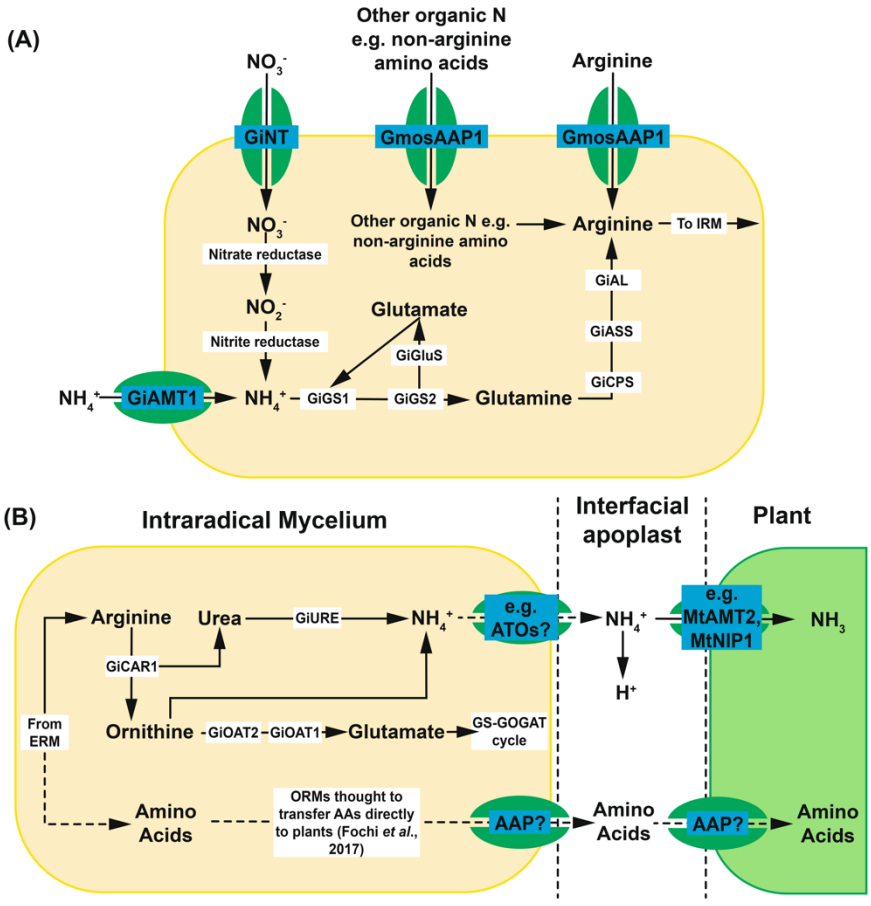

FIGURE 4 (A) Pathways by which nitrogen is assimilated and transformed within extraradical mycelium (ERM) of arbuscular mycorrhizal fungai (AMF) before export to host plants (Govindarajulu et al., 2005; Tian et al., 2010). (B) Pathways by which assimilated nitrogen is transferred from intraradical mycelium (IRM) to plant root cells. Solid arrows indicate known pathways. Dashed arrows indicate potential nitrogen assimilation pathway in orchid mycorrhizas (Chen et al., 2018; Cruz et al., 2007; Fochi et al., 2017; Govindarajulu et al., 2005; Jin et al., 2012)

intraradical mycelium since urease, which is required for the breakdown of arginine in the intraradical mycelium of AMF, is strongly downregulated in *T. calospora* (Fochi et al., 2017). Lack of a genome or transcriptomic data makes it difficult to speculate if arginine is the molecule used by MFRE to transport N from extraradical to intraradical mycelium. Because this appears to be a common mechanism shared by AMF and ErMs and given the phylogenetic proximity of MFRE to AMF, it is plausible that MFRE rely on a similar mechanism of N transport to that used by AMF.

Following conversion into arginine by AMF, N is transported from the extraradical mycelium into the intraradical mycelium to be exchanged with the plant (Fellbaum et al., 2012) (Figure 4B). Labelled substrate studies using ^{13}C, ^{14}C, and ^{15}N have shown that only N is transferred between the fungus and the plant and that the C skeletons of amino acids synthesised by the fungus remain in the fungus (Govindarajulu et al., 2005); therefore, the arginine used to transport N to the intraradical mycelium must be broken down to release ammonium. Given that transcript levels of genes with high similarity to ornithine aminotransferase, urease accessory protein, and ammonium transporters are upregulated in the intraradical mycelium of *Rhizophagus irregularis* (Chen et al., 2018; Govindarajulu et al., 2005; Jin et al., 2012), arginine break down is likely to occur via the urea cycle, with amino acids recycled via the GS-GOGAT cycle once ammonium has been liberated (Chen et al., 2018; Jin et al., 2012). Urease and arginase activity is increased in the mycorrhizal root compartment when compared with the extraradical mycelium, suggesting that the catabolic arm of the urea cycle is more active in the intraradical than the extraradical mycelium (Cruz et al., 2007; Jin et al., 2012).

A similar mechanism for arginine breakdown and ammonium release has been proposed for EcM fungi (Nehls & Plassard, 2018). Upregulation of plant ammonium importer expression in EcM mycorrhizal root tips indicates that ammonium is the principal form of N transferred between the two organisms (Stuart & Plett, 2020). Ammonium is also thought to be the principal form of N transferred by the ErM fungi to the plant, as shown in *Rhododendron fortunei* plants in symbiosis with *Oidiodendron maius* (Wei et al., 2016). When nitrate was provided as the N source, the expression of the plant ammonium transporter (*RfAMT*) increased threefold in colonised roots suggesting that, in a similar manner to AMF, nitrate is absorbed, reduced and converted into arginine before it is broken down in the intraradical mycelium to release ammonium for transfer to the plant (Wei et al., 2016). However, this does not appear to be the case for OrM. In colonised protocorms of *Serapias vomeracea* in symbiosis with *T. calospora*, both plant and fungal amino acid transporters were upregulated, whereas plant ammonium transporters were not strongly upregulated (Fochi et al., 2017). This suggests that an amino acid-based, as opposed to ammonium-based N transfer system, is used in this symbiosis.

In AMF, fungus-to-plant N transfer occurs at the periarbuscular interface between plant and fungal cell membranes (Figure 4B) although the mechanism by which fungal hyphae release N into the interfacial apoplast is currently unknown. Aquaporins may be involved as suggested by the identification of two fungal aquaporins expressed in the extraradical mycelium and arbuscules of maize roots inoculated with *R. irregularis* (Chen et al., 2018; Li et al., 2013). Recently, two *R. irregularis* genes with homology to *Saccharomyces cerevisiae AMMONIA TRANSPORT OUTWARD PROTEIN 3* (*ATO3*) have been identified; these could also be candidates for export of ammonia into the interfacial apoplast (Chen et al., 2018).

Uptake of nitrogen from the interfacial apoplast into the plant occurs via transport proteins such as the ammonium transporter GmAMT4.1 expressed in soybean root cortical cell membranes when in partnership with *Glomus intraradices* (Kobae et al., 2010). There are four other AM-induced AMT genes identified in soybean, two in sorghum, two in tomato, and three in *Medicago truncatula* (Chen et al., 2018; Jin et al., 2012). In addition to ammonium transporters, nitrate transporters in the NRT2 family have been identified as AMF-induced in tomato, *M. truncatula*, and *Lotus japonicus*, although their subcellular localisation and transport activities remain unknown and potential transfer of nitrate in the symbiosis is poorly understood (Hogekamp et al., 2011). Some organic forms of nitrogen may also potentially be transferred; AMF-upregulated amino acid transporters have been observed in *L. japonicus* (Chen et al., 2018) although the labelled substrate studies mentioned above (Govindarajulu et al., 2005) would dispute this as a mechanism for transfer. In EcM, evidence of amino acid transport and the exchange of organic compounds in the apoplastic space also exists, with fungal amino acid exporters being upregulated in mycorrhizal root tips colonised by *Laccaria bicolor* or *Pisolithus microcarpus* (Stuart & Plett, 2020).

6 | CONCLUSIONS AND FUTURE PERSPECTIVES

The reclassification of fine root endophytes as symbiotic Mucoromycotina fungi (Orchard, Hilton, et al., 2017) together with the significant differences in lifestyle and function when compared to AMF (Field et al., 2019; Lin et al., 2014), and developments in molecular identification (Bidartondo et al., 2011) throw into question much of what was assumed about the role of AMF in host plant N nutrition. Although structurally and phylogenetically closer to AMF (Hoysted et al., 2019; Spatafora et al., 2016), the apparent saprotrophic capabilities of MFRE (Lin et al., 2014) bring forth the hypothesis that some of the mechanisms these fungi use for nitrogen acquisition, transport and transfer to plants may be more similar to those of saprotrophic lineages of mycorrhiza-forming fungi such as the EcM, ErM and OrM. The relatively wide host range of MFRE, similar to that of AMF (Orchard, Standish, et al., 2017), coupled with their increased access to organic N pools in the soil, suggests that their contribution to plant N nutrition across whole ecosystems could be vastly more significant than previously considered. Functional complementarity between MFRE and AMF (Field et al., 2019) with co-colonisation by both symbionts commonly occurring across many plant species and ecosystem types (Hoysted et al., 2018; Orchard, Standish, et al., 2017) opens the possibility

that in natural ecosystems with high levels of organic N MFRE may facilitate a significant proportion of plant N uptake.

The isolation and axenic culture of MFRE, and subsequent recolonisation of plants by the fungus (Field et al., 2015), provides an exciting potential experimental system by which the precise nutritional role of MFRE in symbiosis with plants both singly and in dual colonisations with AMF may be investigated. It also gives scope to providing the molecular and genetic tools to explore the mechanisms underlying nitrogen acquisition and processing, for example comparing the suites of enzymes MFRE may secrete to degrade organic matter with those of other saprotrophic mycorrhizal fungi, such as the EcM (Shah et al., 2013).

The interactions of MFRE with other soil microorganisms, such as rhizobacteria, add a further layer of complexity when exploring the role of MFRE in plant N nutrition. Likewise, AMF phosphate acquisition from organic sources is likely dependent on interactions with phosphate-mineralizing bacteria associated with the hyphae (Jiang et al., 2021). Recent research has shown that different species of AMF colonising the same root system recruit distinct microbiomes around their extraradical mycelia with unknown impacts on soil nutrient cycling (Zhou et al., 2020). It is likely that MFRE also recruit their own distinct microbiomes, although the extent to which saprotrophic lineages require their microbiome to break down organic matter and transfer resulting nutrients on to the host plant requires further investigation. Again, isolation and axenic culture of MFRE, which do not have microbiome associates, will facilitate future avenues of research.

MFRE have a wide distribution across plant lineages and ecosystem types, from lycophytes in European heathlands (Kowal et al., 2020) to legumes in Australian pastures (Albornoz et al., 2021), with a potentially prominent role in host plant N nutrition (Field et al., 2019). Because of this, as well as experimental possibilities opened up by isolation and axenic culture experiments, future research into MFRE promises exciting new insights into plant N acquisition both at individual, community, and potentially even agricultural (Albornoz et al., 2022) levels. How this fits mechanistically and ecologically with other forms of mycorrhizas, involving both saprotrophic and obligately biotrophic fungi, is a key area for further study that can be facilitated by new molecular and bioinformatic tools.

AUTHOR CONTRIBUTIONS

Katie J. Field, Silvia Pressel, and Tim J. Daniell conceived the concept and framing for the paper. Nathan Howard and Ryan S. Kaye wrote the first draft, Silvia Pressel constructed Figures 1 and 2, Nathan Howard drew Figures 3 and 4, all authors commented and developed subsequent versions of the manuscript and approved the final version.

ACKNOWLEDGEMENTS

NH, RAK, SP, TJD, and KJF are supported by ERC CoG "MYCOREV" (865225) and NERC (NE/S009663/1). We thank the de Laszlo Foundation for additional support for student research. We are grateful to Alex Williams for feedback on a draft of the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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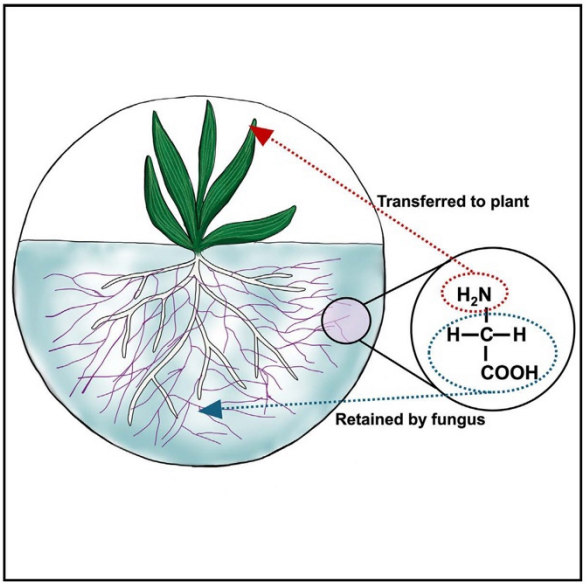
Article

Current Biology

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

Graphical abstract



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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.

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

Howard et al., 2024, Current Biology *34*, 5484–5493
December 2, 2024
https://doi.org/10.1016/j.cub.2024.10.028

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Article

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

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https://doi.org/10.1016/j.cub.2024.10.028

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

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 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). 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

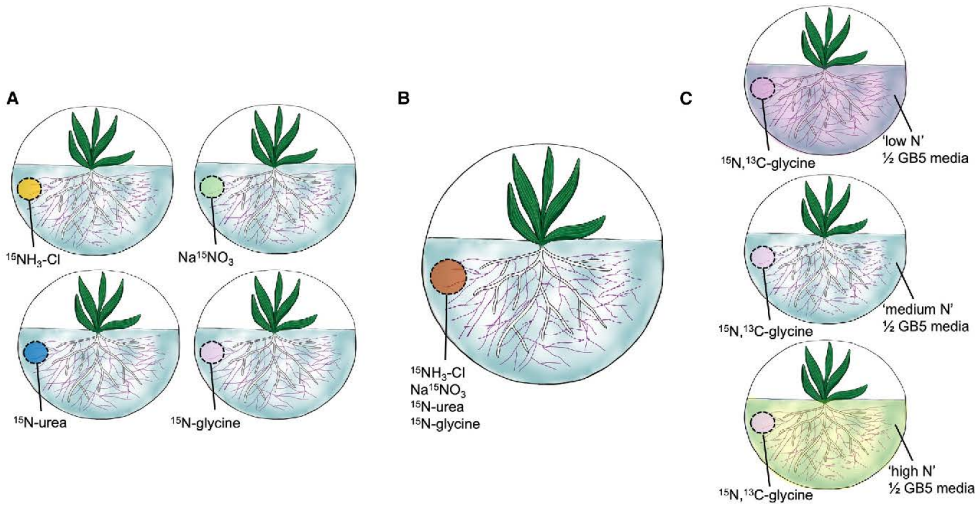

Figure 1. Monoxenic microcosm experiments for tracing movement of C and N between the Mucoromycotina fine root endophyte fungal isolate *Lyc-1* and *Plantago lanceolata*

(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

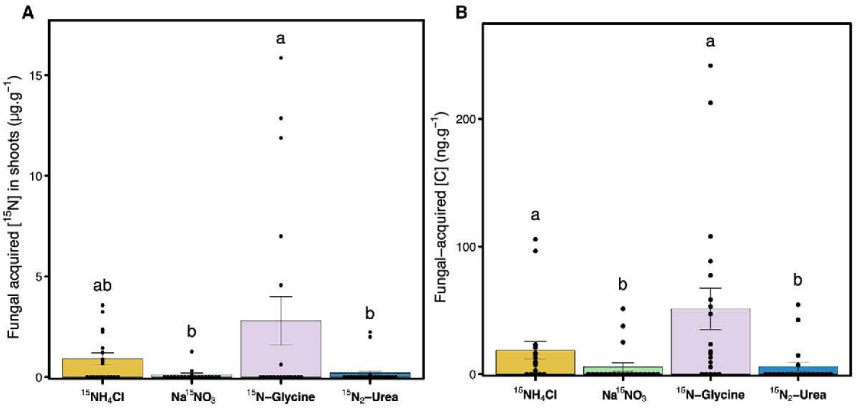

Figure 2. Nutrient-tracing data from N source experiment

(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

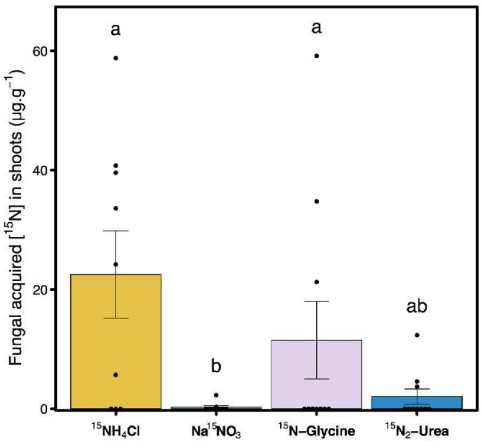

Figure 3. Fungal-acquired ^{15}N content of shoots in fungal choice experiment

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.

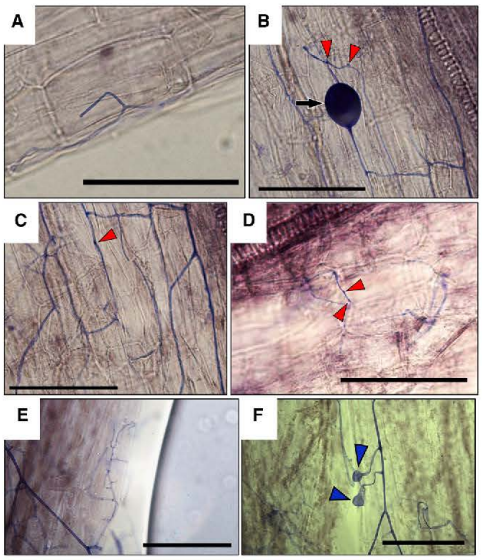

Figure 4. Micrographs of MFRE in *P. lanceolata* roots

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).

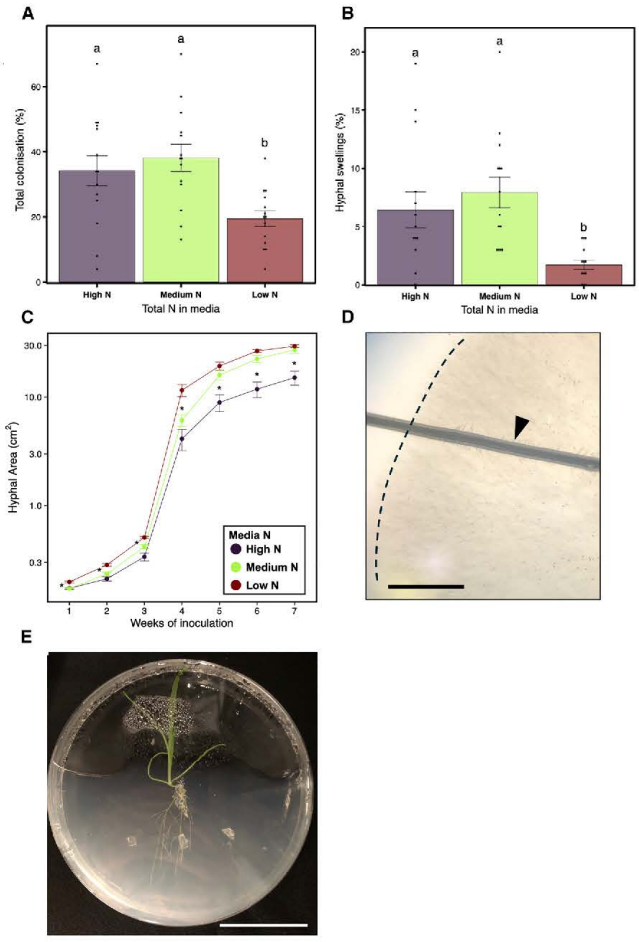

Figure 5. Colonization and mycelial network growth in "N concentration" experiment

(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

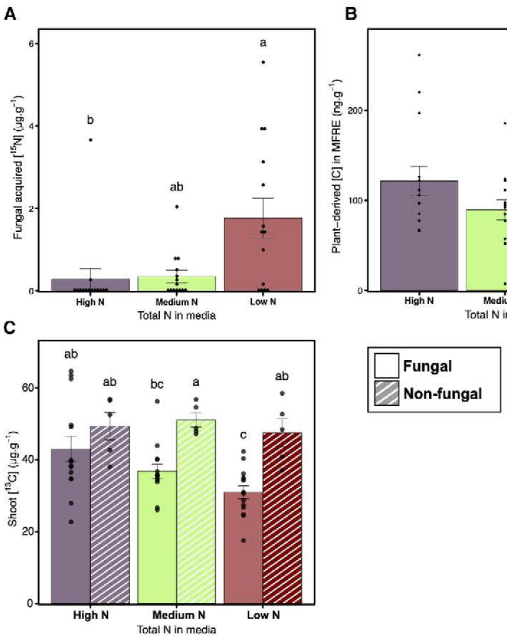

Figure 6. Nutrient-tracing data for the N concentration experiment

(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.

ACKNOWLEDGMENTS

Funding: this work was funded by an ERC consolidator grant (MYCOREV, project ref: 865225) to K.J.F., T.J.D., and S.P. and a NERC grant to K.J.F. (NE/S009663/1). E.D. is funded by an NERC ECORISC CDT PhD studentship (NE/VO13041/1). We thank the De Laszlo Foundation for generously supporting PhD student research.

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:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Plant growth conditions
- METHOD DETAILS
  - Colonisation of P. lanceolata roots by MFRE and mycelial growth
  - ^{15}N, ^{13}C, and ^{14}C isotope tracing
  - Equations
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2024.10.028.

Received: August 28, 2024
Revised: October 2, 2024
Accepted: October 9, 2024
Published: November 19, 2024

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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 70°C, 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

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[80] 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]).