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Review

Compatibility and incompatibility in hyphal anastomosis of arbuscular mycorrhizal fungi

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ABSTRACT: Arbuscular mycorrhizal fungi (AMF), which live in symbiosis with 80 % of plants, are not able to grow when separated from their hosts. Spore germination is not host-regulated and germling growth is shortly arrested in the absence of host roots. Germling survival chances may be increased by hyphal fusions (anastomoses), which allow access to nutrients flowing in the extraradical mycelium (ERM). Perfect anastomoses, occurring with high frequency among germlings and the ERM of the same isolate, show protoplasm continuity and disappearance of hyphal walls. A low frequency of perfect fusions has been detected among co-specific genetically different isolates, although fungal nuclei have been consistently detected in all perfect fusions, suggesting active nuclear migration. When plants of different taxa establish symbioses with the same AMF species, anastomoses between ERM spreading from single root systems establish a common mycelium, which is an essential element to plant nutrition and communication. The interaction among mycelia produced by different isolates may also lead to pre-fusion incompatibility which hinders anastomosis formation, or to incompatibility after fusion, which separates the hyphal compartments. Results reported here, obtained by analyses of hyphal compatibility/incompatibility in AMF, suggest that anastomosis formation and establishment of protoplasm flow, fundamental to the maintenance of mycelial physiological and genetic continuity, may affect the fitness of these ecologically important biotrophic fungi.

Keywords: *Glomeromycota*, hyphal fusions, incompatible hyphal interactions, protoplasmic flow, nuclear migration

Introduction

Most plant species form root symbioses with arbuscular mycorrhizal (AM) fungi (AMF), an ecologically and economically important group of soil microbes fundamental to soil fertility and plant nutrition (Smith and Read, 2008), belonging to *Glomeromycota*. AMF are obligately biotrophic fungi which obtain sugars from their hosts, in exchange for soil mineral nutrients, absorbed and transferred by the extraradical mycelium (ERM), a three-dimensional structure growing indefinitely in the soil. As AMF have a low host specificity, ERM may interconnect different host plants, and distribute soil nutrient resources within plant communities (Giovannetti et al., 2004; Mikkelsen et al., 2008).

Both asymbiotic and symbiotic AMF mycelia show a highly branched and interconnected structure, produced by means of fusions (anastomoses) between hyphae which, coming into contact, can connect and share cytoplasm and nuclei (Giovannetti et al., 1999; 2001) (Figure 1A and B). Anastomosis is an important cellular process in fungi, entailing different essential functions: sexual fusion, protoplasmic flow and genetic material exchange, nutrient flow, intra-hyphal communication and mycelial homeostasis, exploration of the environment with increased foraging and absorbing ability and recovery of network integrity when physically damaged thereby enhancing mycelial fitness (Carlile, 1995). In AMF, studies on cellular events preceding successful anastomoses opened the way to genetic tests on compatibility/incompatibility, which showed genetic exchange between different genotypes (Croll et al., 2009). Here, we review the different stages of hyphal interactions involved in anastomosis formation, self and non-self recognition and compatible and incompatible responses among hyphae of closely related vs. genetically distant AMF taxa.

Compatible hyphal interactions leading to perfect anastomosis

Asymbiotic mycelium

In AMF, spores germinate and grow under appropriate physical, chemical and microbiological conditions and give rise to a coenocytic mycelium, 8-70 mm long, which stops growing within 20 days of germination (see Giovannetti et al., 2010). Anastomosis between hyphae derived from germinated AMF spores (Figure 1A), hereinafter defined as germlings, was first investigated in *Funneliformis mosseae* (formerly *Glomus mosseae*) *Funneliformis caledonius* (formerly *Glomus caledonium*) and *Rhizoglomus intraradices* (formerly *Glomus intraradices*), using time-lapse microscopic studies (Giovannetti et al., 1999). Anastomoses were observed in the same germling, where perfect fusions were formed at rates of 51-57 % in *F. mosseae*, 34-54 % in *F. caledonius* and 58-68 % in

R. intraradices. Such high interconnectedness levels were made possible by the large number of fusions, 0.6-1.3 cm^{-1} in hyphal length. Additionally, anastomoses widely occurred also between hyphae derived from germlings within the same AMF isolate (34-55 % and 59-90 % in *F. caledonius* and *R. intraradices*, respectively) (Giovannetti et al., 1999). In *F. mosseae* fusions ranged from 40 to 85 %, depending on isolates (Giovannetti et al., 2003). These results had also been confirmed by studies performed on germlings of *Rhizoglomus clarus* and *Rhizoglomus irregulare* (Croll et al., 2009; Cardenas-Flores et al., 2010; De la Providencia et al., 2013). Successive experiments increased communal knowledge of the extensive occurrence of perfect fusions in different species of tropical AMF isolates, showing that the frequency of anastomosis differed in hyphal contacts within the same or different germlings, which were 42/73 %, 33/14 %, 75/64 %, 80/91 % and 9/24 %, in isolates of *Acaulospora scrobiculata*, *Acaulospora spinosa*, *Claroideoglomus etunicatum*, *Glomus formosanum* and *Rhizoglomus manihotis*, respectively (Novais et al., 2013). A recent study revealed different anastomosis behavior amongst AMF genotypes in Glomeraceae, such as *F. coronatus* germlings which had a poorly interconnected mycelium (average anastomosis frequency, 4 %) (Pepe et al., 2016). Anastomoses were never observed in *Gigaspora rosea* nor in *Racocetra castanea* germlings (Giovannetti et al., 1999; Novais et al., 2013) (Figure 2).

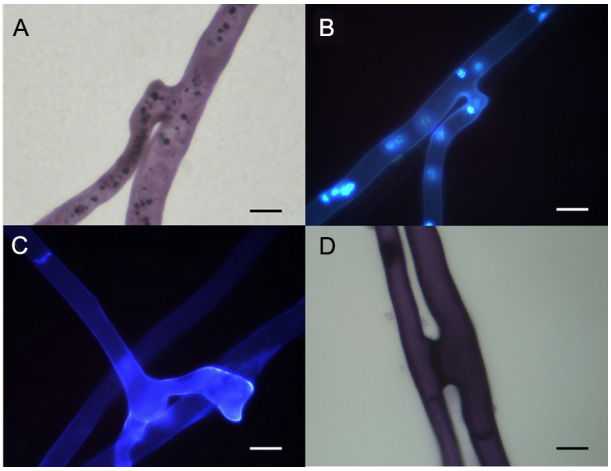

Figure 1 – Light and epifluorescence micrographs of hyphal interactions in arbuscular mycorrhizal fungi. A) perfect anastomosis formed by *Funneliformis mosseae* hyphae, after staining for Succinate dehydrogenase and with Trypan blue; Scale bar = 5 µm. B) perfect anastomosis formed by *F. mosseae* hyphae, showing nuclear mingling after staining with 2,4-diamidinophenylindole; Scale bar = 7 µm. C) pre-fusion incompatibility in hyphae of *Funneliformis coronatus*, showing a septum in a tip-swollen hypha after staining with Calcofluor; Scale bar = 6 µm. D) incompatibility occurring after fusion in *F. coronatus*, showing septa in empty fused hyphae; Scale bar = 6.5 µm.

Perfect anastomoses showed a very high degree of cellular compatibility, encompassing the disappearance of hyphal walls, merging of cytoplasm and nuclei and their migration through fusion bridges, leading to protoplasmic continuity between fused hyphae and integration between different mycelia (Giovannetti et al., 1999, 2003; Croll et al., 2009; Novais et al., 2013; Purin and Morton, 2011, 2013). Such cellular events were detected by histochemical localization of succinate dehydrogenase (SDH) activity in hyphal fusions, while time-lapse light microscopy allowed for the monitoring of anastomosis formation, which was achieved in 35 min, and the visualization of protoplasm streaming through fusion bridges, where a mass of cellular particles (possibly nuclei, mitochondria, granules, vacuoles) migrated at the rate of 1.8-2.6 µm s^{-1}. The compatibility between fusing hyphae was supported by evidence of nuclei and associated cytoplasmic microtubules through fusion bridges obtained using 2,4-diamidinophenylindole (DAPI) staining, epifluorescence and immunofluorescence microscopy (Ästrom et al., 1994). Such events of nuclear mingling, originating also from different germlings, indicate that anastomoses allow for the formation of a physiologically and genetically interconnected mycelial network (Figure 1B). Indeed, Croll et al. (2009) demonstrated that anastomoses allowed for genetic exchange between different genotypes and the transfer of genetic markers to the progeny, while Jany and Pawlowska (2010) detected the intermingling of different nuclear lineages within coenocytic hyphae of *C. etunicatum*. Such data were supported by the findings of nuclear and mitochondrial DNA diversity and by recent evidence of mitochondrial gene horizontal transfer and recombination events in *Rhizoglomus* sp. isolates (Bever and Wang, 2005; Raab et al., 2005; Börstler et al., 2008; Beaudet et al., 2013). Actually it has long been known that in fungi hyphal fusions may give rise to heterokaryons during the parasexual cycle, which have not yet been detected in AMF (Pontecorvo,

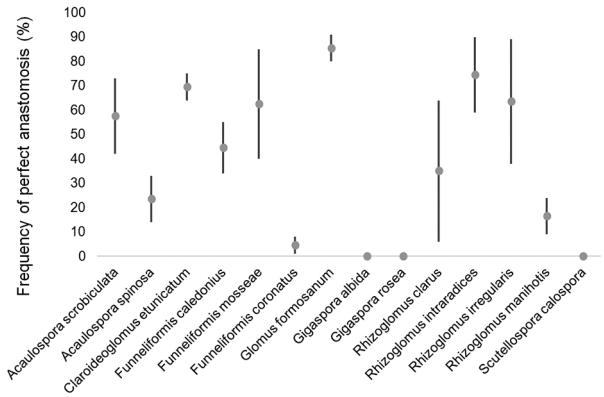

Figure 2 – Graphic summary of bibliographic data reporting frequencies of hyphal contacts resulting in perfect hyphal anastomoses, in spore germlings (asymbiotic stage) of different arbuscular mycorrhizal fungal taxa.

1956). A large body of molecular studies revealed different patterns of DNA heterogeneity and allowed for the reconstruction of phylogenetic relationships in Glomeromycotan species (Souza et al., 2004, 2005), though the detection of recombination events within single isolates entails multilocus molecular analyses and genomic sequencing in its entirety. Recently, *R. irregulare* transcriptome revealed transcripts putatively involved in meiotic recombination and meiosis-specific proteins (Tisserant et al., 2012), while 51 genes similar to *Saccharomyces cerevisiae* meiosis genes were described in *Glomus* spp. (Halary et al., 2011), suggesting that sexual reproduction in AMF could be possible. A putative multi-allelic mating-type locus, containing genes with structural and evolutionary similarity with mating-type loci of other taxa, was found in the genomes of five isolates of *R. irregulare* (Ropars et al., 2016). Additional studies should be carried out in order to investigate whether anastomosis between different AMF genotypes may prelude parasexual cycle or sexual recombination, and thus affect their reproductive success.

A number of authors have detected chemotropism and growth re-orientation before hyphal fusion in *A. scrobiculata*, *F. mosseae*, *F. caledonius*, *G. formosanum*, *C. etunicatum* and *R. manihotis*, (Giovannetti and Sbrana, 2001; Novais et al., 2013), a phenomenon also described in fungi other than AMF, such as *Phanerochaete velutina* and *Stereum* spp. (Ainsworth and Rayner, 1989) and *Neurospora crassa* (Hickey et al., 2002), suggesting that specific recognition signals may be able to modulate anastomosis formation. Nevertheless, information is still lacking on the cytology and biochemistry of the successive developmental steps leading to perfect anastomoses, including cell wall fusion. Further studies should investigate whether specific chemical signals may be active in the control of events preceding contact, such as hyphal fusion competence and positive tropism (Hickey et al., 2002).

Symbiotic mycelium

Symbiotic mycelium or ERM is the fungal structure wherein mineral nutrients are translocated from soil to plants and sugars transferred from plants to the fungal colony. Thus, a high level of interconnectedness between its hyphae is fundamental to its functional efficiency. Anastomosis occurrence was first visualized in intact ERM formed by *F. mosseae*, using an in vivo model system, which also allowed for the quantification of its growth rate, 738-1067 mm d^{-1} (Giovannetti et al., 2001). In such a system the frequency of fusions was 67-77 %, while the number of fusions along hyphae was very high, 0.46-0.51 mm^{-1}. Histochemical localization of SDH activity detected protoplasmic continuity among fusing hyphae, which were 100 % viable, and nuclear migration in anastomosing bridges (Giovannetti et al., 2001). A recent study revealed that AMF genotypes in Glomeraceae differ significantly in perfect anastomosis formation, suggesting fungal symbiont modulation of ERM interconnectedness, as one isolate of *F. coronatus* consistently showed anastomosis frequencies lower than 8 %, when colonising different host plants (Pepe et al., 2016).

The same experimental system provided evidence of anastomosis formation among *F. mosseae* ERM originating from plant roots of different taxa growing nearby. Their frequencies varied according to the different pairings, and ranged from 44 % to 62 % in different combinations between *Allium porrum* and *Solanum melongena* or *Gossypium hirsutum* (Giovannetti et al., 2004). Also in this case perfect anastomoses were confirmed using SDH and DAPI stainings.

An *in vitro* culture approach, using Ri tRNA-transformed root organ cultures (ROC), detected low anastomosis in the ERM of *Glomus hoi*, *Rhizoglomus proliferus*, *R. irregulare* and *R. intraradices*, reaching a maximum of 17 m^{-1} of hypha in *R. proliferus* (De la Providencia et al., 2005). Gigasporaceae ERM produced rare anastomoses *in vitro*, confirming previous data obtained in asymbiotic mycelium (Giovannetti et al., 1999). However, 95 % of such intra-hyphal fusions may possibly have represented the result of a healing mechanism (Gerdemann, 1955; De la Providencia et al., 2005).

Further studies are needed in order to understand the relationships between structural ERM traits, such as extent, density and interconnectedness, and the differential fungal foraging ability, functional to efficient nutritional flows in ERM. Indeed, molecular works detected a differential expression of phosphate uptake genes in ERM (Harrison and van Buuren, 1995; Maldonado-Mendoza et al., 2001), showing the structural and functional importance of ERM in phosphate absorption from the soil. A correlation between ERM interconnectedness and plant P content was found in plants of *Medicago sativa* colonised by different AMF, confirming the important functional role of highly interconnected ERM in the efficient exploitation of soil resources and differential performances of diverse AMF (Avio et al., 2006). As many other genes encoding proteins for mineral nutrients transport are expressed in ERM, studies focused on ERM functional significance would reveal possible relationships between the expression of mineral transporters in different AMF genotypes and their differential performance.

Asymbiotic vs. symbiotic mycelium

Recent experimental studies have shown that asymbiotic hyphae are able to fuse with ERM hyphae growing from plants colonized by the same AM fungus, whose frequency was 5-24 % (Sbrana et al., 2011). This phenomenon may affect survival, viability and the reproductive success of such ancestral obligate biotrophic organisms, which, though lacking a host-regulated spore germination, have survived for 980 million years. Indeed, when germling hyphae do not come into contact with the roots of potential host plants, growth is arrested and protoplasm is withdrawn from the tips to the moth-

er spores, in order to save precious energy for further germination (Siqueira and Sylvia, 1985). Such evolutionary weakness is compensated for by the capability of germling mycelium to plug into the appropriate ERM and drain plant-derived sugar resources flowing in the mycelial network.

Incompatible hyphal interactions

Vegetative incompatibility, described in diverse fungal species, occurs when anastomosis formation leads to the intermingling of different nuclear lineages, and involves the death of the relevant common hyphal compartment (Worrall, 1997). Experimental evidence obtained with AMF revealed events of non-self-discrimination leading to incompatibility between AMF hyphae belonging not only to different taxa, but also to co-specific isolates. Such responses occurred either before or after anastomosis.

Pre-fusion incompatibility

Studies performed on hyphal encounters between AMF germlings of different taxa showed no fusions in combinations among isolates belonging to the genera *Funneliformis*, *Gigaspora* and *Racocetra* (Giovannetti et al., 1999).

When different *F. mosseae* isolates were analysed, most hyphal contacts showed no interference and no anastomoses were formed, while incompatibility preceding fusions, detected in certain interacting hyphae, hindered hyphal fusions (Giovannetti et al., 2003). In such pairings, *F. mosseae* isolates IN101, BEG25 and AZ225C showed chemotropism, growth re-orientation and branching in the approaching hyphae, while differentiation of lateral tips were observed in the contacted ones. Simultaneously, after growth arrest, the contacting hyphae underwent a series of incompatible responses, such as tip swelling, cell wall thickening, hyphal protoplasm vacuolization and septa formation, separating live from dead hyphal compartments (Giovannetti et al., 2003) (Figure 1C). Nevertheless, the early recognition signals and clues regulating hyphal attraction, tropism and directional growth involved in compatibility/incompatibility remain to be understood.

Pre-fusion incompatibility was detected also in germlings and ERM encounters (Sbrana et al., 2011), indicating that chemical signals leading to incompatible responses are active when hyphae produced at different stages in the AMF life cycle come into contact.

Post-fusion incompatibility

Incompatible interactions following hyphal fusion were described in Ascomycota, where heterodimers of het (heterokaryon incompatibility) or vic (vegetative incompatibility) proteins (Glass et al., 2004) led to septa formation and protoplasm retraction in anastomosed hyphae.

Post-fusion incompatibility was first detected in AMF during *in vitro* studies, which revealed that *R. irregulare* genetically different clonal lineages could anastomose and exchange specific markers (Croll et al., 2009). The incompatible responses consisting of protoplasm retraction and cross-wall formation in hyphal fusions (Figure 1D), were similar to those described in Ascomycota. Recently, incompatibility following fusion was confirmed in pairings of *R. irregulare* isolates of the Department of Agriculture (Mycology) Ottawa (DAOM) collection (De la Providencia et al., 2013) and during contacts between ERM hyphae and germlings (Sbrana et al., 2011). Differences in gene expression, physiological characteristics and host recognition ability between germling hyphae and ERM, representing highly different developmental phases of AMF, may account for these findings, although determinants of incompatibility are still to be unravelled in AMF. However, such an event does not hinder genetic exchange, since Croll et al. (2009) have shown viable protoplasmic connections allowing for the migration of nuclei even when fusion rates were very low. In such interactions, a peculiar cytological event was observed, namely, the growth of intrahyphal hyphae, also described within damaged hyphae and as double-walled structures in the intraradical mycelium of *Glomus fasciculatum* (Lim et al., 1983).

An *F. coronatus* isolate, forming low-interconnected mycelium, showed within-isolate incompatible reactions following fusions in 1 to 14 % of hyphal interactions, occurring at both the asymbiotic and symbiotic stage (Pepe et al., 2016), whereas different rates were observed at the same developmental stages in *Rhizoglomus clarus* (Purin and Morton, 2013). At the symbiotic stage, self-incompatibility may affect AMF fitness and reduce the development of common ERM, with possible impacts on nutrient resources flow and AMF survival chances in soil.

Final Remarks

Hyphal anastomoses represent key structures for the formation of efficient and interconnected AMF mycorrhizal networks, which are fundamental to AMF survival, to plant/soil nutrient flow and to the maintenance of genetic diversity. Moreover, anastomoses formed between ERM originating from the root system of different host species allows for the formation of wide hyphal networks interconnecting an indefinite number of diverse plants living in the same ecosystem. So far we know almost nothing about the cellular and environmental background allowing two hyphae to anastomose and form an interconnected mycelium. Reported findings suggest a complex hyphal signaling pattern, with common steps and outcomes when hyphal interactions involve similar genotype/fungal life stages (hyphal fusion) or dissimilar genotype/different fungal life stages (hyphal incompatibility). Such variability in AMF anastomosis behaviour suggests the involvement of multiple gene (or multiple allele) variation in determining the degree of compatibility in this group of fungi.

Further studies should be aimed at improving communal knowledge of AMF hyphal recognition systems and of the impact of different anthropogenic and environmental factors, which may possibly interfere with fungal metabolism, on the formation of highly interconnected and functional ERM.

In particular, the impact of compounds of anthropogenic origin which are commonly found in soil - chemical fertilizers, pesticides and heavy metals - on the anastomosing ability of different AMF should be assessed, in order to detect the best performing AMF strains, characterized by functional traits fundamental to the maintenance of interconnected and effective mycorrhizal networks, to be exploited as suitable biofertilizers and bioenhancers in sustainable agriculture and natural ecosystem management.

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