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Forum

Letter

Critical research challenges facing Mucoromycotina 'fine root endophytes'

Mucoromycotina 'fine root endophytes' form globally widespread, nutritional mutualisms with plants

Mucoromycotina 'fine root endophytes' (MFRE), referred to previously as *Glomus tenue* (Greenall) or more recently *Planticonsortium tenue* (Walker *et al.*, 2018), are a globally distributed group of soil fungi (Orchard *et al.*, 2017a) that form endosymbioses with plants from across most of the land plant phylogeny (Hoysted *et al.*, 2018, 2019; Rimington *et al.*, 2019). Despite much progress having been made in characterizing plant–MFRE symbioses in the last decade, significant challenges remain. Here, we mark out these challenges and discuss future directions for promoting research in this rapidly developing field.

MFRE, within Endogonales (Mucoromycotina, Mucoromycota), are recognized as phylogenetically (Bidartondo *et al.*, 2011; Spatafora *et al.*, 2016; Orchard *et al.*, 2017b) and functionally (Field *et al.*, 2015, 2019; Hoysted *et al.*, 2019) distinct from the more commonly studied arbuscular mycorrhizal fungi (AMF), which belong to the Glomeromycotina (or Glomeromycota) (Spatafora *et al.*, 2016). Research using isotope tracers has shown that MFRE exchange both phosphorus and nitrogen for plant-fixed carbon when in association with liverworts (Field *et al.*, 2015, 2016, 2019) and with the vascular plant *Lycopodiella inundata* (Hoysted *et al.*, 2019, 2021b), while a cryo-scanning electron microscopy (SEM) and X-ray microanalysis study suggests MFRE may play a role in phosphorus assimilation in *Trifolium subterraneum* (Albornoz *et al.*, 2020). Where it has been measured, MFRE have been shown to transfer a significant amount of nitrogen to their host plant (Field *et al.*, 2016, 2019; Hoysted *et al.*, 2019, 2021a), suggesting that there may be a complementary role for these fungal symbionts alongside AMF. In contrast to their well-established role in plant phosphorus nutrition, the extent to which AMF contribute directly to host plant nitrogen nutrition has been subject to some debate (Smith & Smith, 2011; Hodge & Storer, 2015; Thirkell *et al.*, 2016) which is now pertinent given the widespread misidentification of fungal endosymbionts, including MFRE, as AMF (Orchard *et al.*, 2017a; Field *et al.*, 2019). A meta-analysis of the literature on MFRE revealed that many past studies have neglected to focus on MFRE due to difficulties in distinguishing between MFRE and AMF morphologies (Orchard *et al.*, 2017a), the challenge of isolating MFRE, and the absence of MFRE from plant specimens as a result of degradation brought about by sample storage conditions and duration (Orchard *et al.*, 2017c). As the importance of MFRE in plant nutrition is increasingly recognized, further research into their form and function has become critical for understanding of the flows of carbon and nutrients through plant and soil communities. Such findings may have potentially important implications for applications of mycorrhizal fungi in sustainable agriculture (Thirkell *et al.*, 2017).

The choice of plant host for MFRE in experiments represents a critical consideration for researchers, particularly given that relatively little is known about compatibility and variability in function of MFRE symbionts across plant clades. To date, the majority of experiments have been conducted using a relatively limited range of plant hosts, focusing on species where MFRE but not AMF have been detected molecularly across multiple wild populations (e.g. *Lycopodiella inundata* and some Haplomitriopsida liverwort species), or those which are readily colonized by MFRE in soil-based inocula (e.g. *Trifolium* spp.). The breadth of host range for MFRE symbionts, inclusive of compatibility, structure and function of plant–MFRE associations, warrants further investigation (Sinanaj *et al.*, 2020). Experiments involving the use of plants, particularly those where genomes are available, that might be considered as models for symbiosis research (e.g. *Medicago, Lotus*) would be especially valuable in unpicking the molecular and physiological mechanisms underpinning the symbiosis.

Using light microscopy, MFRE are generally recognizable by their fine hyphae (< 1.5 μm diameter) with small intercalary and terminal swellings and 'fan-like' branching structures (Thippayarugs *et al.*, 1999). These contrast with the relatively coarse hyphae (> 3 μm diameter) of AMF (or 'coarse root endophytes') (Field & Pressel, 2018). Arbuscules (highly branched intracellular fungal structures) are characteristic of plant–AMF symbioses; however, their occurrence and appearance in MFRE symbioses across host plants and even plant lifecycles (Hoysted *et al.*, 2021a), is variable (Orchard *et al.*, 2017b; Hoysted *et al.*, 2019). Morphological plasticity has also been noted in transmission and scanning electron micrographs of the ultrastructure of symbioses in plants where only MFRE were detected (Field *et al.*, 2015; Hoysted *et al.*, 2019), making it challenging to distinguish them *in planta* in co-colonizations with AMF (Field *et al.*, 2016). In contrast with the generally very well-characterized AMF spores, those of MFRE are poorly documented. Brief descriptions of their appearance and size occur but are unaccompanied by images (Hall, 1977; McGee, 1987); in fact, only a single unvalidated image of an Endogonales MFRE spore has been published to date (Orchard *et al.*, 2017a).

The prevailing symbiotic scenario among mycorrhiza-forming vascular plants is colonization by multiple fungal symbionts (Hoysted *et al.*, 2019; Teste *et al.*, 2020). Over the years, techniques for the detection and characterization of mycorrhizal fungi have been refined, including molecular detection methods using fungal-specific primers that target marker genes (White *et al.*, 1990), the MaarjAM curated database dedicated to AMF sequences (Öpik

et al., 2010), and inoculation methods using either axenic fungal cultures (Mugnier & Mosse, 1987) or fungal spores extracted from soil (Gerdemann & Nicolson, 1963) to generate plants colonized exclusively by specific species of mycorrhizal fungi. This approach is particularly challenging for MFRE, as their spores are poorly characterized and difficult to isolate, and available fungal isolates are few (Field *et al.*, 2015). This represents perhaps the most pressing obstacle to MFRE research progress, highlighting the need for MFRE–plant experimental systems that allow researchers greater control over biotic and abiotic factors that may influence form and function of MFRE symbioses. Here, we discuss the three state-of-the-art approaches currently available to investigate these associations, including use of soil sieving, wild plants and axenic fungal isolates, together with the caveats that should be considered where each method is employed.

Soil sieving

Inoculum production through soil sieving (Gerdemann & Nicolson, 1963; An *et al.*, 1990; Orchard *et al.*, 2017b) is currently the only published technique for colonizing experimental vascular plants with MFRE, while excluding the other arbuscule-forming symbionts, AMF (Albornoz *et al.*, 2020). This method, based on the observation that AMF spores appear to be much larger than those produced by MFRE, involves wet-sieving soil collected from a site known to contain MFRE to obtain the material that accumulates between sieves of pore sizes 200 μm and 50 μm. This is then dried and used as a soil inoculum enriched in MFRE. The inoculum is mixed with autoclaved sand or soil at a ratio of 1 : 81 (Orchard *et al.*, 2017b) or 1 : 162 (Albornoz *et al.*, 2020) to produce a substrate for plant growth (Fig. 1). There are several factors with this method that require consideration (Table 1). The diameters of AMF spores typically range from 91 μm to > 300 μm (Gerdemann & Nicolson, 1963) but can be smaller (see supplementary material of Aguilar-Trigueros *et al.*, 2019). As such, AMF spores and other propagules such as hyphal fragments (Bingle & Paul, 1986) cannot be consistently excluded from inocula produced using the sieve sizes specified earlier, which generate inoculum containing spores and/or hyphae ≥50 μm and up to 200 μm. MFRE spore diameters are reported to range from 10 to 12 μm (Hall, 1977) or 25 to 35 μm (McGee, 1987) and thus could pass through a 50 μm sieve. As a result of this uncertainty and the ambiguity in descriptions of MFRE spore morphologies, it is difficult to determine the quality of a soil inoculum immediately after it is produced. Checks on inoculum quality and/or viability for monoxenic AMF cultures or AMF spores extracted from soil involve the microscopic quantification of spore density and morphological confirmation of spore identities (Daniels & Skipper, 1982). This is currently not possible for MFRE inocula and is a limitation across methodologies for obtaining MFRE-colonized plants (Table 1). The quality of an MFRE-enriched soil inoculum only becomes apparent when it is used in a substrate to grow plants.

Dilution of soil-based inocula aims to ensure a plant will 'more likely encounter a single unit of inoculum and contain a single fungus' (Orchard *et al.*, 2017b). While this may be the case, the unit of inoculum that colonizes the plant is determined largely by chance and can equally be MFRE or AMF if the growth substrate retains AMF propagules *c.* 50–200 μm. As such, this inoculation strategy may not be effective at exposing plants to similar amounts of fungal propagules and generating roots with consistent MFRE colonization, which can be a limitation to ensuring replicable experimental conditions. An improvement to this method, as used in Albornoz *et al.* (2020), is to first use the soil inoculum to produce an MFRE pot culture. Plants are grown in pots containing diluted soil inoculum, after which the substrate within pots containing the highest amount of MFRE and lowest amount of AMF is sieved again to produce soil inoculum to grow more plants. This process encourages the proliferation of MFRE hyphal networks in the substrate and can be repeated until plants grown in the sieved substrate are consistently colonized by MFRE. This process can be labour intensive and time consuming, especially when propagating a pot culture to produce enough material for an experiment with a large number of replicate plants.

Appropriate quality control measures are necessary when using any soil-based inoculation method, regardless of whether the resultant soil inoculum comes directly from wild soil or from a sieved soil pot culture. AMF contamination, which is the biggest issue of this method, should be monitored through molecular and morphological identification of fungi in plants and their substrate. As with other methodologies for obtaining MFRE-colonized plants, the more quality control checks that are carried out, the more accurate the data on the fungal symbionts present. Entire root systems, which inevitably have patchy colonization regardless of inoculation method, cannot be simultaneously analysed molecularly if other informative but destructive techniques, e.g. root clearing and staining for microscopy, are used. This means symbionts may go undetected molecularly due to the limited amount of root material used for sequencing. However, co-colonizations can go undiagnosed due to the morphological plasticity of AMF and MFRE (Field *et al.*, 2016). As such, it is critical that enough plant material is available to adequately sample (multiple plants, multiple pots, multiple time points) for these checks when using soil inoculation methods.

We tested the effectiveness of soil sieving protocols for growing vascular plants predominantly or exclusively colonized by MFRE in four experiments using long-term pasture soil (Supporting Information Fig. S1; see Methods S1). In Experiment 1, we followed the methods of Albornoz *et al.* (2020) and grew *Trifolium repens* in pots containing sieved soil inoculum combined with either autoclaved soil or autoclaved sand. In Experiment 2, we grew *Medicago truncatula* using the methods of Orchard *et al.* (2017b). Given the necessary considerations outlined earlier, in both of these experiments we used smaller sieve sizes than those published. To refine the protocols further and increase the chance of MFRE colonization, we explored the effect of growing *M. truncatula* in substrate containing colonized root fragments taken from Experiment 2, with or without sieved soil inoculum (Experiment 3). In Experiment 4, we grew *Trifolium repens* in substrate containing root fragments and sieved soil inoculum. We hypothesized that supplementing the growth substrate with root fragments (derived from plants grown in MFRE-enriched substrate), in addition to

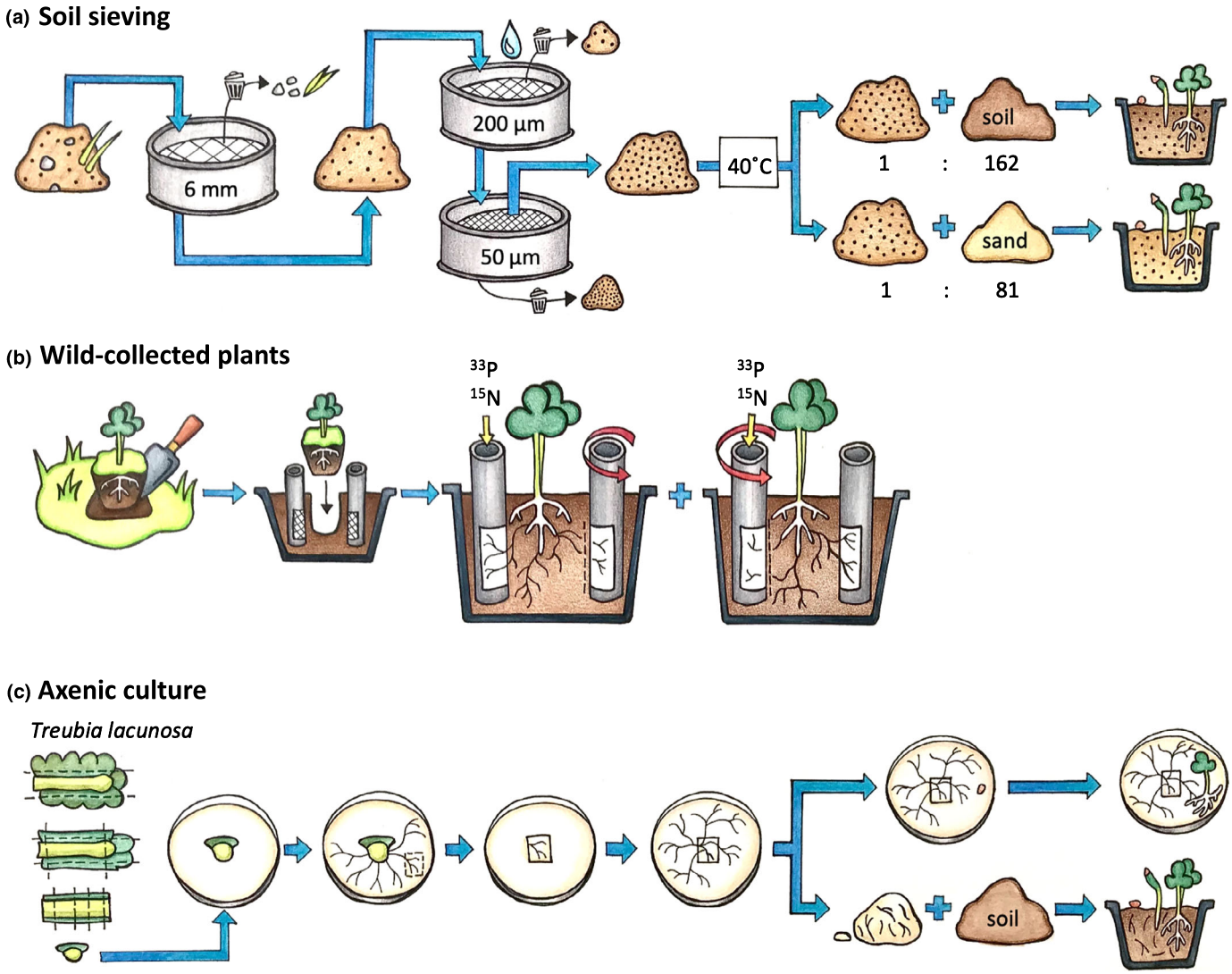

Fig. 1 Current methods used for obtaining plants colonized by Mucoromycotina fine root endophytes (MFRE). (a) Soil is sieved to remove rocks and plant material and then wet-sieved through a couple of sieves to obtain a soil inoculum enriched in MFRE. This is dried in an oven and mixed with soil or sand to produce a substrate for plant growth. (b) Plants are carefully dug up from a site where MFRE are present and transferred into a pot filled with autoclaved substrate and experimental features such as plastic cores with mesh-covered windows accessible to fungal hyphae, which can be filled with isotope tracers and rotated to conduct functional experiments. (c) *Treubia lacunosa* is cut up to obtain a segment of the thallus midrib that is colonized by MFRE. The segment is placed onto sterile media to allow MFRE hyphae to grow out and be isolated onto fresh media where they can proliferate and be used in experiments with plants grown under sterile conditions, or as a blended pure culture mixed with soil for pot-based experiments.

sieved soil inoculum, would increase colonization of plant roots by MFRE through exposure to more MFRE propagules and hyphal networks.

At the harvest stage of each experiment, plant roots were stained with acidified ink to quantify fungal colonization (Vierheilig *et al.*, 1998) and hyphal extractions with Trypan blue staining were carried out on substrate from each pot (Brundrett *et al.*, 1994). In Experiment 1, we found no evidence of fungal colonization in plant roots, while soil hyphal extractions revealed that six pots out of a total of 35 contained few AMF-like hyphal fragments (Fig. S2). As no MFRE were detected in the substrate of any pots, we were not able to generate any inoculum to grow a second generation of plants to recreate the second stage of the methods of Albornoz *et al.* (2020). In Experiment 2, root staining revealed colonization with MFRE and AMF in one pot; colonization with only MFRE in two pots; and no fungal colonization in one pot (Table S1). Values for the percentage total root length (%TRL) colonized by MFRE varied, ranging between 1.4% and 92.9% (Fig. 2a). This wide range for MFRE was similar to results reported in Orchard *et al.* (2017b) where %TRL colonization ranged between *c.* 18% and *c.* 77% between pots ($n = 3$). Using molecular methods (see Methods S1 for details), we detected MFRE in root samples from all four pots using the Endogonales-specific primers EndAD1f and EndAD2r (Desirò *et al.*, 2013). The inclusion of root fragments in the substrates of Experiments 3 and 4 resulted in plant roots with variable levels of MFRE colonization, and increased AMF colonization within roots and in the substrate (Figs 2a–d, S3). Our data confirm methods using soil inoculum obtained from wild

Table 1 Key challenges of the three approaches currently available for obtaining Mucoromycotina fine root endophytes (MFRE)-colonized plants.

Method	Essential considerations
Soil sieving	• Sieve sizes mean arbuscular mycorrhizal fungi (AMF) propagules cannot be consistently excluded. • Soil microbial communities are present in the soil inocula. • Composition of soil inocula may be incompatible with the plant species chosen for pot experiments. • Different starting soils result in different qualities of inocula, making it difficult to draw comparisons between independent experiments. • Plants grown in soil inocula may have patchy colonization, which means fungal symbionts, including MFRE, may go undetected. • Time-consuming.
Wild-collected plants	• AMF propagules are not excluded from substrate. • Only few candidate MFRE specialist plant species are known. • Soil microbial communities are present in the soil inocula. • Plants collected from different sites have different root–microbe associations, making it difficult to draw comparisons between independent experiments. • Patchy root colonization means fungal symbionts, including MFRE, may go undetected. • Ethical considerations when collecting plants (e.g. protected status of species or sites).
Axenic culture	• Microbial communities that occur alongside fungi in soil are absent, thus, little ecological insight. • Only a single axenic MFRE culture has been established to date. • Viability of cultures can change over time. • No available checks for quality/viability of MFRE cultures. • Fungi may evolve phenotypes in culture, which may influence their ability to colonize plant roots. • Time-consuming and costly to establish.

soils are prone to result in inconsistent colonization of plant roots by MFRE fungi.

Experiments with wild-collected plants

Plants colonized by MFRE have been sourced from the wild for use in several experiments examining the nutritional significance of MFRE symbioses (Fig. 1) (Field *et al.*, 2015, 2016, 2019; Hoysted *et al.*, 2019). To do this, a field site is identified where MFRE are prevalent by collecting environmental samples, including plant roots, and identifying the fungal symbionts using staining and molecular methods. Following this, whole plants are carefully removed from the site and transferred into pots containing autoclaved substrate. Although the use of wild plants allows glasshouse experiments to be more representative of natural habitats, there are critical biological considerations that must not be overlooked in such experiments (Table 1).

The soil adhering to the roots of wild plants contains microbial communities that are transferred to the pots, adding a further level of complexity to the experimental system. Rhizosphere bacteria are known to associate with mycorrhizal fungi (Garbaye, 1994; Bonfante *et al.*, 2019); however, the extent of bacterial impacts on fungal fitness and function under different environmental conditions is largely unknown, particularly for MFRE. For researchers wishing to disentangle the effects that MFRE have on plants from those of rhizobacteria, the use of wild-collected plants presents difficulties that can only be mitigated with complex and sometimes expensive experimental features. Custom-made plastic cores with mesh-covered windows accessible to fungal hyphae but not plant roots (Johnson *et al.*, 2001), can be buried within the substrate of pots to account for microbial nutrient cycling (Fig. 1) (Field *et al.*, 2015). Comparisons between the same plant species obtained from different field sites should be drawn with caution, as variations in microbial community, including MFRE diversity, between sites could lead to ambiguity in results. This is also applicable when comparing data from independent experiments on wild plants colonized by different fungal symbionts, e.g. MFRE and AMF may host distinct microbiomes. The types of research questions that wild-collected plants would be suitable for addressing include those exploring the function, recruitment and competition of MFRE in an ecological context where various other biota may be present.

Although staining and molecular identification of fungal symbionts in roots must be carried out as a quality control when selecting sites to source wild plants from and at the end of experiments when wild plants are harvested, this does not guarantee a complete picture of fungal colonization or the absence of AMF. Some stained fungal structures may be only remnant cell walls, lacking cytoplasm and functional capabilities. Patchy fungal colonization is a limitation when DNA sequencing fungi from small segments of root, as primers can fail to detect fungi due to their spatial distribution. The specificity of primers to certain clades of fungi is also an issue (Bidartondo *et al.*, 2011), which can be overcome by using a selection of primer sets to capture fungal diversity. Despite these measures, there is always a chance that fungal symbionts may go undetected, which also brings into question whether plant species, populations or individuals can truly form exclusive MFRE, or AMF, associations in nature. Currently, candidate MFRE-specialist plants include several Haplomitriopsida liverwort species (Field *et al.*, 2015; Rimington *et al.*, 2020) and the lycophyte *Lycopodiella inundata* (Rimington *et al.*, 2015; Hoysted *et al.*, 2019) where, so far, AMF have never been detected across repeated sampling from multiple, geographically separated populations at various time points across a number of years. A single report of rare AMF occurrence in *Haplomitrium mnioides* mainly colonized by MFRE (Yamamoto *et al.*, 2019) was based on limited molecular evidence and without anatomical details and thus requires further investigation (Rimington *et al.*, 2020).

Production of fungal inocula through axenic culture

The gold standard for the production of fungal inocula is isolation and axenic culture of symbiotic fungi, which result in propagules of the desired fungus without contamination with other fungi or bacteria. It is now critical that a rich collection of MFRE isolates, potentially within existing AMF collections, is established,

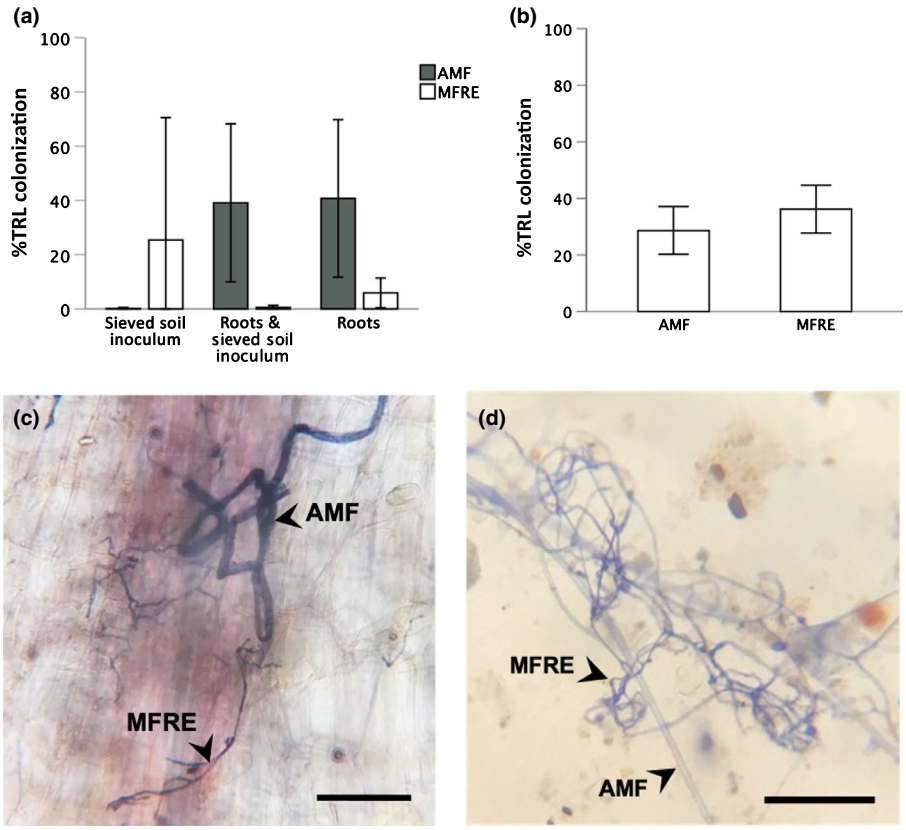

Fig. 2 Fungal colonization in plant roots inoculated with Mucoromycotina fine root endophytes (MFRE) using sieved soil protocols. (a) Percentage total root length (% TRL) colonized by MFRE and arbuscular mycorrhizal fungi (AMF) in *Medicago truncatula* grown in three different substrates (Experiments 2 and 3; $n = 4$ and $n = 5$, respectively). Error bars show standard error of the mean. (b) %TRL colonized by MFRE and AMF in *Trifolium repens* grown in sieved soil inoculum and root fragments (Experiment 4; $n = 6$). Error bars show standard error of the mean. Light micrographs of ink stained fungal structures in (c) the roots and (d) substrate of *Trifolium repens* harvested from Experiment 4. Bars, 50 μm.

inclusive of fungi isolated from the breadth of MFRE plant hosts, across environmental and geographic gradients to truly capture the diversity of symbiotic MFRE fungi. Existing techniques for isolation of MFRE currently remain underdeveloped and require dedicated efforts in developing new protocols and media for their effective isolation and growth.

The first successful axenic culture of MFRE was established and reported in Field *et al.* (2015) where surface-sterilized segments of colonized thallus from the liverwort *Treubia lacunosa* were placed onto sterile fungal media to allow MFRE hyphae to grow out and establish in the media. The cultured fungus was then introduced to and recolonized the axis of another liverwort (*Haplomitrium gibbsiae*) *in vitro*. These MFRE grew in sterile media without a plant host before introduction to *H. gibbsiae* (Field *et al.*, 2015), suggesting they may have facultative saprotrophic capabilities in nature. This trait could give researchers the opportunity, so far unavailable for AMF, to perform *in vitro* experiments to address fundamental questions regarding the function and development of MFRE in isolation; for example, the range of nutrient sources that MFRE are able to access independently and substances such as fungal exudates and enzymes that may be specific to this group. Alternatively, blended pure MFRE cultures could be used as inoculum in pot-based experiments (Fig. 1), but this method remains untested.

As with other methods, care should be taken when interpreting data from experiments using fungal isolates (Table 1). Pure cultures of MFRE do not contain the microbial communities that occur alongside fungi in soil. While this allows determination of the direct impact of MFRE on plant hosts, it may not reflect their true role in natural ecosystems. A full understanding of the diversity of MFRE is needed to ensure that the isolated strains are representative and compatible with the chosen experimental plants. The vitality of cultures is also an important aspect that should be taken into account, as propagule production and the viability of cultures can change over time. For example, it has been suggested that mycorrhiza-forming fungi may evolve particular phenotypes and adaptations as a result of the growth conditions that they are exposed to while in culture, which may influence their ability to colonize plant roots (Rillig *et al.*, 2020).

Future directions

The three experimental methods discussed here have each provided important insights into the form and function of plant–MFRE associations and will continue to do so, particularly if used in combination to determine the direct contributions of MFRE fungi to plants alongside those from interacting rhizosphere microbes. Soil sieving methods have the potential to expand the selection of plant species we are able to study MFRE in, albeit with variable colonization success. Wild-collected plants offer more consistent fungal colonization and are a valuable window into the significance of MFRE-associations in their natural habitat but do not resolve issues with AMF co-colonization in most vascular plants. Fungal isolation and axenic culture techniques will allow the function of plant–MFRE associations to be determined directly in a controlled environment free of additional biota. Although this system may constrain ecological insights, it is likely to be the only feasible avenue by which we may study the fundamental biology of MFRE–

plant symbioses (e.g. signalling and development). Developing the appropriate tools, including a suite of MFRE isolates from a wide variety of plants, is essential for the future development of the field.

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Author contributions

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

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Methods used to carry out soil sieving Experiments 1–4.

Fig. S2 AMF-like coarse hyphal fragment observed in the soil-based substrate of Experiment 1.

Fig. S3 Light micrographs of ink-stained roots of *Trifolium* spp. from Experiment 4.

Methods S1 Full materials and methods for Experiments 1–4.

Table S1 Percentage total root length (%TRL) colonized by fungi in *Medicago truncatula* from Experiment 2.

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Key words: arbuscular mycorrhizal fungi (AMF), endogonales, glomeromycotina, inoculum, mucoromycotina fine root endophytes (MFRE), soil sieving, *Trifolium*, vascular plant.

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