Examining nuclear transfer between homokaryotic and dikaryotic strains of *Rhizophagus irregularis*

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Abstract

Arbuscular mycorrhizal fungi (AMF) are an ancient group of obligate symbionts, colonizing the roots of over 72% of land plants, increasing the uptake of nutrients from the soil, and providing many fitness benefits to their host plants. The multinucleate and coenocytic nature of AMF have interested researchers for decades, leading to many theories of the evolution, and genetic organization of these organisms. Recent findings propose that AMF carry two types of strains, identified based on putative MAT-loci, as either homokaryotic, carrying multiple and genetically similar nuclei, or dikaryotic with co-existing nuclei deriving from two parental strains. In other fungi, hyphal fusions, or anastomosis, between compatible strains results in nuclear transfer, creating heterokaryotic spore progeny. It has been hypothesized that dikaryotic AMF strains arose from the anastomosis between compatible homokaryons harbouring different nucleotypes. The goal of this research is to determine whether anastomosis events, known to occur in other fungi, like homokaryon-homokaryon, homokaryon-dikaryon (Buller phenomenon), and/or dikaryon-dikaryon nuclear exchanges also occur in AMF. To achieve this, the anastomosis frequencies between 15 crosses of homokaryotic and dikaryotic strains of the model AMF species *Rhizophagus irregularis* were examined using microscopy and droplet digital PCR (ddPCR) to determine if nuclear transfer between strains is possible. Overall, these experiments build on the existing evidence of compatible interactions between strains of *R. irregularis*.

Résumé

Les champignons mycorhiziens arbusculaires (CMA) sont un ancien groupe de symbiotes obligatoires, colonisant les racines de plus de 72% des plantes terrestres, augmentant l'absorption des nutriments du sol et offrant de nombreux avantages pour la forme physique de leurs plantes hôtes. La nature multinucléée et coenocytaire de les CMA intéresse les chercheurs depuis des décennies, produisant à de nombreuses théories sur l'évolution et l'organisation génétique de ces organismes. Des découvertes récentes suggèrent que les CMA porte deux types de souches, identifiées sur la base de locus MAT putatifs, soit comme homocaryotes, portant des noyaux multiples et génétiquement similaires, soit comme dicaryotes avec des noyaux coexistants dérivant de deux souches parentales. Chez d'autres champignons, des fusions d'hyphes ou des anastomoses entre des souches compatibles entraînent un transfert nucléaire, créant une descendance de spores hétérocaryotes. Il a été émis l'hypothèse que les souches dicaryotes de CMA résultaient de l'anastomose entre des homocaryons compatibles hébergeant différents nucléotypes. Le but de cette recherche est de déterminer si des événements d'anastomose, connus pour se produire dans d'autres champignons, comme les échanges nucléaires homocaryon-homocaryon, homocaryon-dicaryon (phénomène de Buller) et/ou dicaryon-dikaryon se produisent également dans les CMA. Pour le faire, les fréquences d'anastomose entre 15 croisements de souches homocaryotes et dicaryotes de l'espèce modèle de CMA, *Rhizophagus irregulis*, ont été examinées à l'aide de la microscopie et de la PCR numérique en gouttelettes (ddPCR) pour déterminer si le transfert nucléaire entre les souches est possible. Dans l'ensemble, ces expériences s'appuient sur les preuves existantes d'interactions compatibles entre les souches de *R. irregulis*.

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Figure 1. Light microscopy photos showing interactions between hyphae of different strains of *Rhizophagus irregularis*. The frequency of interactions between hyphae belonging to the same C2 isolate. Scale bar = 500 µm (A). Non-interacting hyphae of the isolates SL1 and B3. Scale bar = 100 µm (B). Pre-fusion incompatibility between hyphae of A4 and C2. Note the swollen hyphal tip and septation in the hypha. Scale bar = 100 µm (C). Post-fusion incompatibility reaction between hyphae of SL1 and C2 showing septation and protoplasmic retraction in both hyphae. Scale bar = 100 µm (D). Perfect fusion between SL1 and B3 showing full hyphae with protoplasmic flow and mature nuclei near the interaction site. Scale bar = 100 µm (E).

Figure 2. Mean frequencies of hyphal interactions detected within the extraradical mycelial networks between strains of *Rhizophagus irregularis* co-cultured with P86 carrot root. Crosses were created between dikaryon-dikaryon strains (DI-DI), dikaryon-homokaryon strains (Buller), and self-crosses between strains of the same identity (control). The number and type of interactions for each cross were enumerated and divided by the total number of interactions per plate. Mean frequency for each interaction type in each cross is displayed (n=3 for each cross).

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Supplemental Figure 1. Multicompartment experimental plate design. Compartments 1-3 were filled with autoclaved M media. Compartments 1 and 3 contained sucrose (+) M media to promote growth of the fungal and carrot co-inoculants. Compartment 2 contained sucrose (-) M media to prevent the growth of the plant symbiont into the middle chamber. Compartment 4 was left empty. *Rhizophagus irregularis* strains were placed into compartments 1 and 3 along with *Daucus carota* cv. P68 agrobacterium root-inducing (Ri) T-DNA transformed root cultures. Autoclaved filter paper bridges were placed between the compartments to allow hyphal movement into the middle compartment. Here, hyphae can interact and anastomose to exchange nuclei.

Supplemental Figure 2. ddPCR methods to detect spore nuclear identity following anastomosis. After isolation of spores in the surrounding interaction site, individual spores were placed into PCR tubes with 4.8 µL of autoclaved dH_2O and crushed using a sterilized needle (1). For the Buller crosses and the DI-DI crosses, the sample was split into two reaction tubes holding equal volume (2.4 µL). To these reaction tubes, a master-mix containing primers and probes matched to the nuclear identity of the parental strains was added: MAT-A and MAT-B matching to parental strain A were added to reaction tube 1, MAT-C and MAT-D matching to parental strain B were added to reaction tube 2 (2). Samples were encapsulated in approximately 20,000 oil droplets using the droplet generator machine (3). Droplets are assumed to contain either one nucleus or no nuclei. Samples were PCR amplified using a thermocycler (4). Droplets were individually read through the droplet reader for the presence or absence of the target sequence (5). Positive droplets contain fluorescently labelled target nuclei that will fluoresce at a higher amplitude than the negative droplets that do not contain the target.

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Supplemental Table 2. Table of primers and probes used for ddPCR spore analysis between crosses of *Rhizophagus irregularis*. Primers and probes for MAT-1 to 3 are the same as those used by (Kokkoris et al., 2021). Sequences are based on the HD1-like (MAT-1,2,5) and HD2 (MAT-3,6) sequences of the corresponding strains.

Supplemental Table 3. Hyphal interactions detected within the extraradical mycelial networks between strains of Rhizophagus irregularis co-cultured with P86 carrot root.

CHAPTER ONE

Introduction

1.1 Overview of AMF

Arbuscular mycorrhizal fungi (AMF) are a group of obligate, ancient asexual fungal species belonging to the Glomeromycotina (Bonfante and Venice, 2020), that are involved in symbiotic relationships with approximately 72% of land plants (Smith and Read, 2008; Brundrett and Tedersoo, 2018). This 400 million year old relationship between plant and fungi is confirmed through fossil records from Rhynie chert, showing the presence of AMF arbuscules in the root cells of *Aglaophyton major*, a now extinct plant species (Remy et al., 1994). Additionally, evidence of AM symbiosis in liverworts (Humphreys et al., 2010), as well as conserved AM symbiosis genes in all plant groups (excluding mosses) (Wang and Qiu, 2006; Wang et al., 2010), provides some validity to the hypothesis that AMF facilitated the migration of plants from an aquatic environments, onto land (Pirozynski and Malloch, 1975; Pirozynski, 1981; Parniske, 2008; Bonfante and Selosse, 2010; Field et al., 2015; Rimington et al., 2018).

In this symbiosis, plants provide the fungus with photosynthetically-derived carbon, largely in the form of lipids (Luginbuehl et al., 2017), in exchange for nutrients from the fungus (Clark and Zeto, 2000). This symbiosis allows not only for the exchange of important nutrients for the plant such as phosphorus and other macro- and micronutrients (Joner et al., 2000; Allen and Shachar-Hill, 2009; Tamayo et al., 2014), but also provides a multitude of other benefits including improved water uptake (Leake et al., 2004), drought tolerance (Augé, 2001), salinity tolerance (Aroca et al., 2013), as well as protection against pathogens (Schwartz et al., 2006) and parasitic nematodes (Elsen and Swennen, 2008); overall contributing to increased plant fitness (Delavaux et al., 2017).

In the early asymbiotic stage, newly emerged hyphae from a germinated AMF spore searches for a host plant in the soil (Giovannetti et al., 1993). Once plant root exudates, like

strigolactones, are detected by the hyphae, hyphal branching increases as the fungus enters the presymbiotic stage, and transitions into the symbiotic stage as the hyphae meet the compatible plant root (Bécard and Piché, 1990; Giovannetti et al., 1993; Buee et al., 2000; Akiyama et al., 2005; Purin and Morton, 2013). After penetrating the plant root epidermis though a specialized appressorium, the fungus is then able to enter the root cells and form specialized structures, called arbuscules, in the cortical root cells (Bucher, 2007; Parniske, 2008). These arbuscules are enveloped by the peri-arbuscular membrane, a plant-derived membrane that forms around the arbuscules and are the centers of bidirectional nutrient exchange between the symbiotic partners (Pumplin and Harrison, 2009).

Eventually, the plant derived carbon will allow the fungus to extend its network further into the soil and to form abundant viable spores on the extraradical hyphae in the soil, in addition to intraradical vesicles that form in the root which can also act as propagules (Bever et al., 1996).

1.2 AMF genetics

For many years, AMF were thought to be heterokaryotic due to their multinucleate, coenocytic status, which was thought to aid, through complementation of co-existing genetically distinct nuclei, in the purging of deleterious mutations which would have accumulated through the lifetime of this ancient group (Sanders et al., 1996; Sanders, 1999; Kuhn et al., 2001; Hijri and Sanders, 2005; Sanders and Croll, 2010; Angelard and Sanders, 2011; Boon et al., 2015). The genetic organization of these *R. irregularis* strains was highly debated for many years (Corradi and Brachmann, 2017), however gradual evidence demonstrating similarity between individual nuclei began to disprove the heterokaryosis hypothesis (Pawlowska and Taylor, 2004; Tisserant et al., 2013). Whole genome sequencing and individual nuclei analysis by Ropars et al. (2016),

revealed genetic organization of different strains and have subsequently built the current model that explains AMF genetics. The model proposes that rather than containing multiple different nucleotypes, as would be observed in a heterokaryon, AMF carry two dominant life-stages or strains: homokaryotic strains (AMF homokaryons), that carry thousands of nuclei within a mycelium and are all of the same genetic background, and dikaryon-like strains (referred to as AMF dikaryons or heterokaryons), where nuclei deriving from two parental strains co-exist. These different genetic organizations result in different life history traits like spore germination and hyphal growth between the strains (Serghi et al., 2021).

1.3 AMF species and strains

Due to their historical associations with plants, AMF are found ubiquitously in the soil in most continents; except Antarctica (Öpik et al., 2006; Brundrett and Tedersoo, 2018). Although they form symbioses with plants from every major plant group, only a relatively low number of AMF species (approximately 300 species) have been described (Krüger et al., 2011; Lee et al., 2013). To interact with more than 200,000 plant species, these AMF generalists show low host specificity, meaning that a single AMF species is able to interact with multiple plant species simultaneously (Giovannetti et al., 2004; Öpik et al., 2009). Although the interaction between plant and fungus is generally positive, the benefit that a single AMF species provides to each of its hosts is variable, thus, a single AMF species could potentially affect the community composition and biodiversity of plants within an ecosystem (Bever et al., 1996; van der Heijden et al., 1998; Husband et al., 2002; Vandenkoornhuyse et al., 2002; Leake et al., 2004).

To further increase the complexity of the common mycorrhizal network, individual AMF species separate into genetically distinct strains, which are present in varying abundance at each

site. For example, different strains of the AMF species *Rhizophagus irregularis* (Croll et al., 2009; Savary et al., 2018), *Acaulospora colossica* (Pringle et al., 2000), and several species of *Glomus* (Sasvári et al., 2011) were found to coexist within a single region. Similarly, Koch et al. (2004) were able to identify at least five strains of *R. irregularis* from a single field site.

1.4 Somatic vs sexual compatibility in fungi

1.4.1 Somatic incompatibility

In addition to AMF being capable of connecting different plant species through the underground mycelial network, there is a possibility that the simultaneous presence of multiple species and strains at a single site can result in increased physical encounters between the strains via direct hyphal interactions (Glass et al., 2004). In fungi, these hyphal interactions are determined by the somatic, or vegetative, incompatibility between the strains (Glass et al., 2000; Fleißner, 2012). Somatic incompatibility is governed by mating-type loci in some fungi like Basidiomycota, or by a separate set of genes that exclusively control the formation of these stable vegetative heterokaryons (Leslie, 1993). Visually, in AMF, hyphal interactions are characterized in four ways: non-interacting, pre-fusion incompatibility, post-fusion incompatibility, and perfect fusion, also known as anastomosis (Croll et al., 2009). Briefly, interacting hyphae without hyphal fusion are classified as non-interacting (Croll et al., 2009), pre-anastomosis activities such as hyphal tip swelling not followed by fusion but resulting in protoplasm retraction is known as pre-fusion incompatibility (Croll et al., 2009; Pepe et al., 2016) and hyphal fusion followed by protoplasmic retraction in both hyphae and septa formation at the fusion point is indicative of post-fusion incompatibility (Croll et al., 2009; Pepe et al., 2016). During anastomosis, the cell and protoplast

of two interacting hyphal filaments fuse, allowing the dissemination of nutrients (Glass et al., 2004), signals (Fleissner et al., 2009), and organelles (Glass et al., 2000).

1.4.2 Sexual compatibility

In sexual fungi, like Basidiomycota and Ascomycota, sex generally follows three steps (Fraser and Heitman, 2003; Lee et al., 2010; Fraser et al., 2014). After recognition of compatibility, two mating partners will undergo plasmogamy and fuse their cells. Fusion between the nuclei can then occur in karyogamy (nuclear fusion), either immediately following plasmogamy, or is delayed, leading to a temporary dikaryotic state. Lastly, meiosis occurs after the fusion of the nuclei, resulting in a haploid progeny (Nieuwenhuis et al., 2013). In fungi. the determination of sexual compatibility is through the mating-type (MAT) loci; unique genetic regions that give both identity to the cells and encode genes that allow for the recognition of the mating partner (Maia et al., 2015). Two mating systems are possible: either a bipolar system where two different alleles at a single locus dictate mating; or a tetrapolar system in which two unlinked loci must have different alleles at those loci in order for mating to occur (Lee et al., 2010). Briefly, the fungus releases pheromones into the environment to be detected by the pheromone receptor of the other cell (Butler et al., 2004; Butler, 2010; Lee et al., 2010). In Basidiomycetes, upon compatibility, the homeodomain transcription factors (HD1 and HD2) that are encoded by the MAT loci dimerize and activate developmental steps like hyphal elongation and control the expression of key genes in sexual reproduction (Morrow and Fraser, 2009; Maia et al., 2015).

1.5 Cryptic Sex in AMF and putative MAT-loci

While sexual reproduction is common in nature due to its evolutionary success and the fitness benefits it provides, there are many costs related to reproducing sexually rather than asexually, referred to as the "queen of questions" in evolutionary biology (Bell, 1988). There are several theories that account for the existence of sex; including the ability to prevent the accumulation of deleterious mutations throughout the genome (Muller's ratchet; Muller, 1964), and as an adaption to escape co-evolved parasites (the Red Queen hypothesis; Hamilton et al., 1990). Conversely, asexual lineages have long-been viewed as short-lived and have typically existed at the tips of evolutionary trees (Maynard Smith, 1986). This is thought to be a result of the inability of these lineages to purge deleterious mutations, diversify, or adapt to outcompete with sexual lineages (Butlin, 2002). Despite these traditional views of asexual lineages, the evidence of several ancient asexual lineages that have persisted throughout evolutionary history could possibly help to uncover why sexual reproduction is so ubiquitous (Neiman et al., 2009).

The apparent long-term lack of a sexual cycle in AMF has resulted in their classification as "ancient asexual organisms," along with bdelloid rotifers and Darwinulid ostracods (Judson and Normark, 1996; Normark et al., 2003; Neiman et al., 2009). The persistence of asexual lineages is intriguing as they are expected to be short lived due to the lack of genetic diversification and the accumulation of deleterious mutations in the genome without meiosis (Maynard Smith, 1986; Butlin, 2002; Normark et al., 2003; Neiman et al., 2009). Recent research has started to decipher some of the reproductive cycle of AMF. The discovery of meiosis-related genes (Halary et al., 2011) and homokaryotic and dikaryotic strains (Ropars et al., 2016) provide some evidence that these organisms are undergoing some cryptic or parasexual cycle.

Although no definite sexual cycle has been observed in AMF to date, putative MAT-like loci have been identified in *Rhizophagus irregularis* (Ropars et al., 2016), adding to the mounting evidence that some form of cryptic sexual recombination occurs in these organisms (Halary et al., 2011; Riley and Corradi, 2013; Tisserant et al., 2013; Riley et al., 2014). Specifically, evidence of a HD1-like, HD2, and pheromone sensing region, similar to those found in sexual fungi, are present in model AMF species (Ropars et al., 2016). Each of the homokaryotic strains of *R. irregularis* harbour one of these alleles (labelled MAT-1 through to MAT-6) in all of the nuclei, while dikaryotic strains harbour nuclei with one of two different mating types (Ropars et al., 2016). This supports that *R. irregularis* is heterothallic and uses a bipolar mating system (Ropars et al., 2016), as well as the theory that dikaryotic strains arose from the joining of two compatible homokaryotic strains (Corradi and Lildhar, 2012).

1.6 Anastomosis

Anastomosis, or hyphal fusion, is a routine process for all fungi involved in colony establishment and development (Glass et al., 2000; Glass and Kaneko, 2003; Fleißner, 2012). This process was first observed by Ward (1888), and further detailed by Buller (1930). During anastomosis, the cell and protoplast of two interacting hyphal filaments fuse (plasmogamy), allowing the dissemination of nutrients (Glass et al., 2004), signals (Fleissner et al., 2009) and organelles (Glass et al., 2000). Anastomosis provides a fitness advantage as larger colonies can be formed and improve resource exploitation (Glass et al., 2000; Fleißner, 2012). Usually, anastomosis is observed in the older segments of the mycelium to increase the interconnectedness of the mycelium and ensure that nutrients are evenly distributed (Glass et al., 2000; Fleißner, 2012). When these hyphal filaments belong to the same strain, this phenomenon is known as “self-

fusion" (Read et al., 2009), while anastomosis of genetically different strains is known as "non-self fusion" (de la Providencia et al., 2013). During non-self fusion between somatically compatible strains, exchange of nuclei, of unique genotypes, and mitochondria takes place between the two genetically different strains, resulting in the formation of a heterokaryon (Glass et al., 2000).

1.6.1 Anastomosis in AMF

Anastomosis in AMF is widely studied (Purin and Morton, 2011; Novais et al., 2013; Purin and Morton, 2013) and, similarly to other fungi, it increases the interconnectedness of the AM hyphal networks (Giovannetti et al., 2004). The establishment of successful anastomosis between compatible AMF strains can happen rather rapidly (within 35 min), as observed with time lapse light microscopy, and the cytoplasmic exchange between hyphae takes place at the rate of 1.8-2.6 $\mu m\ s^{-1}$ (Novais et al., 2017). Compatibility has been observed between same strains (self-fusion), and between genetically distinct strains (Giovannetti et al., 2001; Croll et al., 2009; Purin and Morton, 2011; de la Providencia et al., 2013; Novais et al., 2013; Novais et al., 2017). Due to high compatibility, self-fusion anastomosis is the most common type of fusion found in AMF (Croll et al., 2009; de la Providencia et al., 2013; Novais et al., 2017). When anastomosis takes place between genetically different strains, it allows for the exchange of nuclei and mitochondria (Giovannetti et al., 2001; Glass et al., 2004; de la Providencia et al., 2013). This has been confirmed in homokaryon-homokaryon crosses of *R. irregularis*, where dikaryotic spores were created (Mathieu et al., unpublished). Evidence of these interactions between different strains suggests that somatic compatibility between homokaryotic strains may create successful dikaryons.

As we now know that AMF genetics follow universal fungal processes, the study of somatic compatibility between AMF strains offers new venues of research. In conventional fungal groups, special types of anastomosis occur between homokaryotic and dikaryotic strains, namely the Buller phenomenon (also known as DI-MON interactions; Buller, 1930; Leonard et al., 1978) and DI-DI interactions (Wilson, 1991).

1.6.2 Buller phenomenon

The Buller phenomenon takes place between compatible homokaryotic and dikaryotic strains, where the dikaryon donates a nucleotype to the homokaryon following anastomosis, which is then spread throughout the mycelium, transitioning the homokaryotic strain to a dikaryotic strain (Buller, 1930). The Buller phenomenon has been mainly studied in Basidiomycetes (de la Bastide et al., 1995; Callac et al., 2006; Nieuwenhuis et al., 2011; Nieuwenhuis et al., 2013), but never directly in AMF. Before our current knowledge of AMF genetics, Croll et al. (2009) reported perfect fusion between the strain A4 and the strains B3 and C2 of *R. irregularis*. Years later (Ropars et al., 2016) identified the strain A4 as dikaryotic, and the strains B3 and C2 as homokaryotic, exposing the possibility that the Buller phenomenon may occur in AMF. However, it is yet unknown whether an AMF dikaryon can act as donor of a nucleotype to the homokaryon and whether there is a preference on the donated nucleotype, as seen in the basidiomycete *Schizophyllum commune* (Nieuwenhuis et al., 2011) or whether it can lead to the creation of a stable trikaryon as seen with *Heterobasidion parviporum* (James et al., 2009).

1.6.3 Dikaryon-dikaryon interactions

Another type of anastomosis is the crossing between dikaryotic strains (Wilson, 1991; Pilotti et al., 2002; Babasaki et al., 2003) known as DI-DI interactions. To date, DI-DI interaction studies have looked exclusively at the morphological changes in the affected mycelia, and the type of genetic exchange which takes place, if any, between the interacting strains is unknown. For example, Babsaki et al. (2003) observed the development of chimeric fruiting bodies after the fusion of dikaryotic strains of *Pholiota nameko*, displaying morphological traits intermediate to both strains. However, isolation and subculturing of the mycelia from the morphologically chimeric fruiting body suggested that mycelial aggregation of the two strains, rather than genetic exchange, was the result of the mixture of traits observed in the fruiting body (Babasaki et al., 2003). While anastomosis is possible between dikaryotic strains (Pilotti et al., 2002), it is rare, with the majority of hyphal crossings resulting in the formation of a hyphal reaction line (septum) that does not allow plasmogamy, as seen between dikaryotic strains of *Ganoderma boninense* (Pilotti et al., 2002), *Echinodontium tinctorium* (Wilson, 1991), and *Pholiota nameko* (Babasaki et al., 2003). When anastomosis is observed, it is more likely to happen between strains that share a nucleotype carrying a common MAT locus (Pilotti et al., 2002). Further studies need to examine whether DI-DI interactions can occur in AMF, and to determine, if successful, how nuclear transfer occurs between the dikaryotic strains.

1.7 Research Justification and Goals

AMF are used commercially in agriculture in many countries, including the US, Canada, India, Germany, and Italy, in the form of bio-fertilizers (Chen et al., 2018c). AMF based bio-fertilizers aim to improve the yield of economically important crops, such as potatoes (Hijri, 2016;

Ghobadi et al., 2020), wheat (Dal Cortivo et al., 2020), cowpea (Rocha et al., 2020), and tomatoes (Bidellaoui et al., 2019), while simultaneously they reduce the use of fungicides and chemical fertilizers (Ortas, 2012; Rouphael et al., 2015). In addition to improving crop growth, AMF can trap nutrients like excess N, P, and K into a closed nutrient cycle that reduces nutrient leeching resulting from fertilization (van der Heijden, 2010). The overwhelming majority of AMF inocula are produced using one *Rhizophagus irregularis* strains; DAOM-197198.

As AMF form many belowground associations and assist in the preservation of soil quality, it is important to understand how these fungi can interact with the native pre-existing strains, and particularly with those newly introduced as biofertilizers. Furthermore, hyphal compatibility leading to heterokaryon formation between compatible strains of AMF can further challenge the notion that AMF are ancient asexuals. Understanding the factors that control anastomoses, and the potential outcome of such interactions, will improve our knowledge of the common mycorrhizal networks (CMN) and their genetics.

Moreover, recent evidence of sex in ancient asexual lineages, like bdelloid rotifers (Schwander, 2016) has put other ancient asexual lineages under examination for sexual or parasexual reproduction. In addition to the existing evidence of a parasexual or cryptic sexual cycle in AMF (Ropars et al., 2016; Chen et al., 2018b; Yildirir et al., 2020), confirmation of nuclear transfer following anastomosis between genetically different strains would provide further support to the possibility of recombination occurring between isolates.

This study seeks to address our lack of knowledge on AMF strain compatibility by investigating the presence and frequency of anastomosis between theoretically compatible homokaryons and dikaryons. In particular, crosses involving homokaryons, and dikaryon-dikaryon of the AMF *R. irregularis* will be produced, and nuclear transfer between strains will be visually

assessed at the sites of hyphal interaction. These will also be quantified in the spores resulting from the crossing between the strains using molecular techniques. If successful, this work will add evidence to the hypothesis that compatible strains can create stable heterokaryons.

CHAPTER TWO

Examining nuclear transfer between homokaryotic and dikaryotic strains of *Rhizophagus irregularis*

2.1 Abstract

Arbuscular mycorrhizal fungi (AMF) strains are genetically distinct, and often coexist and interact in the soil through anastomosis. These strains are identified based on putative MAT-loci, as either homokaryotic, carrying multiple and genetically similar nuclei within their cells, or dikaryotic with co-existing nuclei deriving from two parental strains. In other fungi, anastomosis between compatible strains results in nuclear transfer, creating heterokaryotic spore progeny. To assess if homokaryon-homokaryon, homokaryon-dikaryon (Buller phenomenon), and/or dikaryon-dikaryon nuclear exchanges also occur in AMF, the anastomosis frequencies between 15 crosses of homokaryotic and dikaryotic strains of the model AMF species *Rhizophagus irregularis* were examined using microscopy and droplet digital PCR (ddPCR). Although rare in comparison to self-fusions, complete anastomosis between different strains was present in all crosses. However, spores analyzed using ddPCR showed limited nuclear transfer between the crossed strains. Future crosses between more phylogenetically similar strains may result in more successful nucleotype transfers which could further confirm the creation of new nuclear combinations.

2.2 Keywords

Arbuscular mycorrhizal fungi, *Rhizophagus irregularis*, anastomosis, hyphal compatibility, nuclear transfer, homokaryon, dikaryon

2.3 Introduction

Ancient asexual organisms have been of great interest to researchers, as their ability to persist and diversify for millions of years contrasted with the assumption that such organisms should be short-lived due to their inability to purge the accumulation of deleterious mutations in the genome via sexual reproduction (meiosis) (Maynard Smith, 1986; Butlin, 2002; Normark et al., 2003; Neiman et al., 2009). However, evidence of sexual signatures within these anciently asexual lineages has provided some clues as to why such organisms managed to survive (Maynard Smith, 1986; Schwander, 2016). The arbuscular mycorrhizal fungi (AMF) represent a supposedly ancient asexual organism that is hypothesized to have facilitated the migration of plants from an aquatic to a terrestrial environment (Pirozynski and Malloch, 1975; Parniske, 2008), and form symbiotic relationships with over 72% of land plants (Smith and Read, 2008; Brundrett and Tedersoo, 2018). Recent findings unveiled the existence of distinct homokaryotic and dikaryotic strains, as well as putative mating-type (MAT) loci that are involved in the sexual identity in these fungi (Ropars et al., 2016). AMF also harbor meiosis specific genes (Halary et al., 2011), and an abundance of transposable elements within the genome (Chen et al., 2018b), along with evidence of inter-nuclear recombination (Chen et al., 2018a). Altogether, these findings indicate that although there may not be direct evidence of a sexual cycle occurring, a cryptic or a parasexual cycle is likely to exist in these fungi.

A proposed mechanism through which asexual lineages of fungi can diversify their genetic content is through vegetative hyphal fusions (Roper et al., 2011). In this case, somatic compatibility between fungal strains allows hyphae to fuse and nuclear migration and mixing to occur (Glass et al., 2000; Fleißner, 2012; Simonin et al., 2012; Roper et al., 2013). Somatic compatibility is governed by either MAT-loci, or vegetative or heterokaryon incompatibility loci

(*vic* or *het* loci, respectively; Leslie, 1993; Glass et al., 2000). Hyphal fusion, or anastomosis, is a regular process that occurs within a fungal colony. This process is important for colony establishment, as well as to increase interconnectivity of the mycelium and ensure the even distribution of nutrients to growing hyphal tips, providing a fitness advantage, as larger colonies can be formed (Glass et al., 2000; Glass and Kaneko, 2003; Fleißner, 2012). Anastomoses between hyphae of the same strains are known as "self-fusions" (Read et al., 2009), while those between genetically different strains as "non-self fusions" (de la Providencia et al., 2013), leading to the exchange of nuclei and mitochondria to form a heterokaryon (Glass et al., 2000).

In Basidiomycota, the Buller phenomenon (Buller, 1930) is frequently observed in the creation of dikaryotic strains (de la Bastide et al., 1995; Callac et al., 2006; Nieuwenhuis et al., 2011; Nieuwenhuis et al., 2013). Briefly, in distant fungal species, hyphal fusion between compatible monokaryotic (one nucleus per cell) and dikaryotic (two nuclei per cell) strains results in the donation of a nucleotype from the dikaryon to the monokaryon, which is then spread cell-to-cell through clamp-connections to the entire mycelium, transitioning the monokaryon to a dikaryon (Buller, 1930). Similarly, dikaryon-dikaryon (DI-DI) interactions have also been observed in Basidiomycota, but with lower frequency (Wilson, 1991; Pilotti et al., 2002; Babasaki et al., 2003). Studies have so far only examined morphological changes, such as the formation or absence of a reaction line (a physical barrier that forms at the colony tips) between the cultures, without investigating if genetic exchange between strains occurs.

Anastomosis has been widely studied in AMF, with both self-fusions and non-self-fusions observed (Giovannetti et al., 2001; Giovannetti et al., 2004; Croll et al., 2009; Purin and Morton, 2011; Sbrana et al., 2011; Novais et al., 2013; Purin and Morton, 2013; Novais et al., 2017; Sbrana et al., 2018; Novais et al., 2019). In the model AMF species *Rhizophagus irregularis*, strains are

either homokaryotic, where the thousands of nuclei flowing through the coenocytic mycelium are all of the same type, or dikaryotic, where the nuclei originate from two parental strains (Ropars et al., 2016; Chen et al., 2018b; Chen et al., 2018a; Chen et al., 2020). Of the 85 strains of *R. irregularis* in the Canadian Collection of AMF, only 4 strains (A4, A5, SL1, G1) are dikaryotic (Ropars et al., 2016; Kokkoris et al., 2021). Due to the identification of the putative MAT-locus in *R. irregularis*, and the semblance to a heterothallic and bipolar mating system (Ropars et al., 2016), it has been hypothesized that these dikaryotic strains arose from the fusion of two homokaryotic strains harbouring different nucleotypes (Corradi and Lildhar, 2012; Ropars et al., 2016).

This study will examine whether anastomosis events known to occur in other fungi, like the Buller phenomenon and DI-DI interactions can occur in *R. irregularis*, leading to the creation of spores containing nuclei from both parental strains. To test this, anastomosis frequencies in crosses between homokaryotic and dikaryotic strains were determined through microscopy. Following the observation of anastomosis and nuclear movement between strains, spores around the interaction zone were isolated and the nuclear identity was analysed through digital droplet PCR (ddPCR). Spores from crosses showing evidence of some nuclear transfer were used to start new cultures, to determine if the new nuclear identity of the spore is maintained in the next generation. These analyses demonstrate limited nuclear transfer between the chosen strains.

2.4 Methods

2.4.1 Fungal material

Three dikaryotic strains A4, A5, and SL1; and five homokaryotic strains B3, C2, 330, A1, and 4401 of the AMF species *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler were used to create the crosses between strains. The strains A4, A5, A1,

B3, and C2 were all initially isolated from the same field in Tänikon, Switzerland, and subsequently cultured in lab (Koch et al., 2004). The strains 4401 and 330 were isolated from La Martinique Îles-de-la-Madeleine, Canada and Terrebonne, Canada, respectively. Fungal stock cultures were obtained from the Corradi lab collection. Genetic similarity between the strains was previously determined through the H^+-ATPase gene (Ropars et al., 2016), concatenated H^+-ATPase and glomalin genes (Kokkoris et al., 2021), and whole genome assemblies (Yildirir et al., unpublished). The genetic similarity between the MAT-loci has also been previously reported (Ropars et al., 2016; Kokkoris et al., 2021).

2.4.2 Fungal crosses

The strains of *R. irregularis* used for the crosses were selected so the MAT-loci between each pair would differ (Supplemental Table 1), excluding the cross between A4 and SL1, where hyphal compatibility between strains sharing a MAT locus was assessed. The crosses were plated on rectangular tetra-compartment split plates containing sucrose (+) M media (Bécard and Fortin, 1988) in the first and third compartments, and sucrose (-) M media in the second, middle compartment, while the fourth compartment was left empty. The first and third compartments were connected to the middle compartment through an autoclaved filter paper bridge, to facilitate the movement of the hyphae between the compartments. Cellophane membranes (Sigma-Aldrich), cut to fit the 3 x 8 cm^2 space of the compartments, were autoclaved and placed on top of the media in the middle chamber, allowing the fungi to grow along the surface for easier visualization. Crosses were started by adding 1 cm^2 of the strain mycelium from stock plates, and 1 cm^2 of *Daucus carota* cv. P68 agrobacterium root-inducing (Ri) T-DNA transformed root cultures to the first and third compartments (Supplemental Figure 1 for plate design). Carrot was selected as the plant host as

both dikaryotic and homokaryotic strains of *R. irregularis* have higher hyphal branching, hyphal length, a more dense hyphal network, and produce more spores when they are cocultured with *Daucus carota* root organ cultures (Serghi et al., 2021). Plates were incubated at 25 ºC in the dark, allowing hyphae from each strain to populate the middle compartment and sporulate. For each experimental cross, six replicate plates were made, for a total of 78 plates. For the control plates, 3 replicates were made, for a total of 24 plates.

2.4.3 Observations

The hyphal compatibility between the crosses was assessed in the middle compartment, four months after hyphal growth had been detected in the middle chamber. Hyphal contacts were classified into four categories. Non-self-fusions were scored when protoplasmic streaming was observed following anastomosis between the strains in each cross. Self-fusions were scored when anastomosis was observed between the hyphae of a strain originating from the same compartment. Hyphal interactions for both non-self-fusions and self-fusions were scored as incompatible when post-fusion incompatibility signs like protoplasm retraction and septa formation were observed following anastomosis. Due to the density of some of the hyphal networks, non-interacting hyphae and pre-fusion incompatibility between the hyphae could not be enumerated but were observed. Interactions were observed at 100x magnification with a Leica DM750 compound microscope. To assess the type of interaction, interacting hyphae were visually traced back to their origin. Non-self-fusions were confirmed using a Zeiss AxioZoom.V16 microscope equipped with a Axiocam 506 camera to allow for the visualization of nuclei at 200x magnification. The hyphal contact frequency for each category was calculated as a percentage of the total number of interactions counted. Sites with non-self-fusion were marked for spore isolation.

2.4.5 Primers

The primers used to detect nuclear transfer between the strains were the same as those designed by Kokkoris et al. (2021), excluding the MAT-5 and MAT-6 primer/probes, which were altered to increase the sensitivity to those specific MAT-loci (Supplemental Table 2). An initial cross-test between primer/probe and DNA from each strain revealed some amplification of the MAT-3 locus by the MAT-5 primer/probe, as well as amplification of the MAT-2 locus by the MAT-6 primer/probe. The MAT-5 primer/probe was designed based on the HD1-like sequence of the *R. irregularis* SL1 strain, and cross-tested on DNA of each strain with different MAT-loci to ensure sensitivity to only the MAT-5 locus. Similarly, the MAT-6 primer/probe was designed on the HD2 locus of the *R. irregularis* strains A5 and C2, and cross-tested to ensure detection of both MAT-6 loci. Primer design was completed in MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura, Stecher, and Kumar 2021) and ordered as probe-based assays from IDT (Iowa, USA).

2.4.6 Spore isolation and ddPCR

To determine if transfer of nuclei between the parental strains occurred, droplet digital PCR (ddPCR) was used to identify the MAT-loci present in the spores of the middle chamber following anastomosis between strains. ddPCR determines the absolute quantification of DNA by dividing a PCR reaction into 20,000 oil-encapsulated reactions and reading the number of FAM or HEX fluorescently labelled targets in each sample. Due to its sensitivity to small amounts of DNA, single nuclei can be detected in a sample.

Media, containing mature spores from the previously marked anastomosis sites, was excised, and dissolved in a citric acid buffer solution (17 % 0.1 M citric acid and 83 % 0.1 M

sodium citrate). After the media was solubilized, spores were isolated from the hyphae using sterilized needles and transferred into individual 0.2 mL PCR tubes along with 4.8 µL of autoclaved, distilled water, as per Kokkoris et al. (2021). Spores were then crushed with a needle under a light microscope to release nuclei into the solution. As up to four MAT-loci need to be analyzed per spore, and only two probes (one HEX and one FAM) can be detected per reaction, the samples were vortexed and centrifuged, and 2.4 µL of the solution was transferred into a new 0.2 mL PCR tube. It was assumed that the nuclei divided evenly in the paired reactions. A master mix, consisting of 1X Supermix for Probes (Bio-Rad), a 500 nM primers to 250 nM probe mixture (IDT, Iowa, USA), and DNase free water, was added to each reaction tube. Droplets were generated with a QX100™ droplet generator and amplified on a C1000 Touch Thermal Cycler (Bio-Rad Technologies, Inc, Mississauga, ON, Canada). Cycling conditions for the PCR are the same as those described in Kokkoris et al. (2021). Droplet data was analyzed with the QuantaSoft™ Analysis Pro (1.0.596; Bio-Rad) software. Manual thresholds were set at points where the positive and negative droplets could be distinguished. The nuclear identity of the spores was determined by the presence/absence of the MAT-loci detected in the droplets of the paired reactions.

2.4.7 Single spore plates

Following detection of both parental nucleotypes in the spore progeny, single spores from the homokaryon-homokaryon experimental plates were isolated and placed (8-12 spores per plate) in a split plate chamber containing M media and incubated at 25 ºC. For these plates, we used spores only from the homokaryon-homokaryon crosses A1xC2 and A1x4401. Spores were monitored weekly for germination. Following germination, spores were cut from the media with

sterilized scalpels and transferred to M media containing 1% (w/v) sucrose on a new split plate. *D. carota* cv. P68 transformed roots were placed close to the spore to encourage symbiotic growth of the spore. A sterilized filter paper bridge between the sucrose (+) and sucrose (-) halves of the split plate allowed the new mycelial growth from the spore to cross into the sucrose (-) half and sporulate. Plates were monitored weekly for growth. Spores from plates with successful growth were analyzed through ddPCR to determine if the new nuclear identity was maintained.

2.4.8 Statistical analysis

Frequency of anastomosis between the crosses was evaluated using the non-parametric Kruskal-Wallis test. The Kruskal-Wallis test was performed in R studio (version 4.2.0 2022-04-22, Vienna, Austria) using the base function kruskal.test. Significance differences between crosses were visualized using the post-hoc Dunn test (1964) from the FSA package, using the function dunnTest.

2.5 Results

2.5.1 Hyphal contacts between strains

Hyphae from the strains began to enter the middle compartment of the plate around 2 to 3 months after initially being plated. At that point, either both strains grew into the middle compartment at relatively the same rate, so that both strains were observed in the middle compartment, or one strain grew at a more rapid rate and colonized the middle compartment, leading to no growth of the other strain into the middle compartment. This was more frequently observed with the SL1 hypahe, which tended to form more dense hyphal networks. Only in plates

where both strains were present in the middle compartment were the number of hyphal interactions enumerated.

After four months of growth in the middle chamber, anastomosis was observed between strains in all DI-DI and Buller crosses. In the HOM-HOM crosses, anastomosis was not detected in the B3xC2 cross, but was present in the other crosses. Movement of protoplasm and nuclei between the strains in the hyphal bridges was verified visually at 200x magnification (Figure 1). The mean number of hyphal contacts varied between the crosses. For example, in the DI-DI crosses, between 365.33±53.90 in A4XA5, and 975.00±338.44 in SL1xA5, interactions were identified; in the Buller crosses, between 125.67±20.07 in A5x330, and 810.67±193.75 in SL1xC2 interactions were identified; and in the control crosses, between 302.67±27.35 in B3xB3, and 629.67±74.59 in C2xC2 interactions were identified (Supplemental Table 3). Across all crosses, there was a significant difference in the number of hyphal contacts between the strains (Kruskal-Wallis, $\chi^2(14)=41.113$, $p<0.001$), but not consistently across the different types of crosses (i.e. Buller, DI-DI, control).

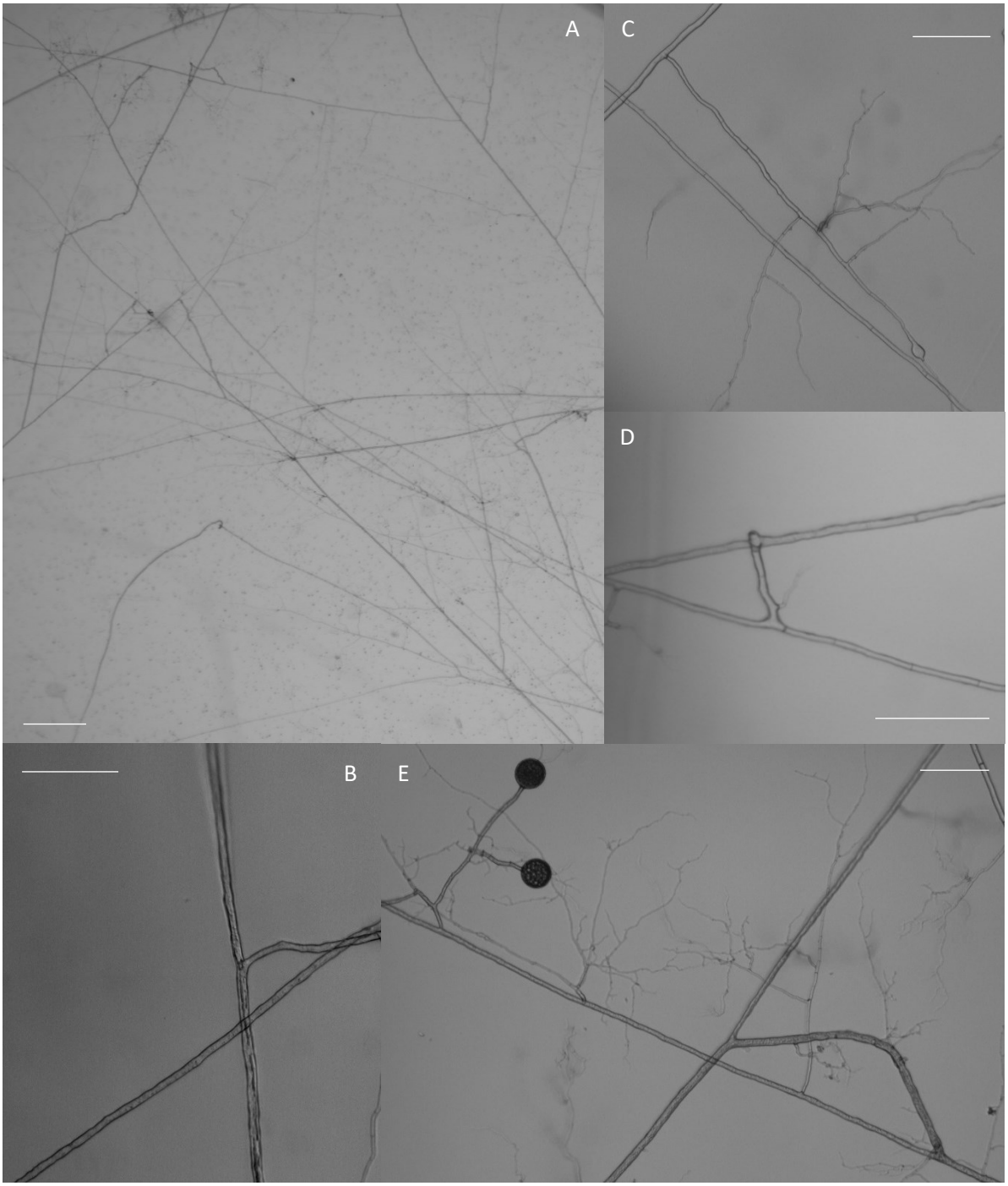


Figure 1. Light microscopy photos showing interactions between hyphae of different strains of *Rhizophagus irregularis*. The frequency of interactions between hyphae belonging to the same C2 isolate. Scale bar = 500 µm (A). Non-interacting hyphae of the isolates SL1 and B3. Scale bar = 100 µm (B). Pre-fusion incompatibility between hyphae of A4 and C2. Note the swollen hyphal tip and septation in the hypha. Scale bar = 100 µm (C). Post-fusion incompatibility reaction between hyphae of SL1 and C2 showing septation and protoplasmic retraction in both hyphae. Scale bar = 100 µm (D). Perfect fusion between SL1 and B3 showing full hyphae with protoplasmic flow and mature nuclei near the interaction site. Scale bar = 100 µm (E).

Among all crosses, the frequency of self-fusions was higher than the frequency of non-self-fusions (Kruskal-Wallis, $\chi^2(3)= 99.037$, $p<0.001$). In the DI-DI crosses, self-fusions occurred at a frequency of 35.69±2.83% in A4xA5 to 60.72±2.44% in SL1xA5, while non-self-fusions occurred at a frequency of 1.21±0.22% in A4xA5 to 1.68±0.05% in SL1xA5. In the Buller crosses, self-fusions occurred at a frequency of 39.91±8.99% in SL1xC2 to 80.65±6.42% in A5x330, and again, the non-self fusions are observed to occur at much lower rates, between 0.34±0.24% in A4xB3 to 4.70±0.23% in SL1xC2 (Figure 2, Supplemental Table 3).

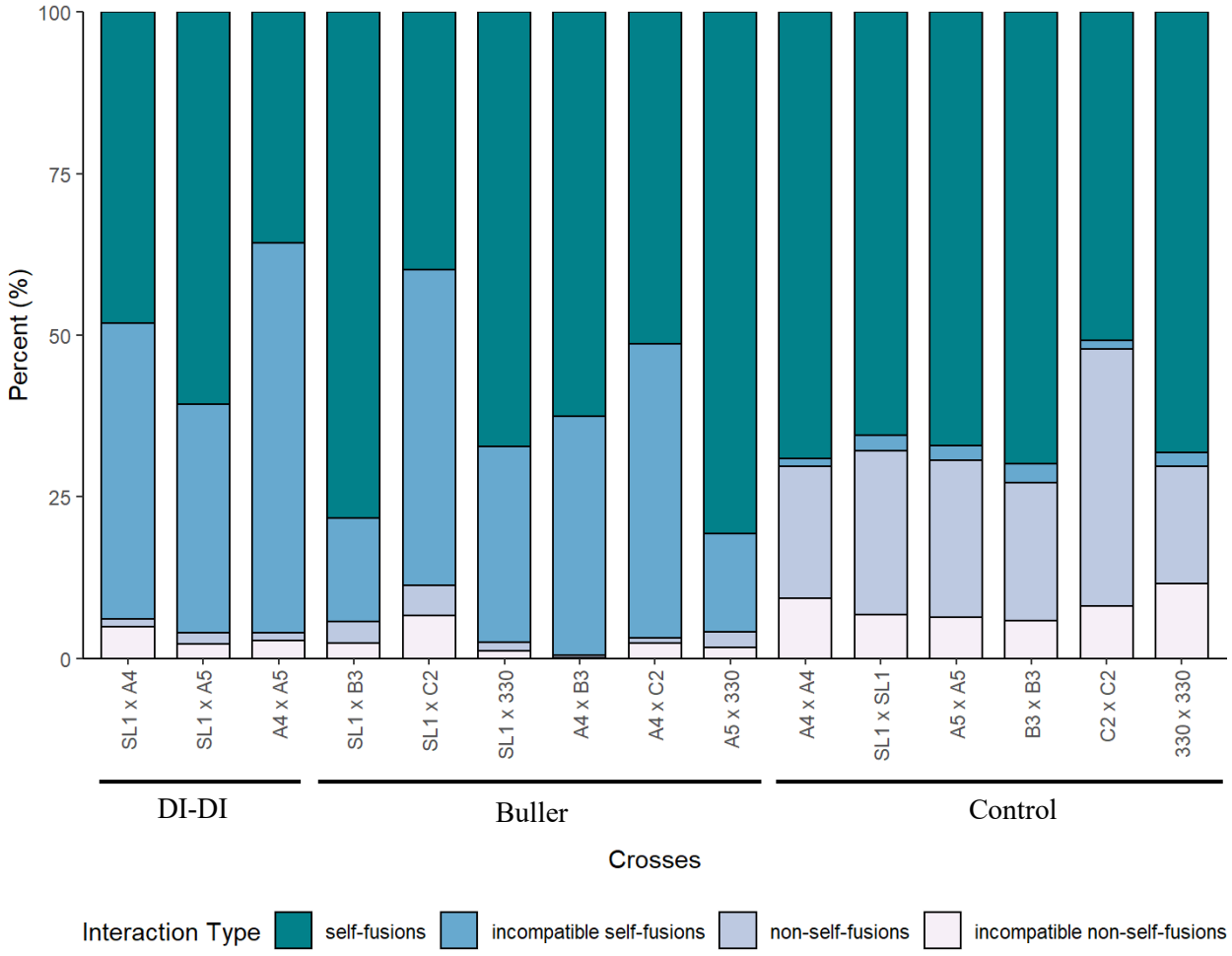


Figure 2. Mean frequencies of hyphal interactions detected within the extraradical mycelial networks between strains of *Rhizophagus irregularis* co-cultured with P86 carrot root. Crosses were created between dikaryon-dikaryon strains (DI-DI), dikaryon-homokaryon strains (Buller), and self-crosses between strains of the same identity (control). The number and type of interactions for each cross were enumerated and divided by the total number of interactions per plate. Mean frequency for each interaction type in each cross is displayed (n=3 for each cross).

Significant differences between the different crosses in the frequency of non-self fusions were found (Kruskal-Wallis, $\chi^2(14)=41.399$, $p<0.001$; Supplemental Table 3). For example, the A4xB3 cross anastomosed at significantly lower frequencies than all of the control crosses, excluding the 330 and A5 control crosses (Dunn's test, $p<0.05$). Similar results were obtained for non-self-fusions between strains (Kruskal-Wallis, $\chi^2(14)=34.475$, $p<0.01$), with the A4xB3 cross having lower rates of anastomoses compared to control crosses (Dunn's test, $p<0.05$).

Self-fusions also differed significantly between crosses (Kruskal-Wallis, $\chi^2(14)=33.534$, $p<0.01$), and were significantly higher in SL1xB3 compared to A4xA5 and SL1xC2 (Dunn's test, $p<0.05$). These, however, did not differ significantly from the control crosses. Incompatible self-fusions occurred at a lower frequency in the control crosses (1.16±0.82% in A4xA4 to 2.98±0.19% in B3xB3) than in the experimental crosses (15.19±7.22% in A5x330 to 48.78±7.98% in SL1xC2).

2.5.2 Nuclear exchange between crossed strains

Between 90 to 100% of the spores collected in the middle compartment and tested using molecular methods had the genetic identify of a single parent strain (Figure 3). As such, the exchange of nuclei between different strains appears to be a very rare event, occurring in only 2.9% (A4xA5) and 3.2% (A1xC2) of the spores tested for each cross. Notably, whenever novel genetic combinations were identified in spores using ddPCR, these resulted from only few droplets (2-3) identified from one parental strain (the donor), while the remaining droplets have the identity of the other parental strain (the recipient).

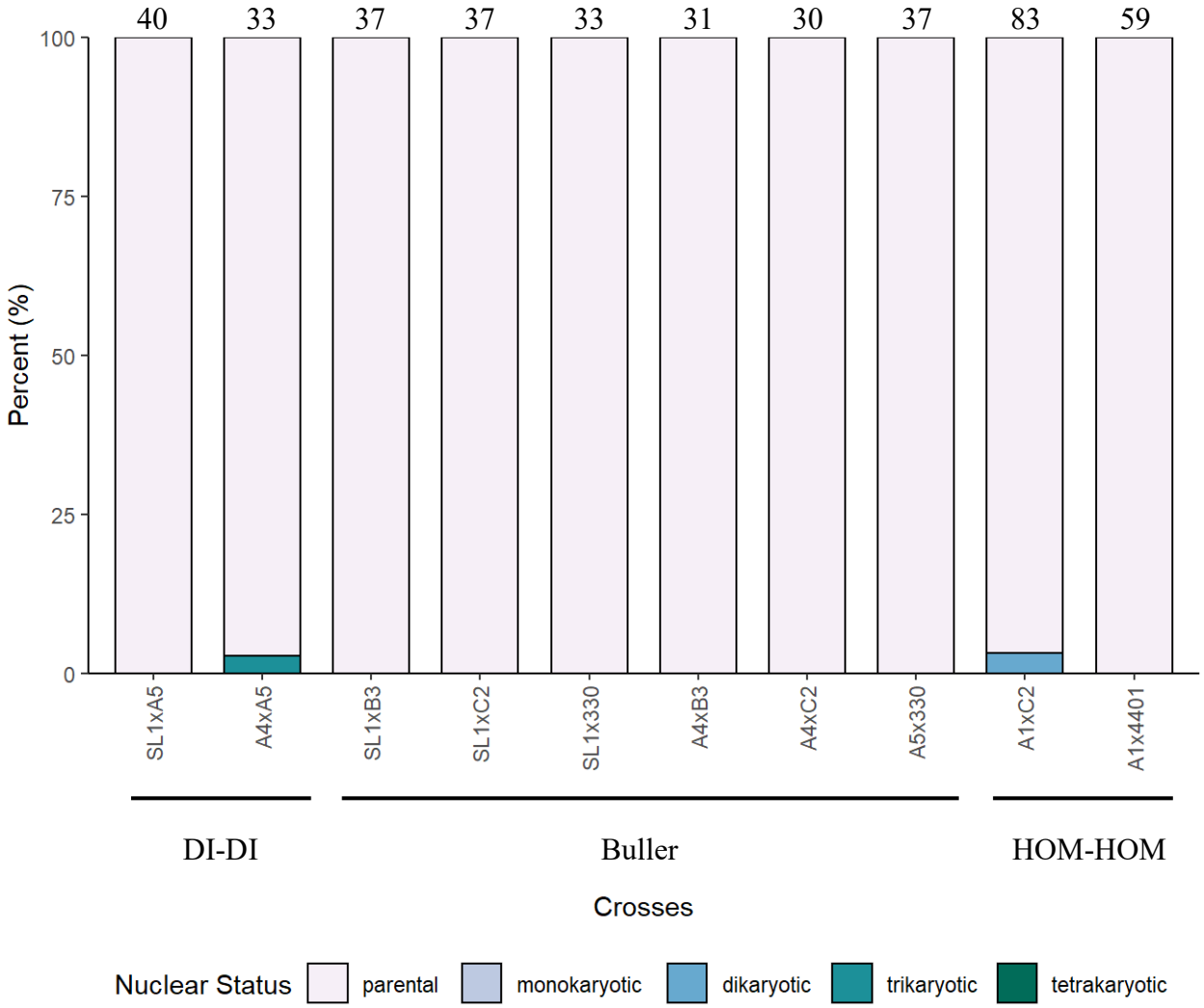


Figure 3. Percentage of spores from each cross displaying each nuclear status based on droplet fluorescence from ddPCR analysis. Nuclear status refers to the identity of the nuclei in the spore, whether they are the same as the parental strains (parental), or a mixture between the parental strains, and the number of different nucleotypes detected within the spore (monokaryotic, dikaryotic, trikaryotic, tetrakaryotic). The number of spores analysed from each cross is displayed above each bar.

In the DI-DI crosses, evidence of genetic exchange was only seen in the A4xA5 cross. Specifically, ddPCR showed evidence of spores carrying three distinct MAT-loci, indicating the production of a trikaryotic spore. For this spore, most of the droplets belonged to the A5 parental strain (droplets had either MAT-3 or MAT-6 identities), while only 3 droplets from the sample had the MAT-2 identity from the A4 strain. No droplets were identified as MAT-1 in these spores, the other possible MAT identity from the A4 strain. In the SL1xA5 cross, all of the droplets had the same MAT identity as the A5 strain (Supplemental Figure 3).

In the Buller crosses, all spores tested using ddPCR maintained the nuclear identity of the parental strains, that is, no evidence of dikaryotic spores carrying new combinations of parental strains, as is expected in the Buller phenomenon, were found. Apart from the A5xC2 cross, in the Buller crosses, all spores tested from the crosses had the genetic identity of the homokaryon strains, rather than the dikaryon strains used in each cross. In the A5xC2 cross, the identity of the spores was the same as the A5 strain.

In the HOM-HOM crosses, dikaryotic spores were formed, that is, spores contained the MAT-loci of both crossed parents. Only the A1xC2 cross produced spores with both MAT-6 (C2) and MAT-3 (A1) genetic identities. In the A1x4401 cross, all spores sampled had the 4401 genetic identity. In all cases, no evidence of sample contamination was observed (negative controls remained negatives).

2.5.3 Nuclear identity of single spore cultures

Following detection of limited nuclear transfer between the crosses of the homokaryotic strains, 600 spores were isolated between the three homokaryon-homokaryon crosses and used to start single spore cultures. Of those spores, 466 germinated, however only one of the resulting

single spore cultures matured to sporulation. Spores analyzed for their nuclear identity from this plate had only the MAT-3 nucleotype. As such, no new dikaryotic culture was produced.

2.6 Discussion

2.6.1 Hyphal interactions

Overall, this work shows that AMF can undergo anastomoses between genetically distinct homokaryons, but also between dikaryons, and between homokaryons and dikaryons, with strains of the same identity (such as the control crosses) forming perfect hyphal fusions more frequently than genetically different strains. Non-self-fusions between strains of the same identity were observed to occur at a frequency of 18-39%, values similar to those reported by Purin and Morton (2011) but lower than those published by Croll et al. (2009) and Pepe et al. 2016) found between crosses of the same strain. The number of incompatible hyphal fusions between strains of the same identity, however, were much higher than those seen in other experiments, ranging from 0.17-11.53% compared to the 0% observed between some strains of *R. irregularis* (Croll et al., 2009). Here, we also observe a large difference between the frequency of fusions occurring between the crossed strains, and the frequency of fusions within a strain. While non-self-fusions ranged from 0.33-39.72%, self-fusions were observed at a frequency of 35.69-85.45%. These values are in agreement to the 4.5-87.5% (Voets et al., 2006) of perfect fusions found within a hyphal network.

These differences in anastomosis frequency between observed and reported values may be due to a variety of factors, including: the life stage of the cultures, nutrient availability, plant host identity, and the physical environment. Asymbiotic spores were found to have a higher anastomosis frequency (Voets et al., 2006; Purin and Morton, 2013; Pepe et al., 2016), as anastomosis is an important process in the establishment of fungal networks and usually occurs in

early developmental stages between germlings (Glass et al., 2000) in order to increase survival of the spores (Sbrana et al., 2011) through nutrient distribution (Fleißner, 2012). By starting the cultures with mature mycelia, the rate of anastomosis and the probability for the different strains to fuse may be reduced as anastomosis appears to be more limited in mature colonies (Simonin et al., 2012), possibly leading to the reduced frequency of non-self-fusions observed in comparison to studies using newly germinated spores. Additionally, variation may be caused by the host plant used in the growth of symbiotic cultures, as the identity of the host plant in the symbiotic association has also been found to affect anastomosis frequency (Pepe et al., 2016). The added space for the mycelia to grow in the media, compared to the growth only along the surface of cellophane, to allow for the co-culturing of the mycelia with the plant host, provides the hyphae with a larger area to explore and thus hyphae may come into contact less frequently, leading to fewer anastomoses (Mosse, 1959; Voets et al., 2006).

As no significant differences were found between the crosses using different strains, and only significant differences between crosses using the same strains versus those using different strains were found, it appears that anastomosis is largely dependent on the similarity between the strains. Compared to the self-anastomosing ability of the strains, the experimental crosses showed a reduced anastomosis frequency. For example, Figure 1 (A) shows the frequency of anastomoses between the strain C2, whereas hyphal contact points between different strains occurred more rarely. Other studies show that strains that are more genetically similar have a higher anastomosis frequency compared to those that diverge (Giovannetti et al., 2004; Croll et al., 2009; Sbrana et al., 2018). This research supports these findings as anastomosis frequency was higher in the control crosses, between strains of the same identity, than the experimental crossed strains with different genetic identities. For example, the crosses with A4xB3 and A4xC2, which have particularly low

rates of anastomosis, are very genetically divergent, based on a new phylogeny (Yildirir et al., unpublished), and this may account for the low anastomosis frequencies observed between these strains.

The SL1xA4 cross, in which both strains share nucleotypes with the MAT-1 locus, does not anastomose more or less frequently than the other crosses, contrary to our prediction that strains sharing a MAT-locus would anastomose more frequently. As these strains are phylogenetically distinct (Yildirir et al., unpublished) and were initially isolated from geographically different locations, these differences are likely to play a role in why these strains do not anastomose more frequently. Occasionally, strains of the same genetic identity that were isolated from different geographic locations have been found to produce incompatible interactions (Giovannetti et al., 2003; Cárdenas-Flores et al., 2010; Sbrana et al., 2018).

Hyphal fusion between genetically different species and strains is generally prevented, to limit virus transmission or exploitation by aggressive genotypes (Glass et al., 2000). In Ascomycota, *vic* or *het* loci govern the compatibility between strains, favouring anastomosis between strains that have the same *vic*/*het* loci (Glass and Kuldau, 1992; Glass et al., 2000; Glass et al., 2004; Fleißner, 2012). Therefore, more similar strains should have a higher anastomosis frequency, as observed in the control crosses, in which the potential *vic*/*het* loci would not differ, because they are identical. Although the genes controlling anastomosis have not yet been identified in AMF, incompatibility loci can be the same as the MAT-loci, and the potential role of incompatibility loci on anastomosis frequency in AMF should be investigated further.

2.6.2 Nuclear transfer

Overall, limited evidence of nuclear transfer between the genetically different strains was observed. Although movement of nuclei was observed in the hyphal bridges during anastomosis between the strains, and cohesive protoplasmic flow throughout the mycelium would dictate the transportation of nuclei to all parts of the mycelium, ddPCR data did not support the presence of overwhelming nuclear transfer in the crosses. When such evidence was found, only 2-3 nuclei of the average 30.6 to 174.2 nuclei per spore were found to originate from the "donating" strain, while the majority of the nuclei belonged to the "receiving" strain. Notably, spores surrounding the anastomosis sites were generally much smaller than those growing close to the root symbiont, and therefore nuclear counts were lower than the average of 200 nuclei per spore reported for some of these strains by Kokkoris et al. (2021). The rarity of spores present close to anastomoses sites, combined with the low germination rates of spores isolated from the crosses for single spore cultures, may explain why none of the new single spore cultures produced using such spores contained the genetic material of crossed strains. More generally, the evidence of nuclear transfer we found between strains indicates that nuclear transfer between strains occurs rarely in AMF, at least with the current experimental setup and strains we used in this study.

Previous anastomosis studies observed the transfer of genetic elements between strains that were detected in the spores following anastomosis, and that were maintained in the subsequent generation following single spore isolation and growth (Croll et al., 2009). Here, the limited nuclear transfer correlates to the low anastomosis frequencies observed, which may limit nuclear movement between the strains. As a result of the low number of spores found to contain a nuclear identity corresponding to that of a mixture between the parental strains, the success of the Buller phenomenon or DI-DI interactions in AMF cannot be conclusively supported. As AMF contain

thousands of nuclei that constantly move bidirectionally throughout the mycelium (Bago et al., 1999; Jany and Pawlowska, 2010), and nuclear movement into the spores occurs through mass nuclear migration (Marleau et al., 2011), it is predicted that successful nuclear migration should lead to a higher proportion of spores found with an altered nuclear identity. This view is not supported by our work, as most spores isolated from crosses had the same nuclear identity as one or the other of the crossed parental strains. As such, there seem to be mechanisms restricting the free movement of nuclei between strains, even when physical barriers such as septal plugging are absent following anastomosis, that was clearly identified in this experiment. Alternatively, following anastomoses the newly passed on nuclei are eliminated from the mycelium, presumably via inter-nuclear competition. Higher genetic similarity between the parental haplotypes, or genetic complementarity may increase the nuclear movement between strains by reducing the vegetative incompatibility.

The presence of meiosis specific genes (Halary et al., 2011; Ropars et al., 2016), internuclear combination in dikaryotic strains (Chen et al., 2018a), and the large number of transposable elements found in AMF strains (Chen et al., 2018b) all provide support for how this anciently asexual lineage has diversified and persisted through time. As these fungi have not been observed to form sexual structures, nuclear transfer through vegetative hyphal fusion may contribute to the evidence of a cryptic or parasexual cycle occurring in AMF (Yildirir et al., 2020).

2.7 Conclusion

While this study demonstrates limited evidence of nuclear transfer between genetically different strains of *R. irregularis*, it provides a baseline of anastomosis frequencies between strains that are frequently used in the field, when cultured on M-media. Nuclear transfer between strains

should further be investigated through the tracking of nuclear movement to determine the fate of individual nuclei throughout the mycelium. Additionally, the effect of different environmental conditions, particularly stressful environments such as low nutrients, should be assessed to determine if these changes can trigger an increase in anastomosis frequency and nuclear transfer between strains.

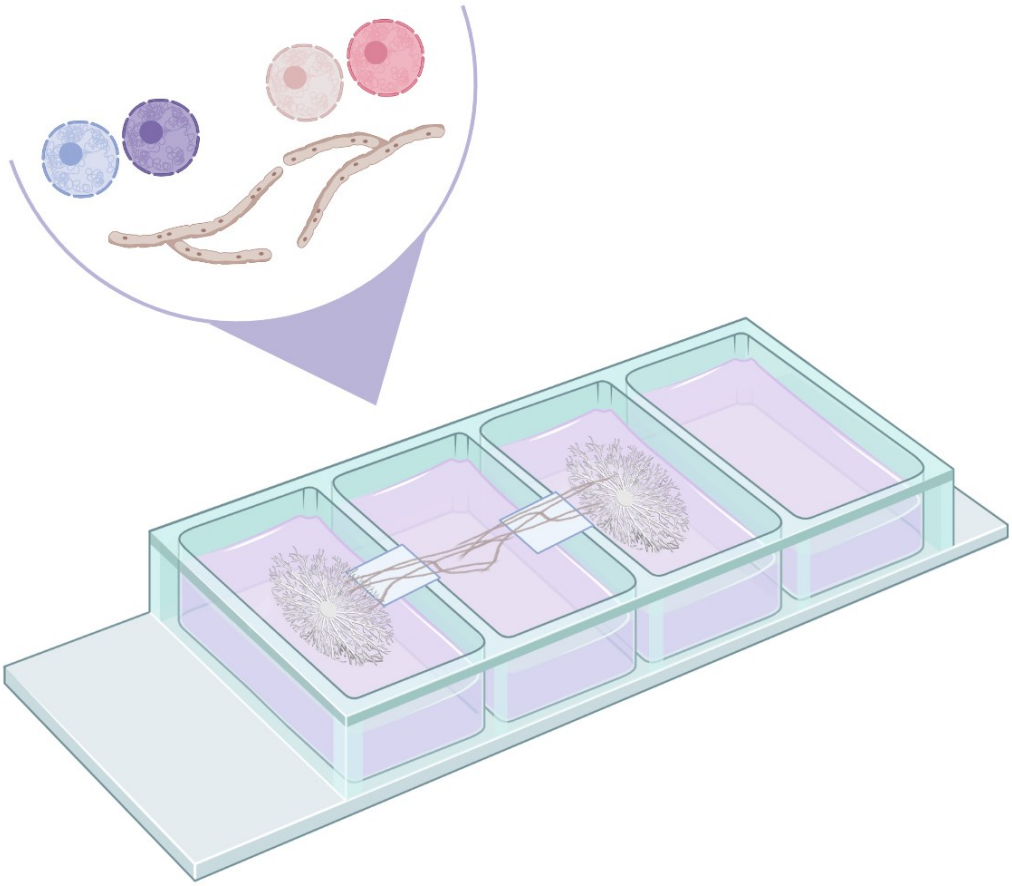
Supplementary Figures and Tables

Supplemental Table 1. Table of crosses created between strains of *Rhizophagus irregularis*. DI-DI and DI-HOM (Buller) crosses were used to determine both hyphal contact frequencies and spore analysis. Control crosses used only to determine anastomosis frequencies. HOM-HOM crosses used only in spore analysis. N = 6 for each of the HOM-HOM, DI-HOM, and DI-DI crosses. N = 3 for the control crosses.

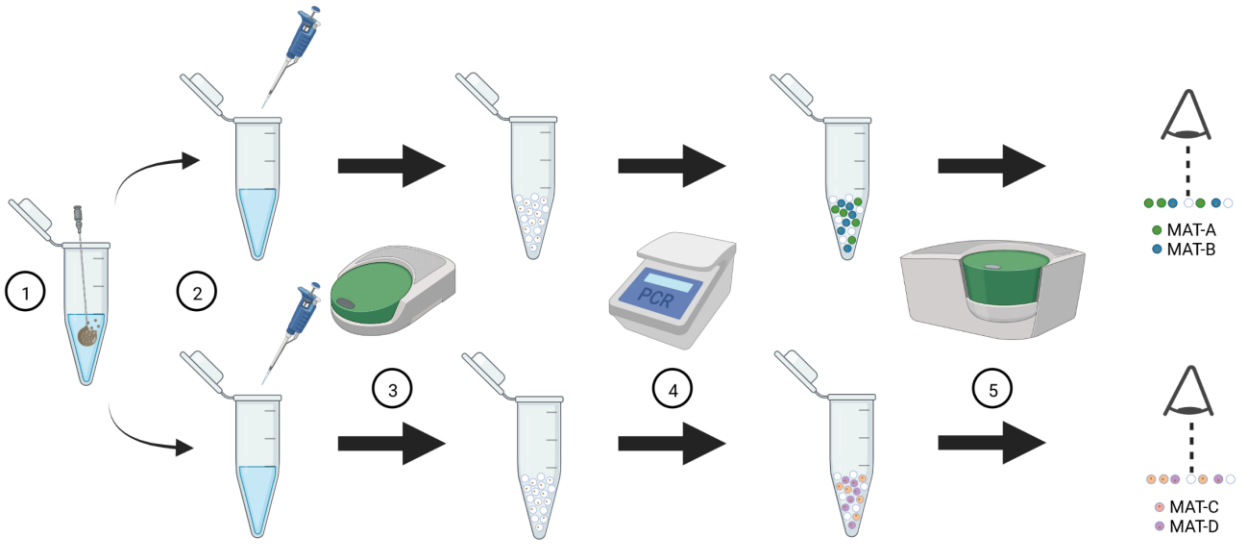
Experiment	Strain A	MAT	Strain B	MAT
Control	B3	MAT-3	B3	MAT-3
	C2	MAT-6	C2	MAT-6
	330	MAT-2	330	MAT-2
	SL1	MAT-1 & MAT-5	SL1	MAT-1 & MAT-5
	A4	MAT-1 & MAT-2	A4	MAT-1 & MAT-2
	A5	MAT-3 & MAT-6	A5	MAT-3 & MAT-6
HOM-HOM crosses	A1	MAT-3	C2	MAT-6
	A1	MAT-3	4401	MAT-6
	B3	MAT-3	C2	MAT-6
DI-HOM crosses	SL1	MAT-1 & MAT-5	B3	MAT-3
	SL1	MAT-1 & MAT-5	C2	MAT-6
	SL1	MAT-1 & MAT-5	330	MAT-2
	A4	MAT-1 & MAT-2	B3	MAT-3
	A4	MAT-1 & MAT-2	C2	MAT-6
	A5	MAT-3 & MAT-6	330	MAT-2
DI-DI crosses	SL1	MAT-1 & MAT-5	A4	MAT-1 & MAT-2
	SL1	MAT-1 & MAT-5	A5	MAT-3 & MAT-6
	A4	MAT-1 & MAT-2	A5	MAT-3 & MAT-6

Supplemental Table 2. Table of primers and probes used for ddPCR spore analysis between crosses of *Rhizophagus irregularis*. Primers and probes for MAT-1 to 3 are the same as those used by (Kokkoris et al., 2021). Sequences are based on the HD1-like (MAT-1,2,5) and HD2 (MAT-3,6) sequences of the corresponding strains.

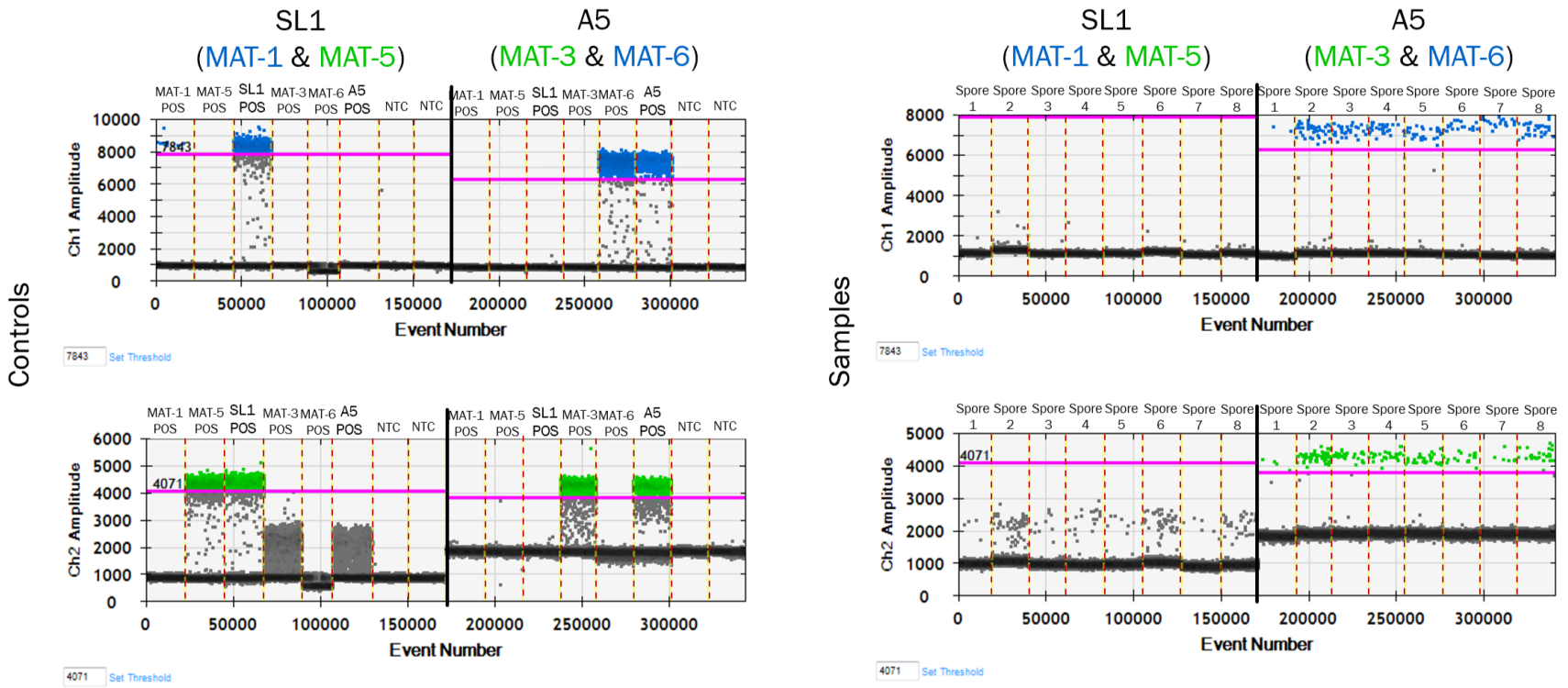
MAT type	Sequences (5' – 3')	
MAT-1	Forward	CAT CAA CAA GTC AAC GAT TTA T
	Reverse	GTG GAT ACA TGA CAT GGT GT
	Probe	CAG AAA CAT TTA ATA ATA ATA ATA CAC GTT
MAT-2	Forward	TAC ACA ACA AGT CAA CGA TG
	Reverse	CAT GAT GCT CAA TAT TAA GTG
	Probe	GTA ATG AAA TTA TAG AAG GAA ATA TTA G
MAT-3	Forward	CGT CAA AGA ATC ACG ACA CTC
	Reverse	CAT TAT TCA CAA TTG CGT TCG G
	Probe	CAT ATA AGA AAC AAA AAT GCC TTA ATT CAA G
MAT-5	Forward	ATA TGA TTG TGA TTA TTT CTT CG
	Reverse	TTC TGA CGA ATA TTT TTG GAC
	Probe	AGG GTT ACC ATA TTC CTC TGC CAG T
MAT-6	Forward	CAA ACC AAA CAT TGA TAA TAC C
	Reverse	CTA ACT TTT TCA ATC TCT TTG C
	Probe	CGA TAA AGA CTT GAG AAT CGA AAT AC



Supplemental Figure 1. Multicompartment experimental plate design. Compartments 1-3 were filled with autoclaved M media. Compartments 1 and 3 contained sucrose (+) M media to promote growth of the fungal and carrot co-inoculants. Compartment 2 contained sucrose (-) M media to prevent the growth of the plant symbiont into the middle chamber. Compartment 4 was left empty. *Rhizophagus irregularis* strains were placed into compartments 1 and 3 along with *Daucus carota* cv. P68 agrobacterium root-inducing (Ri) T-DNA transformed root cultures. Autoclaved filter paper bridges were placed between the compartments to allow hyphal movement into the middle compartment. Here, hyphae can interact and anastomose to exchange nuclei. Created with biorender.com



Supplemental Figure 2. ddPCR methods to detect spore nuclear identity following anastomosis. After isolation of spores in the surrounding interaction site, individual spores were placed into PCR tubes with 4.8 µL of autoclaved dH_2O and crushed using a sterilized needle (1). For the Buller crosses and the DI-DI crosses, the sample was split into two reaction tubes holding equal volume (2.4 µL). To these reaction tubes, a master-mix containing primers and probes matched to the nuclear identity of the parental strains was added: MAT-A and MAT-B matching to parental strain A were added to reaction tube 1, MAT-C and MAT-D matching to parental strain B were added to reaction tube 2 (2). Samples were encapsulated in approximately 20,000 oil droplets using the droplet generator machine (3). Droplets are assumed to contain either one nucleus or no nuclei. Samples were PCR amplified using a thermocycler (4). Droplets were individually read through the droplet reader for the presence or absence of the target sequence (5). Positive droplets contain fluorescently labelled target nuclei that will fluoresce at a higher amplitude than the negative droplets that do not contain the target. Created with biorender.com



Supplemental Figure 3. ddPCR reader output following nuclear analysis of spores following a dikaryon-dikaryon interaction. Positive droplets are coloured in either blue or green, matching to the fluorophore attached to the MAT-specific probe. Pink lines are manual thresholds set to distinguish between positive and negative droplets. Thresholds were set just under the positive "cloud" of droplets for the control samples. (A) shows control samples using extracted DNA of all MAT used in the reaction, to test against cross-amplification of the primers with the target DNA. On the left, samples contained probes amplifying MAT-1 and MAT-5 DNA, while on the right, samples contained probes amplifying MAT-3 and MAT-6 DNA. In both cases, the primers only amplify the matching DNA, without amplifying the other DNA that could possibly be found in that sample. (B) shows the amplification of MAT present in each spore tested. Here, spores with the same number are matched to identify both MAT-1 and MAT-5 on the left, and MAT-3 and MAT-6 DNA on the right. As only the DNA matching to the A5 strain is present in these spore samples, the spores must therefore be of A5 origin, with no nuclear transfer from the SL1 strain detected.

Supplemental Table 3. Hyphal interactions detected within the extraradical mycelial networks between strains of Rhizophagus irregularis co-cultured with P86 carrot root.

Experiment	Cross	Mean number of hyphal contacts (±SD)	Non-self-fusions (%±SD)	Incompatible non-self-fusions (%±SD)	Self-fusions (%±SD)	Incompatible self-fusions (%±SD)
DI-DI	SL1xA4	578.00±27.90 abd	1.26±0.17 abd	4.89±0.51 abd	48.10±3.64 abc	45.74±3.48 bc
	SL1xA5	975.00±338.44 abcd	1.68±0.05 abcd	2.28±0.42 abcd	60.72±2.44 abc	35.32±2.78 abc
	A4xA5	365.33±53.90 abd	1.21±0.22 abd	2.83±0.41 abd	35.69±2.83 b	60.26±2.83 c
DI-HOM	SL1xB3	490.67±51.55 abcd	3.30±1.81 abcd	2.36±1.16 abcd	78.27±9.61 ac	16.05±6.68 abc
	SL1xC2	810.67±193.75 abcd	4.70±0.23 abcd	6.62±0.84 abcd	39.91±8.99 b	48.78±7.98 bc
	SL1x330	318.33±49.47 abd	1.39±0.23 abd	1.15±0.51 abd	67.24±10.84 abc	30.22±10.63 abc
	A4xB3	195.00±13.64 b	0.34±0.24 b	0.17±0.25 b	62.52±3.27 abc	36.96±3.28 abc
	A4xC2	473.33±10.34 bd	0.92±0.10 bd	2.32±0.31 bd	51.40±0.55 abc	45.37±0.63 bc
	A5x330	125.67±20.07 abcd	2.46±0.43 abcd	1.70±1.54 abcd	80.65±6.42 ac	15.19±7.22 abc
CONTROL	A4xA4	334.00±57.80 ac	20.41±8.33 ac	9.37±4.32 ac	69.06±12.98 abc	1.16±0.82 a
	SL1xSL1	577.00±67.45 ac	25.34±4.69 ac	6.78±0.96 ac	65.50±6.25 abc	2.37±1.76 ab
	A5xA5	474.33±29.91 ac	24.22±3.04 abd	6.40±3.76 ac	67.11±5.06 abc	2.27±1.04 c
	B3xB3	302.67±27.35 acd	21.24±0.32 acd	5.91±2.09 acd	69.87±2.22 abc	2.98±0.19 abc
	C2xC2	629.67±74.59 c	39.72±5.07 c	8.15±3.12 c	50.74±8.01 abc	1.39±0.13 a
	330x330	348.33±46.02 abcd	18.13±1.17 abcd	11.53±6.07 abcd	68.17±6.07 abc	2.17±0.39 ab

Values represent means of three replicates (±SD)

Values within the same column followed by a different letter denote significance ($p<0.05$; Dunn's test)

CHAPTER THREE

Discussion

3.1 Summary of Findings

3.1.1 Anastomosis frequency

Anastomosis frequencies between strains was evaluated in the first part of the experiment to determine which crosses formed compatible interactions. I used multicompartment petri dishes to create crosses both between different strains and the same strains. Overall, anastomoses between hyphae of different strains occurred less frequently than those between the same strain. These results build on observations between strains of *R. irregularis* from other studies (Croll et al., 2009) to gather a more complete picture of the interactions possible. By looking at the frequency of anastomoses occurring within a culture, as opposed to those occurring between cultures in a cross, I observed that the hyphae preferred to anastomose with other hyphae belonging to its own mycelial body rather than those belonging to another mycelium, even when the hyphae are of the same strain.

3.1.2 Nuclear transfer between strains

In the second part of the experiment, I investigated whether the nuclei observed in the hyphal bridges following anastomosis were found in the spores. By using ddPCR, a method used to accurately detect small amounts of DNA in a sample, I determined the identity of the nuclei found within the spores following anastomosis. Overall, nuclear exchange between strains occurred very rarely, with only two crosses showing exchange of nuclei. Additionally, in crosses where anastomosis is observed, the number of nuclei transferred between the strains was very low in comparison to the total number of nuclei per spore. This suggests that the observations of nuclear exchange reflect a transient stage, and that over time one the less abundant nuclei will likely be removed from the spore nuclear population.

3.2 Future Directions

To determine if the creation of new dikaryons is possible through crossing experiments, future work needs to focus on strains that are phylogenetically similar. Due to the low rates of anastomosis observed between the different strains, some somatic incompatibility mechanism is acting to prevent more frequent fusions. Crosses between more genetically related strains, harbouring very similar genomes, but different MAT-loci, should potentially remove the observed incompatibilities and allow for increased nuclear exchange and detection of spores with more balanced nuclei from both parental strains. As of now, no candidate homokaryons have been identified as the potential parental strains, based on phylogenies produced from parental haplotypes of dikaryotic strains. To this end, future sequencing efforts should now focus on increasing genomic sampling of this model species across natural ecosystems and particularly within populations.

Fungi are also known to undergo different stages of their life cycle in times of abiotic stress (Huang and Hull, 2017). Growing crosses on an altered minimal media, with changes to the sugar content, pH, nutrient availability, may help to induce more frequent anastomosis patterns as hyphae seek to restore deficiencies. Increasing the frequency of anastomosis between the different strains allows more chances for nuclear movement to occur between the strains, and therefore increases the chance that nuclei from both parental strains could be found in the spores. With increased identification of potentially new dikaryotic spores, the chance of successfully germinating and growing dikaryotic cultures from these spores also grows.

Tracking nuclear movement through fluorescence in situ hybridization (FISH) may help to understand what occurs to nuclei during the anastomosis process, and why nuclei that were observed in the hyphal bridges are not then found in the spores. In Ascomycota, fluorescent

markers have been used successfully in the tracking of nuclei between anastomosing hyphae (Roper et al., 2011; Roper et al., 2013), and this method should also be applied to the crosses of AMF strains.

3.3 Importance of Research

AMF play important roles both ecologically and agriculturally, in the uptake of nutrients from the soil, and the mitigation of stressful environmental events (Bonfante and Genre, 2010). In the advent of increasing agriculture demands to feed a growing population (Tilman et al., 2001; Godfray et al., 2010) and climate change, leading to intensification of extreme weather events like droughts and heatwaves (Deng et al., 2022), sustainable farming systems are crucial to limit further environment degradation (Tilman et al., 2001; Schmidhuber and Tubiello, 2007; Godfray et al., 2010; Grillakis, 2019). The use of biofertilizers or biostimulants, which act to increase plant productivity, are a potential aid to limiting environmental damages as a result of agricultural intensification (van der Heijden et al., 2015; Rouphael et al., 2015; Chen et al., 2018c).

The role that AMF play in biogeochemical cycles, particularly the carbon cycle, and the cycling of N and P, make them good candidates for biofertilizers (van der Heijden et al., 2015). As ancient plant symbionts, AMF acted as an early roots for plants, playing an essential role in the colonization of land (Pirozynski and Malloch, 1975), and can consequently now form associations both with multiple plant hosts, as well as being one of many possible simultaneous symbionts associated with a plant host (Giovannetti et al., 2004; Jansa et al., 2008; van der Heijden and Horton, 2009; Bahram et al., 2011; Kivlin et al., 2011). Not only are AMF efficient at nutrient uptake from the soil (Bender and van der Heijden, 2015), but through common mycorrhizal networks (CMN) they are able to transport nutrients throughout the connected mycelia

(Giovannetti et al., 2015). The lack of specificity for plant hosts, in addition to the ability of AMF to anastomose, suggests that the formation of CMN in nature is a frequent occurrence.

The creation of CMN's between plants are thought to be a fitness equalizing technique, as resources are shared between connected plants (van der Heijden and Horton, 2009; Bever et al., 2010). As such, CMN provide a large benefit to new seedlings, which are rapidly colonized by AMF and have direct access to the nutrients within the network, generally allowing for increased survival and fitness of the seedling (van der Heijden and Horton, 2009). While findings of the significance of carbon sharing occurring in the CMN between photosynthetic plants are controversial (Simard and Durall, 2004; Giovannetti et al., 2015), the transfer of nutrients like N, P, K, and Zn has been confirmed (Jansa et al., 2003; Jansa et al., 2008; Meding and Zasoski, 2008; Cardini et al., 2021). In the current agricultural process, N and P inputs through fertilizers are often not synchronized to plant demands, leading to the leaching of these nutrients, causing eutrophication in aquatic environments (Matson et al., 1997; Carpenter et al., 1998). The uptake of these excess nutrients by AMF, and their distribution or storage through the CMN has been demonstrated as a method to reduce leaching into the environment (Cavagnaro et al., 2015; van der Heijden et al., 2015).

Plants have also been found to communicate through the CMN, sending defense signal molecules from infected plants to healthy plants, eliciting defense responses in the healthy plant by increasing the expression of defense-related genes (Song et al., 2010; Song et al., 2014). As some mycorrhizal networks can reach an extension of around 10 m from the plant host (Rosendahl and Stukenbrock, 2004), this mechanism of communication can potentially reach plants farther from the infected plant than root exudates, which would not diffuse as far in the soil.

Although AMF networks tend to be disturbed by agricultural tilling, leading to a lower diversity of AMF species (Helgason et al., 1998), *R. irregularis* is more resilient to these disturbances (Avio et al., 2013). Its ability to associate with many plants in an ecosystem (van der Heijden et al., 2015), and ability to vary interactions based on plant host (Kokkoris et al., 2021), make the hyphal interactions that could happen between the strains of this AMF species of interest to researchers. Further investigations into the anastomoses that form between different strains of *R. irregularis* are necessary to understand how a CMN might form between these strains and their host plants. The understanding of these interactions can lead to the formation of better biofertilizers.

3.4 Concluding Remarks

For a long time, the heterokaryosis hypothesis of AMF was thought to explain how these ancient asexual organisms were able to survive for over 400 million years without undergoing sexual recombination. In this hypothesis, it was thought that the co-existing nuclei are genetically highly diverging, and such nuclear diversity would compensate for the various mutations acquired over time to the genome (Sanders, 1999; Hijri and Sanders, 2005; Sanders and Croll, 2010). However, recent evidence of the presence of meiosis-specific genes (Halary et al., 2011; Ropars et al., 2016), distinct homokaryotic and dikaryotic strains (Ropars et al., 2016), and inter-nuclear recombination between the nucleotypes in dikaryotic strains (Chen et al., 2018a; Chen et al., 2020) have challenged this view, suggesting instead that a parasexual, or cryptic sexual life cycle could be occurring in these organisms to generate diversity (Yildirir et al., 2020). This model follows known life stages of Basidiomycota, leading to the hypothesis that dikaryotic strains of AMF arose

through the compatibility of two parental homokaryotic strains (Corradi and Lildhar, 2012; Ropars et al., 2016).

In this thesis, I explored whether crosses between strains of *Rhizophagus irregularis* would lead to anastomosis and the transfer of nuclei, and if these potential nuclear transfers could be detected in the spores. The frequency of anastomosis between the strains explored here are a good steppingstone to further exploration of somatic compatibility between strains of AMF and determine what drives the formation of dikaryosis in AMF within mycorrhizal networks.

Much work is still needed to uncover the origin of the dikaryotic strains of *R. irregularis*, and the role that genetic similarity of strains plays in the somatic compatibility and nuclear transfer between strains. This thesis builds on the existing evidence of anastomosis occurring between strains of *R. irregularis* and shows evidence of compatible interactions between homokaryon-dikaryon and dikaryon-dikaryon strains for the first time. The observations of these interactions help in deciphering the frequency of CMN in the soil, and between which strains they may form. Although limited nuclear transfer was observed between the strains tested in this study, further experimentation with crosses should take place to determine the role of somatic compatibility on the creation of AMF dikaryons.

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