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Molecular Evidence of Mucoromycotina "Fine Root Endophyte" Fungi in Agricultural Crops †

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Abstract: Over 85% of land plants engage in symbiotic relationships with mycorrhiza-forming soil fungi that colonise their roots. These mycorrhizal symbioses, which involve the exchange of fungal-acquired nutrients and water for photosynthetically-fixed plant carbon, are considered a promising nature-based solution to making agricultural practices more sustainable. In order to implement the widespread use of mycorrhizal fungi in agriculture, a more complete awareness of mycorrhizal fungal diversity and range of plant hosts is needed. Mucoromycotina Fine Root Endophytes (MFRE) are a group of mycorrhiza-forming fungi that have recently been shown to be phylogenetically and functionally distinct from Arbuscular Mycorrhizal Fungi (AMF). Here, we provide the first molecular evidence of MFRE colonisation of winter wheat, winter barley, spring wheat and strawberry roots. Fungal symbionts were identified from partial DNA sequences of the 18S ribosomal RNA gene, obtained through a workflow involving molecular cloning and Sanger sequencing. Our findings shed light on the true distribution of plant-MFRE associations and give rise to new questions regarding their functional significance within agricultural plants.

Keywords: Mucoromycotina Fine Root Endophytes (MFRE); mycorrhizal symbioses; wheat; barley; strawberries; vascular plant; molecular identification; 18S rRNA gene

1. Introduction

There is growing concern over the impact that climate change and the depletion of global phosphorus reserves will have on future crop production. Mycorrhizal symbioses, the nutritional mutualisms between filamentous soil fungi and plant roots [1], have the potential to make agricultural practices more sustainable by improving soil structure and reducing the need for chemical fertiliser applications [2]. Phosphorus and nitrogen are amongst the mineral nutrients that mycorrhizal fungi transfer to plants in return for photosynthates [3]. The amount of nutrients exchanged in the associations is dependant on several abiotic and biotic factors, including soil nutrient content, plant genetic background and the identity of the fungus [2,4]. It is therefore essential that we characterise the full diversity of mycorrhizal fungi (or 'mycorrhizas') and ascertain the range of plants that they occur in, so that we can use them successfully as part of a more sustainable approach to agriculture.

Mucoromycotina Fine Root Endophytes (MFRE) are a group of mycorrhiza-forming fungi that, over the last decade, have been recognised as separate from the common Arbuscular Mycorrhizal Fungi (AMF) with whom they were incorrectly grouped for several decades [3]. Fungal morphologies similar to those of MFRE were described in early light microscopy studies on roots stained for mycorrhizae, however, they were routinely misidentified as belonging to AMF and were assigned the group *Glomus tenue* (Greenall) in the Glomeromycotina subphylum [5]. Although morphologies of *G. tenue* (also referred to as 'Fine Endophytes') were reported in numerous plant families across a range of ecosystems, including pastures, they were largely neglected due to limitations in the tools available to study them [6]. In the 1990s, fungal identification and taxonomy were improved through the advent of molecular detection methods using fungal universal primers [7]. Unfortunately, the subsequent rapid development of AMF-specific primers meant that MFRE were left undetected and were overlooked in plants co-colonised by multiple fungi [8]. In 2011, Bidartondo et al. presented the first molecular evidence of MFRE in bryophytes and a fern [8], which led to the molecular confirmation of MFRE in several other plant species [5,9–12].

Research published since then has shown that MFRE differ from AMF in terms of their morphology, their position in the fungal phylogenetic tree and their functional significance in host plants [5,8,9,13–16]. While AMF have relatively coarse (>3 μm diameter) fungal filaments called hyphae, MFRE have fine ones (<1.5 μm diameter) [3,17]. AMF are grouped in the Glomeromycotina fungal subphylum, however the DNA sequences of MFRE are more closely aligned to fungal sequences from the Mucoromycotina subphylum [5,8]. Finally, while it is well established that AMF transfer significant amounts of phosphorus to plants growing in nutrient-deficient soils [3], MFRE have been shown to transfer significant amounts of nitrogen to the early vascular plant *Lycopodiella inundata* in an isotope tracing pot experiment [9]. This finding, along with data from similar experiments using non-vascular plants [10,16], suggest that the nutritional roles of AMF and MFRE in plants may be complementary to each other, with MFRE being important in plant nitrogen nutrition while AMF play a bigger role in P uptake [16]. As such, the last decade has seen a paradigm shift with regards to mycorrhizal diversity, as well as significant advancements in our knowledge of MFRE [13]. The true range of plant hosts colonised by MFRE remains to be investigated further.

Here, we use molecular detection methods to obtain a snapshot of the types of fungi that colonise the roots of key agricultural crops grown in the UK. We avoid detection bias towards AMF sequences by amplifying the 18S ribosomal RNA gene using the NS1/EF3 universal fungal primer set [7,18], and consequently, demonstrate the presence of MFRE in several crop species. Our work adds to the growing knowledge base on the symbioses between MFRE and vascular plants, while providing a selection of plant species as options for experiments investigating the functional significance of MFRE in agricultural plants.

2. Materials and Methods

2.1. Plant Material

Root samples from winter wheat (*Triticum aestivum* cv.), spring wheat (*Triticum aestivum* cv.), winter barley (*Hordeum vulgare* cv.), oilseed rape (*Brassica napus* cv.) and strawberry (*Fragaria x ananassa* cv. Malling Centenary) were collected in April 2019 from a farm in Oxfordshire (UK). Samples were stored at −20 °C in 2X CTAB buffer solution (100 mM Tris at pH 8–9, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 1% PVP-40, and dH_2O) prior to molecular analysis for fungal symbionts.

2.2. Molecular Analyses

Roots were cleaned of external debris using forceps, paintbrushes and distilled water, before healthy 6 mm segments were cut off for analysis. Three segments were analysed for winter barley and spring wheat, two segments were analysed for winter wheat, and one segment was analysed for strawberry. DNA extraction, amplification, cloning, and sequencing were performed based on methods described in Rimington et al. [19]. In brief, chloroform extraction with a purification step using the QBioGene GeneClean kit (Fisher Scientific) was used to isolate genomic DNA, which was

then amplified using the NS1/EF3 [7,18] universal fungal 18S primer set, and cloned using the Invitrogen TOPO TA cloning kit (Life Technologies, Paisley, UK) with homemade competent cells [20]. Four to five colonies per root segment were sequenced using both NS1 and EF3 on an Applied Biosystems genetic analyser (ABI3730). The resulting partial 18S rDNA sequences were edited using FinchTV (https://digitalworldbiology.com/FinchTV). BLASTn searches using NCBI BLAST (National Centre for Biotechnology [21]) identified the closest match to each sequence.

3. Results

A variety of mycorrhizal fungi, including MFRE, were detected in all root segments analysed, except for those of oilseed rape—a non-mycorrhizal plant [22] (Table 1). The MFRE sequences that were detected, aligned best to sequences of MFRE published by Bidartondo et al. [8] and Desirò et al. [11]. One of these published MFRE sequences (GenBank accession no. KC708398.1) was the best match for sequences that were present across all four mycorrhizal plants examined.

Table 1. Mycorrhizal fungi detected in agricultural plants following the molecular cloning of amplified DNA extracted from root segments. Four to five colonies were sequenced per root segment. Fungal symbionts are listed by their fungal subphylum, and accession numbers are provided for all top BLASTn matches for the sequence results.

Plant (Root Segments)	Fungal Symbiont	No. of Colonies	GenBank Accession No.
Winter wheat (2)	Mucoromycotina	3	JF414224.1, KC708405.1, KC708398.1
	Basidiomycota	2	AF518593.1, D85646.1
	Ascomycota	1	AF331939.1
Spring wheat (3)	Mucoromycotina	5	JF414221.1, KC708398.1
	Ascomycota	6	EU940039.1, AF548077.1, U45445.1
Winter barley (3)	Mucoromycotina	9	JF414221.1, JF414224.1, KC708398.1
	Basidiomycota	1	KP322948.1
	Ascomycota	3	AB016175.1, EF638703.1, AF548077.1
Strawberry (1)	Mucoromycotina	1	KC708398.1
	Glomeromycotina	2	AJ306441.1, KC708350.1
	Ascomycota	1	EU883430.1
Oilseed rape (1)	–	–	–

In addition to this, fungi of the Mortierellomycotina subphylum and *Olpidium brassicae*, an obligate plant pathogen, were detected in wheat root segments, highlighting the wide range of fungi targeted by the NS1/EF3 primer set. The data from this experiment confirm the presence of MFRE in agricultural plants that are also co-colonised by mycorrhizae from other fungal subphyla.

4. Discussion

Winter wheat, spring wheat, winter barley and strawberry can now be added to the list of plants that have been molecularly confirmed as hosts of MFRE. These results fit well with the global distribution of MFRE-like morphologies that have been reported in previous literature [6]. As well as shedding light on the true range of plants that MFRE associate with, this study also highlights the value of using fungal universal primers to assess mycorrhizal diversity within root samples, before moving on to further experiments using clade-specific primers. Currently, the only primers designed to target mycorrhizal fungi of the Mucoromycotina are EndAD1F and EndAD2R, which are specific

to the Endogonales order [11]. The development of new MFRE-specific primers is needed to facilitate future research on the diversity of this important fungal group.

The presence of similar MFRE sequences in several of the agricultural plants examined in this study indicates that some species of MFRE may have the ability to associate with the majority of angiosperms and possibly other plant clades. This hypothesis is strengthened by the fact that these sequences best align to published MFRE sequences isolated from a liverwort *(Allisonia cockaynei)* that is endemic to New Zealand [8], and a hornwort *(Phaeoceros laevis)* [11]. The occurrence of particular species of MFRE across land plants brings into question the reason why they are so widespread. MFRE associations have been shown to have a high level of plasticity (e.g., under different atmospheric CO_2 concentrations [10] and throughout plant phenology [23]); it may very well be that this trait is the reason why plants have evolved to accept them. As such, MFRE could have huge implications for the future of agriculture, as they might be able to mitigate the effects that fluctuating environmental conditions could have on crops by maintaining plant-fungal nutrient exchange during adverse conditions.

Future research will need to molecularly confirm MFRE in other important agricultural crops including legumes and cash crops such as coffee and cotton. The isolation of different strains of MFRE in pure culture is also necessary to set up functional experiments in order to examine the nutritional role of each strain on a plant species grown under axenic conditions. MFRE, which are considered facultative saprotrophs, have been isolated successfully onto media using colonised thallus material from the liverwort *Treubia lacunosa* [9]. If the isolation of MFRE strains can be achieved using the same method but with root material from agricultural plants, many new avenues for MFRE research will open up, including the possibility of sequencing the first MFRE genome and using it to study MFRE function, development and regulation.

5. Conclusions

This study confirms that MFRE are widespread among agricultural plants, which are often simultaneously co-colonised with other types of mycorrhizal fungi. The presence of similar MFRE sequences across the plant phylogeny suggests that these fungi have a crucial role in angiosperms. This remains to be investigated as it may have the potential to revolutionise agriculture worldwide.

Author Contributions: B.S., M.I.B., S.P. and K.J.F. conceived and designed the experiments; B.S. performed the experiments, analyzed the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

MFRE	Mucoromycotina Fine Root Endophytes
AMF	Arbuscular Mycorrhizal Fungi

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