2011

Vegetative compatibility in arbuscular mycorrhizal fungi

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**VEGETATIVE COMPATIBILITY IN
ARBUSCULAR MYCORRHIZAL FUNGI**

Sonia Purin

**Dissertation submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Agricultural Sciences**

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Division of Plant and Soil Sciences

**Morgantown, WV
2011**

Keywords: vegetative compatibility, anastomosis, arbuscular mycorrhizal fungi

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ABSTRACT

VEGETATIVE COMPATIBILITY IN ARBUSCULAR MYCORRHIZAL FUNGI

Sonia Purin

The process of anastomosis promotes interconnectivity between hyphal branches during formation of a mycelial network and between fungal colonies. Self anastomosis involves fusion within a mycelium from a germinating spore, and this process is common in all species of fungi. Non-self anastomosis involves fusion between hyphae from different spores or colonies and allows lateral gene transfer, but this process is much rarer. Anastomosis in Ascomycota is well studied, but little is known for asexual arbuscular mycorrhizal fungi (AMF) in Glomeromycota. Because AMF are obligate symbionts in plant roots, studies have focused on anastomosis in asymbiotic hyphae of germinating spores. To study behavior of symbiotic hyphae growing from a mycorrhizal plant, a “rhizohyphatron” was designed which allowed symbiotic hyphae to grow from single or paired containerized mycorrhizal plants onto an agar-coated surface that could be removed and examined microscopically. In Chapter 1, anastomosis was studied in genetically uncharacterized populations of AMF species representing major genera. Hyphal fusions occurred at very low frequencies in *Glomus clarum* and *Glomus intraradices* (<15%) but were absent in *Ambispora*, *Gigaspora*, *Paraglomus* and *Scutellospora*. These results suggest that anastomosis in symbiotic mycelia may not be biologically important for most clades of AMF. In Chapter 2, self and non-self anastomosis were studied in both asymbiotic and symbiotic hyphae of *G. clarum* from single spore cultures genotyped using two anonymous microsatellite-flanking markers. Frequency of self anastomosis was similar in asymbiotic hyphae from germinating spores and symbiotic mycelia from plants, ranging from 8 to 38%. Non-self anastomosis was observed only in asymbiotic hyphae of isolates that were genetically identical as well as different isolates. However, fusions occurred in less than 6% of hyphal contacts. Such low levels of hyphal fusion may still permit gene flow in AMF populations, but anastomosis is restricted to asymbiotic hyphae of isolates from proximate locations. Results from this study show that vegetative compatibility between individuals occurs in AMF, but it is limited to only a few lineages and is constrained to only a small phase of the AMF life cycle.

Dedication

God has made these verses become true in my life as never before:

“The Lord is my shepherd; I have everything I need. He lets me rest in fields of green grass and leads me to quiet pools of fresh water. He gives me new strength. He guides me in the right paths, as he has promised. Even if I go through the deepest darkness, I will not be afraid, Lord, for you are with me”.

Psalms 23: 1-4.

I truly had everything I needed, and I always will.

Acknowledgements

Most importantly, I would like to thank God. His grace has been my source of strength, peace, love and safety. My faith in Him has made this accomplishment possible. Day after day, he has poured out countless blessings and fulfilled my every physical and spiritual need.

I would like to honor my parents, Evanilde and João, for their love. I lost my lovely mother before I started my Ph.D. program, but I believe she would be very happy for this accomplishment if she were still with me.

I want to thank my fiancé, Rob Bills. From picking spores, studying biochemistry, coaching a boy's basketball team, and bird watching; we have shared many wonderful experiences together. I want to thank him for his unconditional love, endless support, and for always being at my side.

I am especially thankful to Rick and Debbie Mullens for “adopting” me and making me a part of their family. They are a blessing and wonderful example of love, friendship, parenthood and discipleship. I will never forget their kindness, generosity and support.

I wish to thank Pastor Casey Shorr and my brothers in Christ from the Pierpont Church of the Nazarene. I can't thank them enough for their companionship and for making me feel at home in this loving community.

I am particularly thankful to my friends Marciela de Souza, Carlos Grippa, Rosane Giacomozzi, Angela Purim and Lourdes Araujo. They have proved to me that true friendship can overcome both distance and time. I am very blessed to have them in my life.

I would like to express my gratefulness to my advisor, Dr. Joseph Morton. His guidance, encouragement and support were essential for this accomplishment. He also supported me financially throughout this program and provided me an assistantship for the last year, and I am grateful for that. I am also extremely thankful for the mentorship received from my committee members: Alan Sexstone, Daniel Panaccione, James Bever and Teresa Pawlowska. A special thank you goes to Dr. Bever and Dr. Pawlowska who always managed their schedule to accommodate my needs in Morgantown. I really appreciate their participation, encouragement and support.

I would like to thank my friend and former lab mate Zola Msiska. Much of my experience with molecular biology comes from her.

I would like to extend my appreciation to Bill Wheeler. Every student who has been in Morton's lab knows that we cannot succeed without him. He is truly our superman.

This Ph.D. program was financially supported by the Brazilian Federal Government (CAPES) and the Fulbright Program. I would like to thank both institutions for this exceptional scholarship, and for the unique opportunity to experience graduate education and life in the United States.

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REVIEW OF LITERATURE

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF, Glomeromycota) are obligate symbionts that colonize roots of most land plant species (Wang & Qiu, 2006). AMF are unique among fungi in that their life cycle can be divided into discrete asymbiotic and symbiotic phases. Spores in soil begin the life cycle because they are dispersed as individual propagules and then germinate to produce a new autonomous individual (Smith & Read, 2008). In the absence of a host plant, survival of a germinating spore rarely exceeds 20-30 days (Bonfante & Perotto, 1995). The hyphae branching from a spore germ tube characterizes the asymbiotic phase, during which the fungus grows to seek a root and establish an obligate mycorrhizal association with a plant.

When an asymbiotic hypha establishes contact with the surface of a root, an appressorium forms on the root epidermis. Infection hyphae are produced from the appressorium and penetrate into the root cortex (Garriock *et al.*, 1989). The establishment of mycorrhizal colonization characterizes the beginning of the dominant symbiotic phase, which may last for as long as the plant host lives. AMF form specialized structures associated with intraradical hyphae that are unique to the mycorrhizal association: intracellular branching arbuscules or terminal intercellular vesicles (Gianinazzi, 1991). Arbuscules are formed between the plant cell wall and membrane, and mediate nutrient exchange between plant and fungus (Harrison, 1997).

Extraradical hyphae grow from roots 2-4 weeks after establishment of colonization (Jakobsen *et al.*, 1992). These hyphae spread through soil aggregates and absorb nutrients (mainly phosphorus) that are transferred to the plant symbiont through the arbuscules (Harrison, 1999). Spores are produced either isolated or in aggregates from different parts of vegetative hyphae in soil or roots within weeks to months of mycorrhizal development (Smith & Read, 2008). Throughout the entire fungal life cycle, no regular partitioning of hyphae is observed nor are any specialized structures produced to control migration of multiple nuclei through the fungal thallus or to spores (Jany & Pawlowska, 2010). As a mycelium grows and expands, hyphae that establish physical contact with each other may undergo fusion (anastomosis). This phenomenon is poorly understood in AMF, but it has been well described in Ascomycetes.

Hyphal fusion in Ascomycetes

Of all fungal groups, anastomosis is best understood in Ascomycetes. Phenotypic events associated with anastomosis are similar across species. Events include (i) initiation, (ii) recognition and either (iii) maintenance of the heterokaryon or (iv) rejection of the heterokaryon (Fig. 1).

During initiation, hyphal tips or pegs grow towards each other (Fig. 1i, 2a) and establish physical contact. Parts of the walls from both hyphae are broken down by enzymes. A fusion pore then is formed, through which protoplasmic continuity is established (Fig. 1ii, 2b, c). All steps during initiation occur independent of the genetic identity of vegetative incompatibility loci in either of the interacting hyphae.

During recognition, protoplasmic contents from both fungi are intermixed in a distinct zone that becomes a heterokaryon (Fig. 1ii). This condition is defined as heterokaryosis because interacting fungi likely differ at one or more loci in the genome. The newly formed heterokaryont sector may or may not be stable, depending on the genetic identity of recognition genes from both hyphae. Several genes determining stability of the heterokaryon are identified as **vegetative incompatibility** (*vic*), **heterokaryon incompatibility** (*het*) or **partner incompatibility** (*pin*). Proteins produced by *vic*, *het* and *pin* loci interact and form dimers. If all alleles are identical between anastomosing hyphae, homodimers are formed and the heterokaryon is stable. If only one allele is different at these loci, a heterodimer is formed that triggers destruction of the heterokaryont mycelium (Glass & Kuldau, 1992; Glass & Dementhon, 2006).

For maintenance of the heterokaryon, alleles of all vegetative incompatibility loci must be identical (Fig. 1iii, 2d). After fusion, the apical compartment undergoes cell division and a new heterokaryont mycelium sector is created (Newhouse & MacDonald, 1991; McCabe *et al.*, 1999). The result is vegetative (or somatic) compatibility. The heterokaryon is stable until nuclear segregation eventually occurs through asexual sporulation or hyphal branching and restores homokaryosis.

Rejection of the heterokaryon occurs when hyphae that differ genetically at any incompatibility loci fuse and then are killed (Fig. 1iv, 2e). This process is indicative of vegetative (or somatic) incompatibility. Degradation of the heterokaryont mycelium occurs by programmed cell death (PCD). PCD during anastomosis occurs by autophagy (Marek *et al.*, 2003; Paoletti & Clave, 2007), which contrasts with apoptosis, a form of PCD in animal cells (Pinan-Lucarré *et*

al., 2003). In apoptosis, cells shrink and fragment but are not completely degraded. Organelles that are not harmful can be phagocytosed and recycled by the organism. With autophagy, however, cell contents are totally destroyed and cellular compartments are not recycled (reviewed by Bursch, 2001).

The first step of PCD in heterokaryons is the plugging of septal pores by proteins, which compartmentalizes the hyphal region undergoing an incompatible reaction. Autophagosomes engulf protoplasmic components and target them for degradation in vacuoles (Fig. 2e). Proteases and laccases are produced to degrade fungal cell walls and proteins (Boucherie & Bernet, 1978; Paoletti *et al.*, 2001). Three of these enzymes have been genetically characterized. Because of expression only during autophagy, these genes are classified as *idi* (induced during incompatibility) genes (Saupe, 2000). Progressive loss of cytoplasmic density is observed during autophagy until the entire heterokaryon is degraded, followed by mycelium death and cell lysis (Marek *et al.*, 2003). The zone between two incompatible colonies is manifested phenotypically as a dark line devoid of mycelium (Fig. 3).

Vegetative incompatibility in Ascomycete fungi is regulated by two genetic systems, one allelic and the other non-allelic. In the allelic system, proteins produced by the **same** locus interact to form dimers. In non-allelic systems, interaction occurs between products of alleles from two or more closely-linked or unlinked loci (Fig. 4). Regardless of the type of interaction, all alleles must be identical for fungi to be vegetative compatible.

The number of genes involved in vegetative incompatibility and the type of allelic interaction varies with fungal species (Table 1). Based on the number of genes and alleles, it is possible to estimate the number of vegetative compatibility groups (VCGs) in fungal populations. Every unique allelic combination constitutes a VCG (Fig. 5).

Vegetative incompatibility genes are highly polymorphic, increasing the number of possible VCGs in fungal populations. When polymorphic, all incompatibility genes but one (*het-s*) have a HET domain, suggesting that this specific domain is involved in dimer formation during heterokaryon formation (Paoletti & Saupe, 2009). Although several species have been reported to possess vegetative incompatibility systems, genes have been cloned and characterized genetically only in *Podospora anserina* and *Neurospora crassa*.

Incompatibility systems in *Podospora anserina*

At least 17 loci are involved in both allelic and non-allelic heterokaryon incompatibility in *P. anserina* (Glass *et al.*, 2000). Of those interactions, the *het-s* locus is unique in that the HET domain is absent.

The *het-s* locus can exist as either *het-s* and *het-S* alleles. The *het-s* allele expresses two phenotypes: prion-free [Het-s*] and prion-infected [Het-s]. Prion-free fungi acquire prions from infected individuals upon physical contact. Fungi expressing the *het-S* allele are prion-free, with the phenotype [Het-S]. When undergoing hyphal fusion, [Het-s*] and [Het-S] individuals are able to anastomose. Vegetative incompatibility and cell death occur only when hyphae of a [Het-s] phenotype fuses with that of the [Het-S] phenotype. The prion in [Het-s] induces misfolding of various proteins and triggers programmed cell death (Saupe, 2007).

The best described non-allelic system comprises three closely-linked loci: *het-c*, *het-d* and *het-e*. Four *het-c*, four *het-e*, and three *het-d* alleles have been described. Each allele in *het-c* is incompatible with a specific allele of *het-d* and *het-e* alleles, respectively (Saupe, 2000; Glass & Dementhon, 2006). The gene *het-c* encodes for a glycolipid transfer protein (Saupe *et al.*, 1994). The genes *het-d* and *het-e* encode signal transduction ATPases with numerous domains (STAND proteins). These proteins have three domains: a N terminal HET domain, a central NACHT domain and a C terminal WD-repeat domain. (Saupe *et al.*, 1995; Paoletti *et al.*, 2007). HET-C proteins also have cellular functions other than vegetative incompatibility. These proteins appear to be incorporated into the hyphal cell wall, so that formation of heterodimers in heterokaryon incompatibility interferes with wall synthesis and contributes to cell death (Saupe *et al.*, 1994).

The loci *het-r* and *het-v* are not linked and interact via a non-allelic mechanism. The *het-r*, *het-d* and *het-e* loci all encode STAND proteins (Chevanne *et al.*, 2009). The *het-v* has yet to be cloned and characterized.

Incompatibility systems in *Neurospora crassa*

At least 11 vegetative incompatibility genes have been identified in *N. crassa*, including one mating type gene (*mat*). All of the characterized heterokaryon incompatibility reactions appear to function through an allelic mechanism, with the exception of *mat*.

The *het-C* locus has three allelic forms: *het-C*^{OR}, *het-C*^{PA} and *het-C*^{GR}. These alleles encode glycine-rich proteins associated with the fungal cell walls. Heterodimer formation therefore affects normal growth and morphology (Saupe *et al.*, 1996; Saupe & Glass, 1997).

The locus *het-6* has two alleles: *het-6*^{PA} and *het-6*^{OR}. The similarity between those two alleles is only 68%, the lowest level reported between alleles of a vegetative incompatibility locus. *het-6*^{PA} and *het-6*^{OR} mediate heterokaryon destruction through a non-allelic interaction with *un-24* alleles (Smith *et al.*, 2000). However, how the interaction triggers cell death is not clear.

The locus *un-24* has two characterized alleles: *un-24*^{OR} and *un-24*^{PA}. In other fungi, *un-24* encodes the large subunit of ribonucleotide reductase. Interestingly, this enzyme in *N. crassa* has a unique carboxy-terminal domain that confers the heterokaryon incompatibility function (Smith *et al.*, 2000).

Two alleles are identified in the *het-I* locus: *het-I* and *het-i* (Pittenger & Brawner, 1961). Interaction of these alleles does not produce a full heterokaryotic mycelium sector. Instead, heterokaryons are lost by the destruction of only one nuclear component.

The *mat* gene is the only known example of non-allelic interactions in *N. crassa*. This gene is also involved in sexual incompatibility, conferring different mating types: *A* and *a*. Mating type *A* has three possible idiomorphs: *mat A-1*, *mat A-2* and *mat A-3*. Mating type *a* has only one idiomorph, *mat a-1*. All idiomorphs are involved in sexual incompatibility, but only *mat A-1* and *mat a-1* are involved in vegetative incompatibility. During mating, all alleles must be different. Successful vegetative interactions, however, require that all alleles are identical. Vegetative incompatibility is silenced during heterokaryon formation through proteins produced by an unlinked locus, *tol*. Transcription of *tol* makes the different nuclei tolerant to genetic differences (*tol* = tolerant). This is the only molecularly characterized mediator of allelic incompatibility in fungi (Shiu & Glass, 1999).

Occurrence and significance of anastomosis in Ascomycetes

Anastomosis occurs frequently between hyphae of a same colony and also between different colonies. A fungal colony produced by a single spore is considered a *ramet*, which is loosely equivalent to an individual (Buss, 1983). Therefore, hyphal fusion within a colony is considered “self anastomosis”. Fusion between hyphae produced by two separate colonies, or

“individuals”, is considered “non-self anastomosis” (Glass & Kaneko, 2003; Glass *et al.*, 2004). Non-self anastomosis can occur between both genetic similar or genetic different colonies.

Several hypotheses have been proposed to explain the occurrence of vegetative compatibility. Aanen *et al.* (2008) examined costs and benefits of vegetative compatibility, and they proposed hypotheses that seem consistent with patterns observed for most fungal species studied to date, especially those in the Ascomycetes. They suggest that self anastomosis is beneficial and selects for compatibility. Self compatibility within a mycelium is important for redistribution of water, nutrients and cytoplasmic contents (Gregory, 1984). On the other hand, non-self anastomosis is costly and therefore selects for incompatibility. Non-self anastomosis might represent fitness loss for one of the partners because of frequent nuclear parasitism (Debets & Griffiths, 1998) and transmission of viruses or other harmful cytoplasmic elements (Biella *et al.*, 2002). However, non-self anastomosis is not always costly. Exchange of nuclei between fungi is important to create genetic variability by mitotic recombination in the heterokaryont condition. This event might not be critical for fungi that alternate sexual and asexual stages, because heterokaryons are formed in the sexual stage and meiotic recombination generates variability (Coppin *et al.*, 1997). Yet vegetative non-self compatibility might be very important for exclusively asexual fungi such as those in Glomeromycetes. In the absence of sexual reproduction, anastomosis between individuals would provide the only means for gene flow between populations.

Vegetative compatibility in AMF

Anastomosis has been studied mostly in the asymbiotic phase of the AMF life cycle, which consists of all events and processes from spore germination to contact with a host plant. Studies on anastomosis during the symbiotic phase, or events and processes after establishment of a mycorrhizal association, are rare because of methodological constraints. Mycelium behavior in colonized plants (Giovannetti *et al.*, 2001; 2004) is difficult to observe and measure because hyphae are very sensitive to damage when manipulated. *In vitro* root organ cultures (Bécard & Fortin, 1988) are useful for observation of hyphal fusions, but a majority of AMF species cannot be successfully cultivated *in vitro*. In this dissertation, a new cultivation system (rhizohyphatron) was invented to allow evaluation of anastomosis in symbiotic mycelia without constraints that are typically observed in other systems. The rhizohyphatron has two variations; one designed to

evaluate self anastomosis in mixed populations of six AMF species (Chapter 1) and the other designed to evaluate both self and non-self anastomosis of defined genotypes in the species *Glomus clarum* (Chapter 2).

The definition of “self” or an AMF “individual” has been subjected to intense and endless debate. Some researchers have considered all spores from one accession to represent a *ramet*. All hyphal fusions observed within a mycelial network originating from a sampled population were described as self anastomosis (Giovannetti *et al.*, 2003). However, this definition is questionable because one sample might contain two or more different genotypes so that the term “self” is not valid. More recently, the term “self” has been applied only to a single spore or a culture derived from a single spore inoculum (same *ramet*). The term “non-self” is used to denote interactions between different spores or between different single spore derived cultures (Croll *et al.*, 2009). These will be the definitions adopted throughout this dissertation. When multiple spores are used as an inoculum source to study anastomosis, the interactions observed will be described as a “population” rather than an “individual”.

Vegetative compatibility within populations

The first study of vegetative compatibility in AMF was documented by Tommerup (1988). She reported the occurrence of anastomosis between isolates of *Acaulospora laevis*, *Archaeospora trappei*, *Glomus caledonium*, *G. monosporum*, *G. fasciculatum*, *G. tenue*, *Gigaspora decipiens* and *Scutellospora calospora*. Because data concerning origin of these isolates, frequency of anastomosis, and experimental growth conditions were not documented, interpretation of results was of limited value. After 15 years, other studies were carried out, but inocula still consisted of multiple spores.

Evidence for hyphal fusion in the asymbiotic phase has been found only in *Glomus* species (Giovannetti *et al.*, 1999; 2003; Cardenas Flores *et al.*, 2010). In this group, the frequency of anastomosis ranges between 40-90%. No anastomosis has been found in the mycelia of *Scutellospora* and *Gigaspora* species (Giovannetti *et al.*, 1999), and it has not been studied in genera such as *Paraglomus* and *Ambispora*.

Anastomosis in symbiotic mycelium has been studied only in *Glomus*. In root organ cultures, 100% of hyphal contacts in mycelia of *G. proliferum*, *G. intraradices* and *G. hoi* had resulted in fusions (de la Providencia *et al.*, 2005). In whole plant associations with *G. mosseae*,

the frequency of anastomosis is considerably lower (36-78%) (Giovannetti *et al.*, 2001; 2004). Anastomosis frequency was similar within the mycelium of a single plant or between mycelia of paired plants.

Anastomosis has been studied in populations of only three out of 15 AMF genera. Two main constraints have limited advancements in research on anastomosis in symbiotic mycelia. The first is reliance on cultivation systems which do not reflect whole plant-fungus interactions. *In vitro* root organ cultures constitute atypical growth conditions, where the plant host consists of immortalized root explants subjected to high nutrient levels (de la Providencia *et al.*, 2005; Voets *et al.*, 2006). The second is the low number of species capable of being cultured *in vitro*. The main reason little is known about anastomosis in groups other than *Glomus* is their lack of compatibility with the root organ culture environment.

Self and non-self anastomosis

Self anastomosis in the asymbiotic phase has been studied only in *G. intraradices*. When germinating spores were paired, 46-51% of the hyphal contacts led to fusion (Croll *et al.*, 2009). Self anastomosis in symbiotic mycelium has been studied in a few species of Glomeraceae and Gigasporaceae using the *in vitro* culture system. In *Glomus proliferum* and *G. intraradices*, percentage of anastomosis in hyphae produced by a mycorrhizal root organ was of 80 and 88%, respectively (Voets *et al.*, 2006). In *Gigaspora* and *Scutellospora*, the frequency of hyphal fusion is less than 10% (de Souza & Declerck, 2003; de la Providencia *et al.*, 2005; Voets *et al.*, 2006). When fusion is evaluated between hyphae from two mycorrhizal root organs, the percentage of anastomosis decreases dramatically in *Glomus* and is completely absent in *Gigaspora* and *Scutellospora* (Voets *et al.*, 2006). These observations suggest that recognition phenomena differ in a single mycelium versus paired mycelia of an AMF.

Non-self anastomosis has been studied only in germinating spores of defined genotypes of *G. intraradices* (Croll *et al.*, 2009). Frequency of non-self anastomosis ranged between 0 and 10%, and some concomitant variation in phenotypes of mycorrhizal plants suggests that lateral gene transfer occurred. Information on non-self anastomosis in the symbiotic phase has yet to be obtained on any AMF species, even though this phase is lengthy and crucial to success of the symbiosis.

Morphology of hyphal interactions

When two colonies of Ascomycete fungi are paired, anastomosis events either lead to successful fusion or destruction of the heterokaryon. When colonies meet, there is a typical tip-to-tip fusion (Fig. 1) and if individuals are incompatible a zone of hyphal destruction is formed (Fig. 3). Therefore, anastomosis in Ascomycetes is usually scored as either present or absent. When AMF mycelia are paired, no typical tip-to-tip fusion occurs, so hyphae often overlap. Hyphal contacts occur in both the center and periphery of colonies, and any response is not absolute. Some contacts may be incompatible while others result in fusion. Therefore, anastomosis cannot be scored using the same methods used for Ascomycete fungi. Instead, every hyphal contact must be evaluated under a microscope.

Compatible hyphal fusions in AMF mycelium are mainly characterized by the formation of short and long bridges (Giovannetti *et al.* 2001; 2004; Fig. 6a, b). While vegetative incompatibility is regulated after hyphal fusion in Ascomycetes (Fig. 1, 2), it seems to be evident both before and after fusion in AMF. Post-fusion incompatibility is identified when, following anastomosis, the protoplasm of one hypha withdraws and a septum formed between the two hyphae (Fig. 6c, d). Low levels of post-fusion incompatibility have been observed only in germinating spores of *G. intraradices* and *G. mosseae* (Croll *et al.*, 2009; Sbrana *et al.*, 2010). However, no post-fusion incompatibility has been observed when the fungus is in a symbiosis with its host (Giovannetti *et al.*, 2001; 2004; de la Providencia *et al.*, 2005; Voets *et al.*, 2006; Sbrana *et al.*, 2010). Vegetative incompatibility in AMF also is regulated at the pre-fusion stage. Before or during physical contact between hyphae, germ tubes swell, branch and form septa (Fig. 7). Physical changes in germ tubes prior to any contact suggest that specific recognition signals are involved during this stage.

Significance of anastomosis in arbuscular mycorrhizal fungi

Anastomosis is functionally important for AMF for underground resource allocation as well as for healing of wounded or broken hyphae. Fusion between mycelia of *Glomus mosseae* isolates produces common mycorrhizal networks (CMNs) that allow nutrient transfer between plants (Mikkelsen *et al.*, 2008). So far, the formation of CMNs has been reported only in *G. mosseae*, so the universality of this phenomenon remains unknown. Anastomosis behavior in other AMF species and phylogenetically diverse groups would contribute to a better

understanding of the genetics of population and community-level interactions. After hyphal damage, new lateral or apical tips are formed and fuse to re-establish cytoplasmic flow (de la Providencia *et al.*, 2005). Hyphal fusion is involved in healing processes in three genera studied so far (*Glomus*, *Scutellospora* and *Gigaspora*).

Knowledge of anastomosis also has implications at the genetic level. The genetic organization of AMF is unclear from available evidence, and two alternative hypotheses have been proposed, one involving homokaryotic and the other heterokaryotic organization of the AMF genome (Fig. 8). Anastomosis would not be important for maintenance of homokaryosis (Pawlowska & Taylor, 2004), but it would be required to compensate genetic drift created by bottlenecks if heterokaryosis is the dominant process (Bever & Wang, 2005).

Non-self anastomosis represents the only possibility for gene flow among individuals of asexual fungi. Non-self anastomosis between genetically different germinating spores of *G. intraradices* has been measured at low levels (Croll *et al.*, 2009). However, non-self fusion between genetically different individuals cannot be generalized across AMF lineages for two reasons. First, data so far is confined to results from the study of only one species, *G. intraradices*. Second, no information is available on non-self fusion in symbiotic mycelia. Broader understanding of non-self anastomosis in a range of AMF species and in different stages of the AMF life cycle will help to understand mechanisms of inter-individual interactions.

The present dissertation is divided in two chapters. The objective of the study presented in the first chapter was to assess vegetative compatibility in symbiotic hyphae of populations of species from representative AMF genera. The hypothesis to be tested was that vegetative compatibility is observed in all taxa. The objective of the second chapter was to evaluate self and non-self anastomosis in germinating spores and symbiotic hyphae of *Glomus clarum*. This study was conducted to test the hypothesis that anastomosis is observed in all phases of the AMF life cycle.

Table 1. Number of vegetative incompatibility genes, vegetative compatibility groups, and type of interaction in some species of Ascomycete fungi. Fonts: Glass & Kuldau (1992), Glass *et al.* (2000).

Species	Vegetative incompatibility genes	Vegetative compatibility groups*	Type of interaction
<i>Cryphonectria parasitica</i>	7	128	Allelic
<i>Aspergillus nidulans</i>	8	256	Allelic
<i>Neurospora crassa</i>	11	2048	Allelic and non-allelic
<i>Podospora anserina</i>	17	131072	Allelic and non-allelic

* calculated on the assumption that each gene has two alleles. Therefore, a species with seven genes and two allelic forms of each gene has 128 vegetative compatible groups (2^7).

Figure 1. Events during hyphal fusion (anastomosis) between hyphae of filamentous fungi. (i) initiation, (ii) recognition, (iii) maintenance, (iv) rejection. Adapted from Glass & Kaneko (2003).

Figure 2. Events during anastomosis between hyphae of *Neurospora crassa*. a) Homing of hyphal tips. b) Fusion of walls and creation of a fusion pore (arrow). c) Intermixing of cytoplasmic contents. d) Movement of a vacuole between compatible hyphae through a fusion pore. e) Evidence of heterokaryon rejection. Note intense vacuolization (black arrowhead) and hyphal degradation (white arrowhead). Bars = 10 μm. Images from Saupe (2000), Hickey *et al.* (2002), and Glass & Kaneko (2003).

Figure 3. Pairings between vegetative compatible (a) and vegetative incompatible (b) colonies of *Cryphonectria parasitica*. The dark line in (b) reflects the zone of anastomosis between the two colonies where anastomosis fails and the heterokaryont mycelium is destroyed. Source: personal image by author.

Figure 4. Three types of interactions between vegetative incompatibility genes. For fungal individuals to be compatible, all alleles must be identical (top). If one allele is different, interacting individuals are vegetative incompatible (bottom). Adapted from Glass & Dementhon (2006).

Figure 5. Four possible vegetative compatibility groups (VCGs) considering 2 vegetative incompatibility genes (*het-c* and *pin-c*), each one with two alleles (*c1* and *c2*). Source: personal image by author.

Figure 6. Compatible and incompatible hyphal fusion in AMF. a) Long bridge in *G. mosseae*, anastomosis is evidenced by formazan salt depositions. From Giovannetti *et al.* (2001), bar = 10 μm . b) Short bridge in *G. mosseae*, notice fusion of hyphal walls and establishment of protoplasmic continuity. From Giovannetti *et al.* (2004), bar = 10 μm . c) Post-fusion incompatibility in *G. intraradices*; protoplasm withdrawal and septum formation (arrow) in one of the fused hyphae after anastomosis. From Croll *et al.* (2009). d) Post-fusion incompatible interaction in *G. mosseae*, evidenced by formation of septa separating fused compartments. Reprinted with permission from Mycologia. ©The Mycological Society of America. From Sbrana *et al.* (2010), bar = 4.5 μm .

Figure 7. Evidence of pre-fusion vegetative incompatibility between isolates of *Glomus mosseae*. Note swelling, retraction and formation of septa before fusion of hyphae. From Giovannetti *et al.* (2003). Bars = 35 μm in a; 9 μm in b.

Figure 8. Sorting of genetic variation in AMF according to contrasting (a) heterokaryotic and (b) homokaryotic models. From Pawlowska & Taylor (2004).

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CHAPTER 1

***IN SITU* ANALYSIS OF ANASTOMOSIS IN REPRESENTATIVE GENERA OF ARBUSCULAR MYCORRHIZAL FUNGI¹**

Abstract

Arbuscular mycorrhizal fungi (AMF) form obligate symbiotic associations with plants. As a result, the role of hyphal interactions in the establishment and maintenance of common mycorrhizal networks is poorly understood because of constraints on methods for *in situ* analysis. We designed a rhizohyphatron that allows the examination of intact mycelia growing from whole mycorrhizal plants. Plants preinoculated with spores were cultivated in a compartment with a connecting tube from which hyphae extend through a fine nylon mesh onto agar-coated slides. Species selected from each of the five AMF genera were used to assess and characterize the anastomosis behavior in the rhizohyphatron. Hyphal networks of *Paraglomus occultum*, *Ambispora leptoticha*, *Scutellospora heterogama*, and *Gigaspora gigantea* growing on the agar coated slides showed no evidence of hyphal fusion. In contrast, anastomosis occurred in the hyphal networks of *Glomus clarum* and *Glomus intraradices* at an average frequency of less than 15% for both species. The rhizohyphatron developed in this study will provide knowledge of the biology and genetics of self/non-self recognition in AMF and help to better understand Glomeromycotan life history strategies.

¹ Purin S, Morton JB. 2011. *In situ* analysis of anastomosis in representative genera of arbuscular mycorrhizal fungi. Mycorrhiza. doi: 10.1007/s00572-010-0356-9. Reproduced with permission from the publisher.

Introduction

Fungi are modular organisms that can grow until the nutritional resources are exhausted (Andrews, 1992). The continuous growth of mycelia permits fungal individuals to explore vast volumes of substrate. However, this hyphal network also poses a challenge to maintaining a continuous flow and exchange of genetic information and cytoplasmic material. A continuity of mycelia is maintained by hyphal fusion (anastomosis) that reconnects sectors that grow apart or become separated by mechanical injuries (Bago *et al.*, 1999; de la Providencia *et al.*, 2005). Hyphal fusions also provide a mechanism for gene flow between genetically distinct individuals within a population (Glass *et al.*, 2000).

Anastomosis has been studied and characterized extensively in species of Ascomycete fungi (Saupe *et al.*, 2000; Smith *et al.*, 2000). After hyphae fuse, protoplasmic intermixing occurs in a confined sector named a heterokaryon (Pontecorvo, 1956; Glass *et al.*, 2000). The expression of vegetative incompatibility genes (*vic* genes) determines heterokaryon stability. Allelic differences between hyphae trigger the destruction of the heterokaryon and the onset of vegetative incompatibility (for reviews, see Leslie, 1993; Glass & Kaneko, 2003). Colonies with identical *vic* alleles are vegetatively compatible, and they maintain a stable heterokaryont mycelium capable of indefinite propagation (Glass & Dementhon, 2006).

Although the mechanisms and the significance of anastomosis are well understood in Ascomycetes, understanding of hyphal fusions in populations of arbuscular mycorrhizal fungi (AMF) in Glomeromycetes is rudimentary. AMF colonize the roots of many plant species so that mycelial connectivity contributes to common mycorrhizal networks (CMNs) potentially capable of interconnecting roots of the same or different plant species (Giovannetti *et al.*, 2004). Little empirical evidence is available to explain the potential ecological significance of CMNs because of difficulties in making direct *in situ* observations of growing mycelia. AMF are obligate biotrophs that can only be cultured in the absence of a plant host for a brief period before a mycorrhizal association is established (Giovannetti *et al.*, 1994; 1996). For this reason, AMF hyphal growth and development have been studied mostly in this asymbiotic phase or in monoxenic cultures of transformed roots.

Early studies reveal that different lineages of Glomeromycota exhibit diverse patterns of hyphal interactions, with unique differences in how these fungi behave during a asymbiotic versus a symbiotic phase. In the asymbiotic phase, anastomosis between germination tubes from

a population of spores does not occur in *Gigaspora* and *Scutellospora* species but ranges from 34% to 90% in *Glomus* species (Giovannetti *et al.*, 1999; 2003). In the symbiotic phase, a similar, albeit less dramatic, pattern of anastomosis is observed. The percentage of anastomosis in extraradical hyphae associated with root explants of *Gigaspora* and *Scutellospora* species is 4–10%, while anastomosis in *Glomus* species reaches 80–100% (de la Providencia *et al.*, 2005; Voets *et al.*, 2006). By comparison, anastomosis between the hyphae of *Glomus mosseae* growing from colonized plants is lower, ranging in frequency from 46% to 78% (Giovannetti *et al.*, 2001; 2004). Hyphal fusions in the symbiotic phase have not been studied in any other AMF species.

Clearly, the interpretation of anastomosis behavior among external hyphae produced after the establishment of a mycorrhizal symbiosis is complicated by the experimental environment. The *in vitro* root organ culture system (Bécard & Fortin, 1988) is atypical because immortalized root explants grow as autonomous organs in a medium with high soluble nutrient levels. The compatibility between fungi and the root host could be altered from the norm, as evidenced by the limited number of species that so far are maintained in culture (Declerck *et al.*, 2005). To overcome these complications as much as possible, a cultivation system was invented in which the behavior and dynamics of hyphae generated from intact mycorrhizal plants growing in a solid substrate could be studied *in situ*. To test this cultivation system, we evaluated anastomosis within individual populations of a representative species from five glomeromycotan genera.

Materials and methods

Rhizohyphatron and growing conditions

A “rhizohyphatron” (Fig. 1) was designed to permit the direct observation of AMF hyphae actively growing in association with roots of intact host plants growing in a solid medium. This cultivation system consisted of two compartments: (1) a plant growth compartment within which mycorrhizal plants were grown for 7 to 8 weeks and (2) a slide compartment to hold two horizontally placed agar-coated glass microscope slides that could be added, removed, or replaced at any time (Fig. 1a). The plant growth compartment consisted of a 20-cm-long PVC tube. When upright, the bottom end of the tube was sealed with a plastic cap having three small holes for water drainage. A 3.4-cm circular opening was cut approximately

8.5 cm from the top of the tube to allow the lateral insertion of the slide compartment. The slide compartment consisted of a 7.0-cm long PVC tube. Plastic supports for slides were cut from 1.0×7.5-cm plastic pot labels and attached to the wall of the tube using 100% silicon sealant (Silicon II, General Electric Co.). The two supports in each tube were spaced 7.0 mm apart in the center of the tube (Fig. 1c). The plant and slide compartments were separated by a nylon mesh partition with 41- μ m openings (Sefar America Inc., Depew, NY, USA) between the plant and slide compartments. The nylon mesh and plastic cap sealed the end of the slide chamber and provided enough resistance so that the slides were in tight contact with the nylon mesh at the juncture of the plant compartment (Fig. 1d). All plastic parts consisted of Schedule 40 PVC pipe fittings, with tubing of 3.4-cm outside diameter.

Before assembly, all components were surface-sterilized by immersion in a 10% sodium hypochlorite solution for 30 min. Plastic components were washed with distilled water, washed again in 70% ethanol, and then exposed to ultraviolet light in a laminar flow chamber for approximately 30 min.

To assemble the rhizohyphatron, a 5.0×5.0-cm nylon mesh section was held manually in place at both ends of the slide compartment, with one end inserted into the plant compartment and the other end capped and sealed (Fig. 1d). The top opening of the slide compartment was sealed with transparent tape presterilized by UV irradiation. The plant compartment was filled with Terragreen (Oil Dri Corp., Chicago, IL, USA) that had been premoistened and autoclaved by incubation at 121°C for 30 min. Two 10–12-day-old sorghum (*Sorghum sudanense* L.) seedlings were inoculated with 100–200 spores of a selected fungal isolate (Table 1) pipetted directly and evenly along intertwined roots. Before inoculation, spores were surface-cleaned by sonication and stored at 4°C for 2 days. Spores showing any signs of degradation or microbial contamination were removed. After inoculation, seedlings were transplanted into Terragreen in the plant compartment. The slide compartment was covered with a layer of aluminum foil to ensure darkness. This entire protocol was completed in a sterile laminar chamber.

Rhizohyphatrons were placed in a growth room with a light intensity at 320 μ E m⁻² s⁻¹ for a 14-h period and air temperature ranging from 25°C to 27°C. Plants were watered *ad libitum* daily with double-distilled deionized water and grown for 5 weeks to allow mycorrhizal development and formation of external hyphae. After 5 weeks, each rhizohyphatron was moved

to a laminar flow chamber. The cap was removed from the slide compartment, two agar-coated slides were inserted, and the tube resealed with the plastic cap.

To prepare microscope slides for insertion into the rhizohyphatron, a 22×50-mm cover slip was first affixed to the center of each slide with a 3×3-mm strip of autoclave tape. A cover slip was required to observe and/or measure the details of hyphal interactions under an inverted light microscope. Slides were first autoclaved for 20 min at 121°C and then transferred to a laminar flow chamber, where they were evenly spaced in the bottom of a flat 11.5×20-cm plastic tray, with 14 slides per tray. The slides were covered with a thin, uniform layer of agar by gently pouring 22 ml of autoclaved agar to cover the bottom of the plastic tray. The 1.5% water agar contained 0.1 mg kg⁻¹ P (added as KH₂PO₄) and was adjusted to pH 5.5. Once the agar solidified, each slide was carefully excised with a sterile dissecting blade and placed immediately into the slide compartment. After the slides were positioned, the slide compartment was capped and the rhizohyphatron transferred to the growth room.

AM fungal isolates

Globally distributed species representing five of the major genera in Glomeromycota were chosen for this study (Table 1). One productive accession of each species was selected from the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM; Morton, 1993). Each accession had been started initially from many spores to adequately capture genetic diversity, and then a large sample of each pot culture was used in at least six successive propagation cycles to minimize any possibility of genetic drift. Given the history of these cultures, the possibility of different genetic lineages coexisting in any one of them could not be excluded.

Microscopic evaluation of hyphal contacts

The spread of AMF external hyphae from the plant growth compartment was monitored by placing the rhizohyphatron under a stereomicroscope and inspecting the top slide through the transparent tape that covered the opening in the slide compartment. Observations began 5 days after the slides were inserted and they were repeated thereafter at 3-day intervals. When a hyphal front had advanced close to midway across the cover slip, the slide and plant compartments were gently separated from each other in a laminar flow chamber (Fig. 2a). Cover slips were detached

from each slide with forceps and immediately placed under an inverted microscope (Nikon Eclipse, TE2000-S) for examination.

On less than 25% of the slides, the agar surface became contaminated with bacteria or saprophytic fungi. These units were removed and dismantled. The source of the contaminants was traced to the plant compartment but origins never were identified definitively. Other coatings, such as Millipore membranes, were tested in attempts to reduce contamination. However, hyphae failed to grow from the plant compartment onto these membranes.

Hyphae present on cover slips were verified as being of AMF origin based on the structures of each species that typically formed concomitant with hyphal development, such as spores, auxiliary cells, or saccules. To facilitate the observation and identification of hyphal contacts, each cover slip was overlaid on a 1.0×1.0-mm grid. The hyphal front served as the base reference point (Fig. 2b). Hyphal contacts were evaluated in alternate columns distal to the hyphal front for a distance of 11 mm. Therefore, the columns in the grid were evaluated at 1, 3, 5, 7, 9, and 11 mm from the hyphal front (Fig. 2b). Preliminary observations had indicated that most variations in hyphal anastomosis occurred in this region (data not shown). Within each column, alternate squares were examined, totaling an area of 11 mm² per column. This approach was used for the isolates of *Ambispora*, *Glomus*, and *Paraglomus* species. Because hyphal contacts were much less frequent among hyphae formed by the isolates of *Gigaspora gigantea* and *Scutellospora heterogama*, all columns and all squares in each column were examined up to 11 mm from the hyphal front. One rhizohyphatrons and two cover slips were evaluated per AMF isolate.

Hyphal contacts were classified as “compatible,” “incompatible,” or “no recognition” based on published descriptions of vegetative interactions in AMF. Compatible hyphal interactions were identified when interacting hyphae formed bridges with complete wall fusion and protoplasmic continuity (Giovannetti *et al.*, 1999; 2001; 2004). Interactions suggestive of incompatibility were recognized when interacting hyphae formed septa, changed shape, or both (Giovannetti *et al.*, 2003). Hyphal contacts were characterized as no recognition when hyphae physically overlapped without any morphological changes (Giovannetti *et al.*, 1999; de la Providencia *et al.*, 2005). The number of hyphal contacts in each category (compatible, incompatible, or no recognition) was divided by the total number of observed hyphal contacts in order to calculate relative frequencies.

Results

Rhizohyphatron utility

The rhizohyphatron provided a suitable environment to conduct *in situ* observations of hyphal interactions in the symbiotic mycelium of AM fungi. With the exception of *Acaulospora*, the extraradical mycelium of all species extended into the slide compartment where the hyphal network could be examined microscopically. In most cases, the rhizohyphatron remained free of bacterial and fungal contamination for the duration of an experiment (7–8 weeks). Fungal contamination occurred in less than 25% of rhizohyphatrons and varied in both composition and abundance depending on the fungal isolate used as inoculum. Fungal contaminants always grew from the plant compartment.

Patterns of hyphal interactions in AM fungi

For all AMF species, the slide compartment was opened and cover slips were removed for microscopic analysis when the mycelia had grown an average distance of 40 mm on the agar surface. The mycelia of *Ambispora leptoticha*, *Glomus clarum*, and *Glomus intraradices* reached this distance within 7 days after the slides were placed in the rhizohyphatron. A similar distance for mycelial growth of other species was observed at 14 days. The mycelia of *A. leptoticha*, *G. clarum*, *G. intraradices* and *Paraglomus occultum* produced spores branching from older hyphae, especially near the nylon mesh partition separating the plant and slide compartments. The hyphae of *G. gigantea* and *S. heterogama* did not produce any spores, but they did form abundant auxiliary cells. Fast and bidirectional protoplasmic streaming was observed in the hyphae of *A. leptoticha*, *G. clarum*, and *G. intraradices*. In the hyphae of other species, bidirectional streaming was observed but the rate of streaming appeared to be markedly slower.

Evidence of hyphal fusion was observed only in the *Glomus* species analyzed. The hyphae of both *G. clarum* and *G. intraradices* fused by producing bridge-like structures. Bridges shorter than 50 μm were classified as “short bridges” (Fig. 3a, b), and bridges longer than 50 μm were classified as “long bridges” (Fig. 3c). Protoplasm flow between two hyphae via hyphal bridges confirmed mycelium continuity. Short and long bridges formed with similar frequency between hyphae of both species (Table 2).

A unique anastomosis behavior was observed in *G. clarum* that did not involve hyphal tips or projections. Cell wall and membrane fusion occurred between some hyphae growing in such close proximity to each other that no bridges were formed (Fig. 3d). Protoplasmic streaming was observed in regions where hyphal walls in direct contact had dissolved, thus confirming that anastomosis had occurred.

Three morphological changes in interacting hyphae were hypothesized to indicate vegetative incompatibility: (1) septation, (2) change in shape, and (3) septation accompanied by change in shape (Table 2). Septation without any change in shape occurred most frequently in the hyphae of most of the AMF species tested and it often was localized near the tips and in coarser hyphae. Septation was the only indication of vegetative incompatibility among hyphae of *G. gigantea* and *S. heterogama*. For all AMF species, mycelial compartments created by septa were devoid of cytoplasm (Fig. 4a).

Changes in the shape of the hyphae were observed in all genera except *Gigaspora* and *Scutellospora*, regardless of the occurrence of septa formation. When a hyphal tip or lateral projection contacted a putative incompatible hypha, the morphology of the former was altered (Fig. 4b). Tips became swollen with lateral expansions, and anastomosis did not occur between the interacting hyphae (Fig. 4b). This response occurred most frequently as indicative of vegetative incompatibility in the hyphae of *A. leptoticha* and *G. intraradices*. In some instances, septation occurred with a change in hyphal shape and the flow of cytoplasm became blocked (Fig. 4c).

Hyphal contacts classified as no recognition occurred most frequently among the hyphae of all AMF species studied. Hyphae established physical contact with each other, but no evidence of anastomosis, septation, or change of shape was discernible (Fig. 4d). For *P. occultum*, all hyphal interactions were characterized by no recognition.

The average frequency of compatible hyphal contacts did not exceed 15% among the two species in which anastomosis occurred (Table 3), ranging from 6.7% in *G. clarum* to 13.9% in *G. intraradices*. The frequency of incompatible contacts ranged from 7% to 32% among the six species studied. Among all AMF species, 61–100% of hyphae in physical contact with each other showed no evidence of recognition (Table 3).

Discussion

The rhizohyphatron designed for this study represents a unique tool for the *in situ* analysis of AMF external mycelia. The components of this system are easy to mass produce, and maintenance is straightforward. Slides can be inserted in the rhizohyphatron at any time after the establishment of root colonization so that the mycelium can be evaluated at any stage of the symbiosis or age of the host plant.

In an open system such as this one, contamination always is the most serious concern. Less than 25% of rhizohyphatrons showed evidence of either fungal or bacterial contamination, but the absence of any pattern suggested that the most likely source was spores in the inocula. Even though spores were incubated for 2 days to permit the growth of any internal parasites, some still could have escaped detection at the time of inspection. The 2-day incubation period used in this study to detect these contaminants was probably insufficient for some spore populations. Spore germination assays and inoculation of axenic root organ cultures with a range of isolates in INVAM indicate that even the most visually clean spores may harbor internal fungal or bacterial propagules, and these sometimes are not detected without a longer incubation (results not shown). In this study, the rhizohyphatrons were maintained in a room where several other pot cultures are maintained and so some contamination also from the air cannot be excluded.

The rhizohyphatron successfully allowed the anastomosis behavior in symbiotic hyphal networks to be studied for the first time in ancestral AMF species. The rhizohyphatron may also provide a useful approach in studying hyphal architecture, rates of hyphal development, and other variables *in situ*.

Anastomosis has been studied previously in fungi with membership in *Glomus* group A, *Gigaspora*, and *Scutellospora*. Our study expanded the analysis of anastomosis to include *A. leptoticha* and *P. occultum*, two species in ancestral clades (Redecker & Raab, 2006). In addition to the species listed in Table 1, we also tested *Acaulospora morrowiae* (INVAM accession number WV220), but this species did not produce hyphae in the rhizohyphatron. *Acaulospora* species, in general, tend to be unpredictable even in standard pot culture environments (personal observation). Different species and growth media will need to be screened to resolve the compatibility issues in this genus.

The frequency of hyphal fusions in *Gigaspora* and *Scutellospora* has been reported previously to average less than 10% (de la Providencia *et al.*, 2005; Voets *et al.*, 2006) or not to occur at all (Giovannetti *et al.*, 1999). The results of this study support the findings of the latter study. The absence of hyphal fusion in *Archaeospora* and *Paraglomus* suggests that anastomosis is a derived trait present at least in the *Glomus* group A clade (Schüßler *et al.*, 2001). In other fungi, anastomosis is important for colony formation to homogenize water and nutrient distribution in the mycelium and to reconstitute mycelium continuity when hyphae are injured (Gregory, 1984). However, hyphal fusions are rare or absent when a population harbors deleterious cytoplasmic elements, so their transmission among individuals is avoided (Biella *et al.*, 2002). However, without knowledge of AMF genomes, it is difficult to explain the occurrence of anastomosis in one major clade only. A similar behavior may be found in other *Glomus* clades (B and C), but those species have not yet been tested.

Evidence suggests that anastomosis in *Glomus* mediates the underground resource allocation between plants by the formation of CMNs (Mikkelsen *et al.*, 2008) and is important for hyphae-healing mechanisms (de la Providencia *et al.*, 2005). These benefits would be of positive value to all glomeromycotan fungi and would not be restricted to *Glomus* group A. Whatever historical events or processes involved in the emergence and heritability of anastomosis cannot be inferred from data available at this time.

The dominant type of compatible hyphal interactions observed in *Glomus* species was the formation of hyphal bridges, which have also been described in some ascomycetes. In *Neurospora crassa*, *Tuber borchii*, and *Beauveria bassiana*, for example, short bridges are produced mainly when hyphae grow parallel to each other and in close proximity (Kawamoto & Aizawa, 1989; Hickey *et al.*, 2002; Marek *et al.*, 2003; Sbrana *et al.*, 2007). Long bridges are observed as well in some ascomycete species but at frequencies lower than those measured for short bridges (Ainsworth & Rayner, 1986). The occurrence of long bridges is hypothesized to involve signaling molecules required for attraction (Hickey *et al.*, 2002). In this study, an evidence of the homing of hyphal branches towards each other before fusion supports this hypothesis. In some *Glomus* species, the existence of signaling molecules might be important because short and long bridges were formed with similar frequency.

All fusion events between vegetative hyphae reported to date involve homing of at least one hyphal tip, such as observed in the formation of bridges and “tip-to-side” fusions or pegs

(Ainsworth & Rayner, 1986; Aylmore & Todd, 1986; Ainsworth & Rayner, 1989; Correll *et al.*, 1989; Kues, 2000). The significance of the unique type of anastomosis behavior in *G. clarum*, where fusion occurred between hyphae growing parallel to each other in close proximity, is not known, but the localization of signaling molecules may intensify the opportunity for fusion events.

Both this study and the one by Giovannetti *et al.* (2003) indicate that any vegetative incompatibility response among symbiotic external hyphae is expressed prior to a fusion event. Given the coenocytic nature of AMF mycelium and rapid protoplasmic streaming, chemical signals that trigger vegetative incompatibility likely are expressed before anastomosis occurs. Otherwise, the spread of deleterious genetic elements across coenocytic hyphae would occur rapidly and the entire mycelium could be compromised. In contrast, signal recognition in ascomycete and basidiomycete fungi occurs during a post-fusion stage when a heterokaryont mycelium is established. In these fungi, only a small fraction of the hyphal network is compromised when there is allelic incompatibility because plugging of septa would effectively block the spread of deleterious elements (McCabe *et al.*, 1999; Marek *et al.*, 2003).

The frequency of hyphal fusions observed in *G. clarum* was very low when compared to the results of previous studies on other species in *Glomus* group A. Anastomosis in the symbiotic mycelia of *Glomus hoi*, *G. mosseae*, and *Glomus proliferum* ranged from 44% to 100% (Giovannetti *et al.*, 2001; 2004; de la Providencia *et al.*, 2005; Voets *et al.*, 2006). The type of inoculum likely was not a factor since populations of spores were used in all of these studies as well as the one reported here.

The frequency of anastomosis in *G. intraradices* was 13.9%, which is much lower than the 88% to 100% observed by de la Providencia *et al.* (2005) and Voets *et al.* (2006), respectively. However, the frequency of anastomosis could vary greatly between ecologically and geographically disjunct populations of a species, as has been observed in some basidiomycetes (Sbrana *et al.*, 2007). Differences in cultivation systems may also influence the rate of hyphal anastomosis because of the variation in physiological processes and/or gene expression. Anastomosis in *G. intraradices* has been studied previously only in root organ cultures, which represent a selective and atypical growth environment for AMF. True comparative studies of root organ versus whole plant cultures have yet to be conducted to test the nature and magnitude of those differences.

An overall anastomosis frequency of less than 15% in glomeromycete fungi is low compared to that of fungal species in other groups. Isolates of the basidiomycete *Sclerotium rolfii* show no evidence of incompatibility reactions (Punja & Grogan, 1983). In the ascomycete *T. borchii*, the percentage of anastomosis ranges between 50 and 88% (Sbrana *et al.*, 2007). In *Rhizoctonia solani*, anastomoses occur in more than 50% of hyphal contacts (Hyakumachi & Ui, 1987).

The low frequency of anastomosis in AM fungi might be causally linked to the structure of their mycelia, which is coenocytic coupled with rapid protoplasmic streaming (personal observation; Jany & Pawlowska, 2010). Although little is known about protoplasmic flow within a hyphal network, the absence of septation may allow for the continuous homogenization of cytoplasm in the different parts of a mycelium. Anastomosis then would not impact significantly on the redistribution of nutrients. Gregory (1984) reports that the coenocytic zygomycete fungi lack the ability to anastomose. However, no relationship between the inability to anastomose and mycelium structure or behavior has been established empirically.

In other fungal phyla, anastomosis allows the distribution of extra chromosomal DNA elements across a mycelium (Glass *et al.*, 2000). A majority of studies report the frequency of hyphal fusions per hyphal length or focus on pairing isolates of different vegetative compatibility groups for the transmission of genetic elements such as viruses (Correll *et al.*, 1989; Liu & Milgroom, 1996). At the present time, it is not known if members of any glomeromycete species harbor plasmids or viruses. Anastomosis possibly plays an important role if AMF are heterokaryonts by compensating the effect of genetic drift (Pawlowska & Taylor, 2004; Bever & Wang, 2005), but details of the AMF genetic organization still are not decisively understood.

No sexual stage has been demonstrated in AM fungi; so, the only means for gene transfer among individuals is through the vegetative fusion of mycelia (Croll *et al.*, 2009). Low percentages of anastomosis would suggest that the gene flow among “individuals” of AM fungi also likely is low. Other mechanisms must be active to provide genetic variability and compensate for genetic isolation.

Even though single isolate cultures were used in this study, inocula did not originate from a single spore. Therefore, the symbiotic hyphal interactions observed in all species at present must be interpreted within the context of a population with the possibility that more than one

genotype was present. Even so, the absence of anastomosis in all of the genera tested except *Glomus* group A remains enigmatic.

Acknowledgements

The authors wish to thank Bill Wheeler and Robert Bills for the help in building and maintaining the rhizohyphatrons. We would like to thank Dr. J. Yao for the use of the inverted microscope in his laboratory. We are especially indebted to Dr. Teresa Pawlowska for insightful guidance and mentorship. Funding was provided by a Fulbright/CAPES Ph.D. scholarship to Sonia Purin and NSF grant DEB-0649341 to Joseph Morton.

Table 1. Accessions obtained from the INVAM for the *in situ* evaluation of anastomosis.

Species	Accession code	Origin – contributor
<i>Ambispora leptoticha</i>	CR312	Costa Rica – L. Aldrich-Wolfe
<i>Gigaspora gigantea</i>	MN414D	Minnesota, USA - F. Pfleger
<i>Glomus clarum</i>	WV101	West Virginia, USA - J. B. Morton
<i>Glomus intraradices</i>	ON201B	Ontario, Canada - T. McGonigle
<i>Paraglomus occultum</i>	WY112A	Wyoming, USA – D. Watson
<i>Scutellospora heterogama</i>	SN722	Singapore - I. Louis

Table 2. Type and number of hyphal contacts indicative of compatibility and/or incompatibility of symbiotic external hyphae from the six glomeromycotan species tested, as measured on a 1×1-mm grid overlaying an agar-coated slide.

Vegetative compatibility in species:	Short bridges	Long Bridges	Parallel growth and fusion	Total compatible contacts
<i>Glomus clarum</i>	5	4	2	11
<i>Glomus intraradices</i>	14	12	0	26
Vegetative incompatibility in species:	Septation	Change in shape	Septation + change in shape	Total incompatible contacts
<i>Ambispora leptoticha</i>	5	10	1	16
<i>Gigaspora gigantea</i>	38	0	0	38
<i>Glomus clarum</i>	44	4	5	53
<i>Glomus intraradices</i>	3	7	4	14
<i>Scutellospora heterogama</i>	25	0	0	25
<i>Paraglomus occultum</i>	0	0	0	0

Table 3. Total number of hyphal contacts observed in the six glomeromycotan species tested. The hyphal contacts were classified as “compatible,” “incompatible,” and “no recognition.” The frequency of contacts (calculated as a percentage) was determined from the number of hyphal contacts in each category divided by the total number of contacts.

Species	Number of contacts	Compatible contacts (%)	Incompatible contacts (%)	No recognition (%)
<i>Ambispora leptoticha</i>	149	0	10.7	89.3
<i>Gigaspora gigantea</i>	247	0	15.4	84.6
<i>Glomus clarum</i>	165	6.7	32.1	61.2
<i>Glomus intraradices</i>	187	13.9	7.5	78.6
<i>Paraglomus occultum</i>	118	0	0	100.0
<i>Scutellospora heterogama</i>	189	0	13.2	86.8

Figure 1. Design of a rhizohyphatron used to evaluate interactions among external hyphae of arbuscular mycorrhizal fungi originating from symbiotic mycorrhizal plants. a) Illustration of the assembled unit: a vertical plant compartment containing preinoculated mycorrhizal seedlings, a lateral slide compartment for the placement of two agar-coated glass slides, a nylon mesh with 41- μ m openings so that only hyphae from the plant compartment are able to grow into the slide compartment, and a nylon mesh-and-cap combination to keep the slides in place and seal the slide compartment. b) An assembled unit containing two 4-week-old mycorrhizal sorghum plants. c) The slide compartment consisting of a 3.4 $\times$ 7-cm PVC tube with a centered 3.3-cm opening to observe and monitor hyphal growth on top slide and plastic supports for glass slides made from pot labels and affixed with 100% silicon. d) End cap and nylon mesh with 40- μ m openings to cover each end of the pictured slide compartment.

Figure 2. a) Illustration of AMF mycelia growing on a cover slip coated with agar. b) cover slip overlaying a 1×1-mm grid to evaluate anastomosis. The hyphal tip most distal from the side of the cover slip nearest the plant compartment was identified as the reference point for measurements. Hyphal contacts were evaluated in alternate squares of 1-mm-wide columns (labeled 1–11) starting at the reference edge.

Figure 3. Hyphal contacts in the two *Glomus* species that produced compatible hyphal fusions (anastomosis). a) and b) Formation of a short bridge between the hyphae of *G. intraradices* in close proximity. c) Formation of a long bridge between the more distant hyphae of *G. intraradices*. d) Fusion of walls of *G. clarum* hyphae in direct contact with each other. Bars = 25 μm .

Figure 4. Hyphal contacts suggestive of vegetative incompatibility in two AMF species. a) Septa formation in a hypha of *G. clarum*. b) Change in shape of a hyphal branch of *G. clarum*. c) Septation and change in shape of a hyphal branch of *G. clarum*. d) No recognition between the hyphae of *G. gigantea*. Bars = 25 μm.

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CHAPTER 2

ASYMBIOTIC AND SYMBIOTIC HYPHAE OF THE ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS CLARUM* HAVE UNIQUE ROLES IN NON-SELF RECOGNITION²

Abstract

The life history of arbuscular mycorrhizal fungi (AMF, Glomeromycota) consists of a short asymbiotic phase (germinating spores) and a long symbiotic phase (mycelium associated with a host plant). To date, non-self anastomosis has been studied only in the asymbiotic phase. Two anonymous markers of microsatellite-flanking regions were used to genotype single spore isolates of *Glomus clarum* from different environments and geographic locations. Patterns of self and non-self recognition as measured by hyphal anastomosis were evaluated in genetically identical and different isolates during both asymbiotic and symbiotic phases. An axenic system was used to examine anastomosis in germinating spores. A rhizohyphatron was used to evaluate anastomosis in symbiotic hyphae. Self anastomosis occurred at similar frequencies (8-38%) in germinating spores and symbiotic mycelia. Non-self anastomosis was observed only in germinating spores and occurred between both genetically identical and different isolates, at less than 6% of hyphal contacts. Non-self anastomosis was confined to asymbiotic hyphae of *G. clarum*. This result provides evidence of functional differences between asymbiotic and symbiotic hyphae and that the former provides a short opportunity for gene flow between populations of this AMF species.

² Submitted for publication in the form of a research manuscript to the Journal “Fungal Biology”.

Introduction

Arbuscular mycorrhizal fungi (AMF, Glomeromycota) form mutualistic association with a broad range of terrestrial plant species. Like other fungi, AMF are modular organisms and thus have considerable flexibility in growth and behavior, with no defined birth or death. Hyphae within a mycelial network often come in contact with each other and can interact to fuse in the process of anastomosis. Anastomosis can occur in either an asymbiotic phase, when spores germinate to produce one or more germ tubes that elongate and branch in search of roots to colonize, or in a symbiotic phase, where hyphae grow both within and outside a mycorrhizal root. Growth of the fungus in the absence of a host is of brief duration, rarely extending beyond a few weeks (Bécard & Piché, 1990). External hyphae exist as several forms (Friesse & Allen, 1991) and either grow along root surfaces or expand into the surrounding soil and scavenge for mineral nutrients such as phosphorus and nitrogen (Li *et al.*, 1991; Smith *et al.*, 2001). Self anastomosis occurs within a mycelium produced by one spore and is a universal phenomenon in all fungi studied so far (Glass & Kulda, 1992). This process performs the essential function of interconnecting different parts of the mycelium to redistribute water and nutrients within an expanding colony (Gregory, 1984). Non-self anastomosis occurs when mycelia derived from different spores establish contact. This process is advantageous when different alleles other than *vic* or *het* co-occur in hyphae with a heterokaryotic nuclear condition. For example, different enzyme forms can increase the ability of a fungus to grow on different nutrient substrates (Jinks, 1952; Caten & Jinks, 1966). In AMF, non-self anastomosis could help maintain genetic diversity and compensate for the presumably low diversity caused by absence of sexual recombination (Bever & Wang, 2005). Despite the theoretical advantages of heterokaryon formation, non-self anastomosis occurs with low frequency in most fungi (Aanen *et al.*, 2008). This process is hypothesized to be risky because some populations harbor deleterious cytoplasmic viruses that are transmitted following fusion of hyphae (Liu & Milgroom, 1996; Cortesi & Milgroom, 1998). The incidence of aggressive genotypes and parasitic nuclei in some fungal populations also may explain the rarity of non-self anastomosis (Debets & Griffiths, 1998).

Anastomosis behavior in AMF has been studied mostly by examination of hyphae from germinating spores or mycorrhizal roots that originated from fungal populations of undefined genetic origins (Giovannetti *et al.*, 1999; 2001; 2003; 2004; de la Providencia *et al.*, 2005; Cardenas Flores *et al.*, 2010; Sbrana *et al.*, 2010). Since the AMF organism is of diffuse structure

and organization, an “individual” or “self” can be defined only by a thallus derived from a single spore or a single spore-derived culture (Croll *et al.*, 2009). The term “non-self” refers to interactions between different individuals, whether they are genetically identical or genetically different (Glass & Kaneko, 2003; Glass *et al.*, 2004).

Self anastomosis has been studied in only a few AMF species and the patterns appeared to differ both between the asymbiotic and the symbiotic phase as well as among different lineages of glomeromycotan fungi. During the asymbiotic phase, self anastomosis between paired spores can range from 46 to 51% in *Glomus intraradices* (Croll *et al.*, 2009). During the symbiotic phase, self anastomosis in *G. intraradices* and *G. proliferum* ranges from 80 to 88% within a symbiotic network, but these numbers decrease to 12-20% when mycelia are paired (Voets *et al.*, 2006). In contrast, self anastomosis within a mycelium from mycorrhizal roots ranged between 1 and 10% in *Scutellospora* and *Gigaspora* species, but was absent between paired mycelia (de Souza & Declerck, 2003; de la Providencia *et al.*, 2005).

Non-self anastomosis has been studied only during the asymbiotic phase of the life cycle of one species, *G. intraradices* (Croll *et al.*, 2009). The average frequency of compatible hyphal contacts was 3%, with some evidence suggesting that allelic variation was transmitted to progeny spores. The occurrence of non-self anastomosis during the symbiotic phase remains unknown, even though it comprises most of an AMF life cycle. An experimental constraint has been the absence of a cultivation system that allows for the *in situ* observation of hyphal interactions between mycelia from paired mycorrhizal plants.

In this study, self and non-self anastomosis were examined in both the asymbiotic phase (germinating spores) and the symbiotic phase (mycorrhizal external hyphae) of *Glomus clarum*, a species closely related to *G. intraradices* (Schüßler *et al.*, 2001). We used an axenic Petri dish system (Croll *et al.*, 2009) to measure asymbiotic interactions and a “rhizohyphatron” (Purin & Morton, 2011) to observe *in situ* anastomosis between AMF hyphae in the symbiotic phase. “Self” and “non-self” interactions were evaluated between genetically similar and genetically different isolates of *G. clarum* identified using two anonymous genetic markers. We hypothesized that both self and non-self anastomosis can occur during the asymbiotic as well as the symbiotic phase. Contrary to this expectation, non-self anastomosis was observed only during the asymbiotic phase.

Materials and methods

Sampling sites and establishment of single spore cultures

Single spore cultures of *G. clarum* Nicolson & Schenck (henceforth defined as isolates) originated from two distinct environments: abandoned coal stripmine sites and pasture mixtures. The mine sites were located in Morgantown, WV (39°38'1"N 79°57'2"W), supporting plant communities composed of *Andropogon virginicus*, *Acer rubrum*, *Crataegus* species, *Dicranum scoparium*, *Liriodendron tulipifera*, *Oxydendrum arboretum*, and *Solidago* species. Soil properties were pH 3.0-3.3, 2.8-4.2 mg kg⁻¹ Olsen-extractable P, and 229-466 mg kg⁻¹ Al. Pasture mixtures were located near Logan, WV (37°50'54"N 81°59'16"W) and Cassville, WV (39°39'43"N 80°03'03"W), soils of both characterized by pH of 6.6-6.9, 9.3-12.8 mg kg⁻¹ Olsen-extractable P, and 0.9-1.6 mg kg⁻¹ Al. Plant communities consisted of *Agrostis alba*, *Achillea millefolium*, *Festuca eliator*, *Lolium perenne*, *Phleum pratense*, *Plantago lanceolata*, *Trifolium pratense*, and *Trifolium repens* hydroseeded 5-6 years previously following disturbance. Multiple rhizosphere samples were collected from each location (Fig. 1). Roots and attached soil were collected from the rhizosphere of dominant plants in each mine site and pooled into a 300 mL sample. Isolates from the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM) were used as the source of spores from pasture sites.

AMF spores were extracted from soil by wet-sieving and sucrose gradient centrifugation (Gerdemann & Nicolson, 1963), washed, collected under a dissecting microscope and surfaced cleaned by sonication in distilled water. Fifteen spores of *G. clarum* were selected randomly from the population at each location to start single spore cultures. Each spore was pipetted onto intertwined roots of two 10-12 day-old sorghum (*Sorghum sudanense* L.) seedlings. Plants were transferred to 3.8 x 21 cm (150 cm³) SC10 Supercell "cone-tainers" (Stuewe and Sons, Inc., Corvallis, OR) filled with a mixture of steamed-sterilized soil and Terragreen (Oil Dri Corp., Chicago, IL) in a 1:2 ratio (v/v). Cone-tainers were transferred to a growth room with 420 µE m⁻² sec⁻¹ light intensity for a 14 hour day period and air temperature ranging from 25-28°C. Plants were watered *ad libitum* daily. After 4 months, sporulating cultures were transplanted to 7.5 x 25 cm (600 cm³) D40 "deepots" filled with Terragreen. Sorghum seeds surface-sterilized in 10% bleach for 10 min and numbering 50-60 were placed in each deepot and covered with a thin layer of Terragreen. Plants were grown for four months under conditions described above. Cultures

showing evidence of abundant sporulation were air-dried *in situ* for two weeks, pot contents bagged after removal of plant tops, and stored in sealed zip-loc bags at 4°C.

Molecular markers

Microsatellite-flanking regions were used as molecular markers. PCR was performed using select microsatellite repeats known to occur in the genome of some AMF species as primers (Table 1). Genomic DNA of *Glomus clarum* was extracted from a pooled sample of approximately 2000 spores collected at random from all single spore cultures. Spores were surface-cleaned by sonication in distilled water and transferred to 1.5 mL Eppendorf tubes containing 0.5 mm glass beads. Spores were broken using a mini-bead beater (Biospec, USA). Genomic DNA was extracted using a DNeasy plant mini kit (Qiagen) following the manufacturer's protocol.

PCR was performed in a 50-μL volume containing 5.0 μL of genomic DNA, 1.0 U Taq DNA polymerase (New England Biolabs, USA), 7.0 μL 10× PCR buffer, 3.5 mM MgCl₂, 1.25 mM each of dNTPs, and 1.0 μM primer. Amplification conditions were as follows: initial denaturation at 94°C for 2 min; 30 cycles with denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 1 min; followed by final extension at 72°C for 10 min. The annealing temperature differed for each primer pair (Table 1). Amplicons were visualized by electrophoresis on 1.5% agarose gel stained with ethidium bromide. Bands were excised and purified using a Gel extraction kit (Qiagen, USA). Purified PCR products were cloned using a cloning kit (Qiagen, USA). Recombinant colonies were grown overnight in 5 mL of liquid LB medium with ampicillin at 37°C. Plasmid DNA was purified using a Miniprep kit (Qiagen, USA) and sequenced with M13F and M13R primers using the Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI 3730XL DNA Analyzer (Applied Biosystems). Sequencing was performed by the West Virginia University Genomics Laboratory. Sequences were edited using Bioedit (Hall, 1999). Sequences were subjected to nucleotide query BlastN search (Altschul *et al.*, 1990) of the National Center for Biotechnology Information (NCBI) databases. Sequences with high similarity to bacterial or fungal DNA sequences were discarded from further analyses. Forward and reverse primers were designed based on remaining sequences deposited in Genbank as accession numbers JF430217-JF430226, excluding the 5' and 3' microsatellite repeats to decrease the risk of no amplification if the number of repeats was

variable among single spore cultures. Primers listed in Table 2 were designed using the software Primer 3 (Rozen & Skaletsky, 2000).

Genotyping of single spore cultures

Two single spore cultures from each location (Fig. 1) were selected for genotyping using newly-designed primers (Table 2). Genomic DNA was extracted from 2000 progeny spores from each culture using a DNeasy plant mini kit (Qiagen). PCR was performed under the same reaction conditions described above, except that a proof-reading enzyme (Tgo DNA polymerase, Roche Applied Science) was used. Amplification conditions were as follows: initial denaturation at 94°C for 2 min; 20 cycles with denaturation at 94°C for 30 s, annealing for 60 s, and extension at 72°C for 45 s; followed by final extension at 72°C for 7 min. Annealing temperature differed for each primer pair (Table 2). PCR products were visualized and purified as described above and sequenced directly. Sequences were aligned in Clustal X (Thompson *et al.*, 1997) and inspected for differences.

One primer pair (*Gcla*1155-98F/*Gcla*1155-1058R) failed to amplify DNA of some isolates. Another primer pair (*Gcla*550-80F/*Gcla*550-497R) produced non-specific amplification products in some isolates. Only two primer pairs (*Gcla*690-61F/*Gcla*690-553R and *Gcla*650-32F/*Gcla*650-552R) generated sequences that were polymorphic among isolates. The *Gcla*650-32F/*Gcla*650-552R fragment was highly similar to published sequences from other AMF. A sequence between nucleotide positions 348 and 445 was 94% similar to one from *Glomus intraradices* (NCBI accession AC237359.1). Conversely, no similarity was found between the *Gcla*690-61F/*Gcla*690-553R fragment and Genbank AMF sequences. All other primer pairs produced monomorphic sequences and thus were uninformative. Sequences obtained from all fungal isolates were deposited in Genbank as accession numbers JF430227-JF430260.

Validation of AMF genotypes

Single spore isolates 6AmA#2 and 6AmA#3 were established as *in vitro* root organ cultures (Bécard & Fortin, 1988) to obtain contaminant-free mycelium. *Ri* T-DNA transformed carrot roots were grown in Petri dishes containing M medium in 3% Phytigel at pH 6.0 for 1 week at room temperature. Spores were extracted and surface-sterilized with chloramine T and streptomycin/gentamicin sulfate (Bécard & Fortin, 1988). Approximately 50 spores were placed

close to root tips in each plate and the plates were stored in the dark at 30°C for 8 weeks. The Phytigel was solubilized by adding 10 mM sodium citrate and incubating at 30°C for 15 minutes. Mycelia were filtered from the solubilized medium using a 15 µM nylon mesh and washed with distilled water. DNA was extracted using a DNeasy plant mini kit (Qiagen). PCR was performed using only primer pairs that generated polymorphic sequences in the genotyping of single spore cultures and the same cycling conditions described previously. Sequences from genomic DNA of both *G. clarum* isolates were identical to those obtained from pot culture samples, thus verifying that amplified DNA originated from *G. clarum*.

Phylogenetic analyses

Genetic marker sequences were concatenated and aligned using ClustalX (Thompson *et al.*, 1997). The appropriate model of nucleotide substitution was determined using MODELTEST 3.7 (Posada & Crandall, 1998). The TrN+I and TrN+G+I (Tamura & Nei, 1993) models adequately explained DNA substitutions. Neighbor-joining, maximum likelihood and maximum parsimony trees were constructed using PAUP 4.0b (Swofford, 1998), each with 1,000 bootstrap replications. Genetic distances among single spore cultures were computed in PAUP 4.0b (Swofford, 1998) using the TrN+I substitution model. The Mantel test was applied to verify the correlation between genetic and geographical distances using the software Arlequin 3.1 (Excoffier *et al.*, 2005).

Criteria to establish pairings between isolates

Anastomosis between AMF isolates was evaluated in selected pairings between genetically different and genetically similar cultures. Six pairings were established to evaluate self anastomosis within and between germinating spores of the following isolates: WV310#5, WV123A#6, WV123A#7, 1UnC#7, 6AmA#2 and CRwest#8. Non-self anastomosis was evaluated in three pairings between genetically similar isolates (WV310#5 x WV123A#6, WV123A#7 x WV123A#6 and WV123A#7 x WV310#5) and seven pairings between genetically different isolates (WV310#5 x 1UnC#7, WV123A#6 x 1UnC#7, WV123A#7 x 1UnC#7, 6AmA#2 x 1UnC#7, 6AmA#2 x CRwest#8, 1UnC#7 x CRwest#8 and 1UnC#5 x 1UnC#7).

Self and non-self anastomosis in symbiotic mycelia were evaluated using the same pairings described earlier. Six additional pairings were established to measure non-self anastomosis in a broader sampling of genetically similar isolates: 1UnC#7 x CRwest#8, CRcenter#12 x WV101#5, 6AmA#2 x 1UnC#5, Valley#4 x Valley#18, Valley#4 x 6AmA#3, Valley#18 x 6AmA#3. Another seven pairings were established to measure non-self anastomosis between a wider range of genetically different isolates: 1UnC#5 x CRwest#8, Valley#4 x 1UnC#7, Valley#4 x CRwest#8, Valley#18 x 1UnC#7, Valley#18 x CRwest#8, 6AmA#3 x 1UnC#7, 6AmA#3 x CRwest#8.

Anastomosis in asymbiotic hyphae

Spores were surface-sterilized (Bécard & Fortin, 1988) and placed on cellophane membranes positioned into two-compartment, 10-cm diameter Petri dishes. Two pieces of cellophane membranes were cut to fit the internal shape of each compartment. Membranes were placed on a thin layer of M medium, pH 6.0, lacking sucrose and vitamins (Bécard & Fortin, 1988). To evaluate self anastomosis within a mycelium, one spore was placed in the center of each compartment using sterile reverse-action tweezers. To evaluate self and non-self anastomosis between paired mycelia, two spores were placed 3 mm apart in the center of each compartment (Croll *et al.*, 2009). Fifteen replications were established for each single spore isolate and for each combination of paired spores.

Petri dishes were sealed with Parafilm and incubated at 28°C in the dark. After 14 days, germinated spores were observed for hyphal contacts using a Nikon Eclipse TE-2000S inverted microscope. For individual spores, all hyphal contacts were examined to detect any morphological changes. For paired spores, only the contacts between hyphae originating from different spores were examined. Compatible hyphal fusions were identified when interacting hyphae showed complete wall fusion and protoplasmic continuity (Giovannetti *et al.*, 1999; 2001; 2004). The number of compatible hyphal fusions was divided by the total number of observed hyphal contacts in order to calculate percentage of anastomosis.

Anastomosis in symbiotic hyphae

A modified rhizohyphatron (Purin & Morton, 2011) was used to monitor *in situ* hyphal interactions in the symbiotic phase of the AMF life cycle. Two versions of this system were

used: (i) a single plant compartment to measure self anastomosis within a mycelium (Fig. 3a, b) and (ii) two plant compartments to measure self and non-self anastomosis between paired mycelia (Fig. 3c, d). Both versions consisted of plant compartments containing actively-growing mycorrhizal plants and a compartment housing three agar-coated microscope cover slips on which hyphae from plant compartment(s) could grow. All plastic components of the rhizohyphatron consisted of Schedule 40 PVC pipe fittings. The plant compartment was made using a 4.8 cm (inside diameter) 90° PVC elbow. Two small holes were drilled in the base of the elbow for drainage. The cover slip compartment was a 4.0 x 4.0 cm section of a PVC tube, within which plastic tabs to support cover slips were cut from 1.0 x 7.5 cm pot labels and affixed to the wall of the PVC tube using white General Electric 100% Silicon II sealant. One support (1.0 x 4.0 cm) spanned the center of the tube and the other two supports (1.0 x 3.5 cm) were spaced one cm above and below the center. Before assembly, all components were surface-sterilized by immersion in 10% bleach for 20 minutes followed by rinses with distilled water and 70% ethanol. Component parts then were exposed to ultraviolet light in a sterile hood for 20 minutes. At the time of assembly, the cover slip compartment remained empty. Each plant compartment was filled with premoistened, autoclaved Terragreen. Three 10-12-day-old sorghum seedlings were inoculated with approximately 200 spores by pipetting directly along the length of intertwined roots. Sorghum seedlings were transplanted into the Terragreen and the rhizohyphatrons were transferred to a growth room where they were maintained. Plants were watered *ad libitum* daily with double-distilled deionized water.

After mycorrhizal plants had grown for four weeks, rhizohyphatrons were transferred to a laminar hood to add cover slips. Cover slips (25 x 40mm, Ted Pella, Inc.) were coated with a thin layer of autoclaved 1.5% water agar containing 0.1 mg kg⁻¹ P (added as KH₂PO₄). To regulate thickness of the agar coating, 17 ml of autoclaved agar was poured over 10 cover slips arranged on the bottom of a flat 8.8 x 12.5 cm, 2cm deep plastic tray. Once the agar solidified, cover slips were excised using a sterile dissecting blade and positioned in the compartment. In all units, care was taken to ensure that cover slips were in close contact with the nylon mesh so that hyphae from the plant compartment would migrate easily onto the agar surface of each cover slip. Rhizohyphatrons were returned to the growth room for ten days, at which time cover slips were removed from each unit for microscopic observation.

Hyphae from paired plant compartments overlapped so that interactions were examined in detail in the central 30 mm region of each cover slip. Spores of *G. clarum* formed on all cover slips, thus confirming species identity. In all of the pairings made in this study, no fungal contaminants were observed. Contamination was eliminated by repeated cleaning of all adjacent surfaces with 70% ethanol and watering of plants only with double-distilled water.

All contacts between hyphae originating from opposing plant compartments were evaluated for morphological changes. Hyphal contacts were examined in three cover slips for each pairing (approximately 300 hyphal contacts per pairing). Hyphae from each contact point were tracked visually back to both ends of a cover slip to verify compartment of origin. Compatible hyphal fusions were identified and percentage of anastomosis was calculated as for germinating spores.

Percentages of self anastomosis were compared between single and paired spores using the t-Test. Spearman's rank correlation coefficient was calculated between the following combinations of variables: geographic distance + percentage of non-self anastomosis in germinating spores and genetic distance + percentage of non-self anastomosis in germinating spores. Analyses were performed using the software JMP version 5.0 (SAS Institute 1989-2002).

Results

Genetic diversity of *Glomus clarum* at pasture and mining sites

The 1014-bp concatenated sequences of two anonymous genetic markers *Gcla*690-61F/*Gcla*690-553R and *Gcla*650-32F/*Gcla*650-552R (Table 2) used to genotype 17 isolates of *Glomus clarum* differentiated 15 distinct haplotypes. Isolates collected from Logan and Cassville pasture habitats showed little genetic differentiation despite a distance of almost 200 km between sites (mean pair-wise genetic distance of 0.002 ± 0.002 SD; Fig. 1, 2). In contrast, isolates from habitats disturbed by coal-mining were distinct genetically even though they all originated from sites less than 20 km apart (mean pair-wise genetic distance of 0.008 ± 0.003 SD; Fig. 1, 2). The Mantel test revealed no significant correlation between geographic and genetic distance ($P = 0.678$).

Anastomosis in asymbiotic mycelia

Extensive asymbiotic hyphal networks emerging from germinating spores of *G. clarum* were formed in 14 days. The percentage of self anastomosis in a mycelium from a germinating spore ranged from 6 to 21%. Self anastomosis occurred at higher frequencies in hyphae merging from two spores ($24.9\% \pm 3.7\%$ SE) than in hyphae growing from one spore ($12.6\% \pm 3.7\%$ SE) ($P < 0.05$; Table 3). Non-self anastomosis was detected at low frequency in all pairings between genetically similar isolates from geographically close pasture sites (Table 4). Successful anastomosis was evident by complete wall fusion (Fig. 4a) and protoplasmic flow. When spores of genetically different isolates were paired, anastomosis was observed at an average frequency of 5.8% (Table 4, Fig. 4b, c) only between isolates 1UnC#5 and 1UnC#7 from a mining site. Negative correlations were observed between genetic distance and percentage of non-self anastomosis (Spearman's $\rho = -0.664$; $P = 0.036$) and between geographic distance and percentage of non-self anastomosis (Spearman's $\rho = -0.739$; $P = 0.014$).

Anastomosis in symbiotic mycelia

Self anastomosis within a mycelium growing from a single plant compartment averaged $8.6\% \pm 3.8\%$ SE over all isolates (Table 3), with a range of 2-16%. Self anastomosis between mycelia from two plant compartments was considerably higher, averaging $21.4\% \pm 3.8\%$ SE over all isolates ($P < 0.05$, Table 3). All fusions were characterized by formation of bridges between paired hyphae (Fig. 5). Complete fusion between hyphae was confirmed by continuity in protoplasmic streaming. Non-self anastomosis was absent in all pairings, regardless of whether the paired isolates were genetically similar or different from each other (Table 4).

Discussion

In this study, clear distinctions were evident between asymbiotic and symbiotic phases of hyphal growth in *G. clarum*. Self anastomosis occurred in both asymbiotic and symbiotic hyphae, but non-self anastomosis was confined only to asymbiotic hyphae. Patterns of non-self interactions in symbiotic AMF mycelium, therefore, appear to be unique compared to other fungal groups. In Ascomycetes, hyphal interactions result in either fusion or rejection responses (Glass & Kaneko, 2003). In AMF, neither response occurs once the fungus establishes symbiosis, so that inter-individual recognition is absent.

The two phases of the AMF life cycle are easy to separate methodologically, thus providing the means to assess functional differences. Asymbiotic hyphae are examined exclusively in spore germination assays (Giovannetti *et al.*, 1999; Croll *et al.*, 2009). Symbiotic hyphae require living hosts, and so they were examined in a rhizohyphatron designed to observe interactions of hyphae traceable to mycorrhizal plants (Purin & Morton, 2011).

Isolates of *G. clarum* could not be genotyped using primers that amplified microsatellite repeats in *G. intraradices* (Croll *et al.*, 2008; Mathimaran *et al.*, 2008), even though this species is in the same phylogenetic clade (Schwarzott *et al.*, 2001; Silva *et al.*, 2006). Analysis of the *G. intraradices* amplicons using these primers revealed that 46-90% nucleotide variation is in the microsatellite-flanking regions instead of in the repeat itself (Croll *et al.*, 2008; Mathimaran *et al.*, 2008). Even in ascomycete and basidiomycete fungi, variability in microsatellite-flanking regions is high (Guerin *et al.*, 2004; Gauthier *et al.*, 2007). Only two of ten products of flanking regions amplified by microsatellite primers were polymorphic in *G. clarum*. Similarly, only 18 of 80 primer pairs yielded polymorphic sequences in *G. intraradices* (Mathimaran *et al.*, 2008).

Despite only two markers, distinct genotypes in the two single spore cultures from each of the nine sampling sites were differentiated. Only in the location WV123A were both isolates genetically identical. Differentiation observed in such small sample sizes indicates that any assemblage of spores collected from a location may contain more than one genotype. Therefore, studies using inocula from multiple spores are not likely to distinguish self from non-self interactions (Giovannetti *et al.*, 2001; 2003).

Asymbiotic hyphal networks from germinating spores of *G. clarum* were extensively formed in 14 days instead of the 35 days reported for *G. intraradices* (Croll *et al.*, 2009). An average of eight contacts was present per pairing of spores, compared to an average of one contact per paired spores of *G. intraradices*. Hyphal fusions might be of more limited benefit for initial “colony” formation in *G. clarum* than in other *Glomus* species. The overall frequency of self anastomosis in *G. clarum* (19%) was lower than the 46-51% measured in germinating spores of *G. intraradices* (Croll *et al.*, 2009) and the 36-69% measured in *G. mosseae* (Giovannetti *et al.*, 1999).

Non-self anastomosis was observed between germinating spores of genetically similar and different isolates. Pairings of similar genotypes from neighboring locations showed a frequency of non-self anastomosis of less than 3%. Even though the two markers were identical,

it is unlikely that these fusions were self interactions because self-anastomosis averaged 25% (Table 3). Since similar frequencies of non-self anastomosis were measured between genetically different isolates of *G. intraradices* (Croll *et al.*, 2009), the *G. clarum* non-self anastomoses likely occurred between isolates that differed in other loci of the genome.

Non-self anastomosis was observed only between isolates sampled from geographically close locations with similar environments. Likewise, *G. intraradices* was collected by Croll *et al.* (2009) from locations separated by less than 85 meters in the same field site. Non-self anastomosis in these two AMF species appears to occur only between isolates that are geographically close, suggesting that populations in similar environments are selecting for gene combinations that allow vegetative compatibility. Geographically distant isolates that also were genetically different failed to anastomose, as observed by Giovannetti *et al.* (2003) for *G. mosseae*. Non-self anastomosis between the genetically different isolates 1UnC#5 and 1UnC#7 collected from the same location provides further evidence to support this hypothesis.

Methods to study anastomosis in symbiotic hyphae have relied mainly on transformed root organ cultures (Bécard & Fortin, 1988). Our approach was to use a rhizohyphatron which permitted symbiotic hyphae from plants to grow on an agar-coated cover slip (Purin & Morton, 2011). The plant compartment from which a mycelium originated always could be identified because hyphae in the center of the cover slip were traceable back to a plant compartment.

Self anastomosis in symbiotic hyphal networks emerging from the same plant compartment averaged 8.6% across all isolates, which was only slightly higher than the 6.7% observed *in situ* from a genetically uncharacterized multi-spore culture of *G. clarum* (Purin & Morton, 2011). In both experiments, self anastomosis was considerably lower than the 80% and 88% measured for symbiotic hyphae of *G. proliferum* and *G. intraradices*, respectively, growing from root organ cultures (Voets *et al.*, 2006). This disparity may have a genetic basis, once frequencies of putative self anastomosis in paired symbiotic mycelia of *G. mosseae* range between 36 and 70% (Giovannetti *et al.*, 2004; Sbrana *et al.*, 2010). Alternatively, it also may be caused by differences in the experimental environment.

Non-self anastomosis was never observed between symbiotic mycelia of any isolate of *G. clarum*, regardless of genetic identity. The lack of anastomosis was not an artifact of the experimental environment, because hyphal fusions were always observed when an isolate was paired with itself. The frequency of anastomosis was two-fold higher in hyphae from paired

spores or plants than in hyphae originating from the same source. Germ tubes from some paired spores fused and formed a common hyphal network so fast that the zone of recognition could not be identified. Symbiotic hyphae from paired plants quickly established contact in the center region of the cover slip and produced more frequent bridges between long, thick runner hyphae rather than the fine hyphae on the periphery of each mycelium. These patterns indicate behavior of self recognition within a colony-forming network is distinct from that of hyphae meeting from separate colonies. Self anastomosis between hyphae originating from spatially separated plants has important implications for formation of common mycorrhizal networks (CMNs).

Interconnection of roots and nutrient transfer between plants are possible through CMNs, which can affect population and community-level processes (Giovannetti *et al.*, 2004; Simard & Durall, 2004). Our data suggest that CMNs are formed only between hyphae originated from the same parental spore.

Hyphal fusions were typically characterized by bridges similar in structure to those reported in other studies (Giovannetti *et al.*, 1999; 2001; Purin & Morton, 2011). Although nuclear staining was not performed, perfect fusion was evidenced visually by continuity in cytoplasmic streaming. Non-recognition characterized the majority of hyphal contacts, with no evidence of post-fusion incompatibility reported by Croll *et al.* (2009) in *G. intraradices* or by Sbrana *et al.* (2010) in *G. mosseae*. Septation was observed in approximately 25% of the contacts between asymbiotic hyphae and in 5% of the contacts between symbiotic hyphae. However, septa are observed in hyphal tips and certain regions of the mycelium during normal growth of AMF, and so presence of these structures may not reflect signs of incompatibility.

The occurrence of non-self anastomosis only in germinating spores may not be unusual. Some ascomycete fungi are able to anastomose only after a short period following conidia germination (Read *et al.*, 2009). These species form specialized conidial anastomosis tubes (CATs) that allow fusion between vegetative incompatible individuals. The mechanism underlying fusion between CATs is unknown, but likely involves suppression of *vic* genes (Roca *et al.*, 2005). Similar behavior may occur in AMF, where genes regulating vegetative interactions are differentially expressed in germinating spores. Certain genes involved in AMF metabolism have been reported to be differentially expressed in either asymbiotic versus symbiotic hyphae (Requena *et al.*, 2003; Tamasloukht *et al.*, 2003; Requena *et al.*, 2007), and similar regulation may occur for genes involved in recognition.

Results of this study and those from Croll *et al.* (2009) are complementary. The frequency of self anastomosis in paired spores averaged 25% in *G. clarum* and 48% in *G. intraradices*. In non-self interactions, the frequency of anastomosis decreased in both species to less than 10%. Anastomosis behavior in other glomeromycotan genera has not been studied in detail, but likely differs from *Glomus*. For example, self anastomosis in symbiotic hyphae of representative *Gigaspora* and *Scutellospora* species growing from root organ cultures is less than 10% (de la Providencia *et al.*, 2005; Voets *et al.*, 2006) and completely absent when growing from plants in a rhizohyphatron (Purin & Morton, 2011). In *Paraglomus* and *Ambispora* as well, no self anastomosis is observed in symbiotic mycelia (Purin & Morton, 2011). With rare self anastomosis, non-self anastomosis likely will be even rarer in these lineages. All of the available evidence indicates that non-self anastomosis is a relatively rare phenomenon in glomeromycotan fungi, occurring mostly or possibly even exclusively during the asymbiotic phase of fungal growth and development. However, even the low frequencies of non-self fusions in asymbiotic hyphal networks of both *G. clarum* and *G. intraradices* still is sufficient to permit horizontal gene transfer and produce new genotypes in a population (Croll *et al.*, 2009).

Acknowledgements

The authors wish to thank Teresa Pawlowska for insightful guidance and Jianbo Yao for the use of the inverted microscope in his laboratory. Rhizohyphatrons were built and maintained with the help of Bill Wheeler and Robert Bills. We thank David Douds for the DC2 isolate of *Ri* T-DNA transformed carrot roots developed by Guillaume Bécard. Funding was provided by a CAPES/Fulbright Ph.D. scholarship to Sonia Purin and NSF grant DEB-0649341 to Joseph Morton.

Table 1. Microsatellite repeats used as PCR primers to amplify flanking regions in genomic DNA extracted from a pooled population of *Glomus clarum* spores.

Repeat	Reference	TA (°C)	Approximate amplicon size (bp)
(CA) ₉ *	Longato & Bonfante (1997)	45	-
(GTG) ₅	Longato & Bonfante (1997)	52	500, 600
(GACA) ₅ *	Longato & Bonfante (1997)	48	400, 650
(AAT) ₈	Douhan & Rizzo (2003)	37	-
(AAC) ₈	Douhan & Rizzo (2003)	55	650
(AGC) ₅ *	Douhan & Rizzo (2003)	50	550, 650
(CAT) ₈	Douhan & Rizzo (2003)	55	-
(CCG) ₅ *	Douhan & Rizzo (2003)	58	-
(CAA) ₆	Mathimaran <i>et al.</i> (2008)	43	-
(TAT) ₈ *	Mathimaran <i>et al.</i> (2008)	36	-
(TTA) ₈	Mathimaran <i>et al.</i> (2008)	37	-
(AATG) ₅ *	Mathimaran <i>et al.</i> (2008)	43	400, 600, 690, 770, 1155
(AATGGT) ₅ *	Mathimaran <i>et al.</i> (2008)	55	-

* The number of repeats was modified in order to increase the annealing temperature.

Table 2. Primer pairs used to amplify microsatellite-flanking regions from single spore cultures of *Glomus clarum*. Primers were designed from ten amplicons generated by microsatellite-primers (Table 1).

Origin of sequence: microsatellite – amplicon size (bp)	Primer name	Primer sequence (5'-3')	TA (°C)	Amplicon size (bp)
(GACA) ₅ – 400	<i>Gcla</i> 400-9F	GACAGACAGACAATACCAACCA	55	300
	<i>Gcla</i> 400-308R	ACTGCTGAATTTACGTGCTC		
(GACA) ₅ – 650	<i>Gcla</i> 650-32F	AGCCCATCCGTGTCTTT	55	521
	<i>Gcla</i> 650-552R	GCATTCATTCGAATCTGTTGG		
(AAC) ₈ – 650	<i>Gcla</i> 650-125F	TCATGTAATTCATAATACCTTCCAAT	55	448
	<i>Gcla</i> 650-572R	CGAAGAGAACGATAGTCATGGA		
(AGC) ₅ – 550	<i>Gcla</i> 550-80F	GTATCGGCGGCATCAAAC	57	418
	<i>Gcla</i> 550-497R	TAGCCGATAGCGGAAACG		
(AGC) ₅ – 650	<i>Gcla</i> 650-77F	ACCGTCACTGCCACATACG	56	555
	<i>Gcla</i> 650-631R	GCAGCAGCGATAGTTGGAA		
(AATG) ₅ – 400	<i>Gcla</i> 400-87F	CCCCTTAAGATGAACGGAATC	56	311
	<i>Gcla</i> 400-397R	TGAATGAATGAAATTAGAGGCTACC		
(AATG) ₅ – 600	<i>Gcla</i> 600-52F	TTTCCTTGGTCATCATGTCTG	57	502
	<i>Gcla</i> 600-553R	TTCCATTCATCATCCCAAC		
(AATG) ₅ – 690	<i>Gcla</i> 690-61F	CGACGCTATTCCCATCA	57	493
	<i>Gcla</i> 690-553R	CACCGCCAAATTGAACCT		
(AATG) ₅ – 770	<i>Gcla</i> 770-99F	ATCCGTCATCTCCGAACTTT	57	575
	<i>Gcla</i> 770-673R	GGCCGAATTTGTGACGTTAT		
(AATG) ₅ – 1155	<i>Gcla</i> 1155-98F	AAAATGGCCAATTGGAACC	57	961
	<i>Gcla</i> 1155-1058R	TCACCGTCCAATCAAGCA		

Table 3. Percentage of hyphal contacts that resulted in compatible hyphal fusion (self anastomosis) in relation to the total number of hyphal contacts observed. Fusions were observed in germinating spores (asymbiotic phase) and in extraradical mycelium of *Glomus clarum* growing from mycorrhizal plants (symbiotic phase).

Isolate	Asymbiotic phase		Symbiotic phase	
	Single spore	Paired spores	Single plant	Paired plants
1UnC#7	6.3	8.0	15.6	37.4
6AmA#2	6.6	19.7	10.3	24.5
CRwest#8	17.4	37.5	11.7	32.4
WV123A#6	13.4	30.3	2.4	5.4
WV123A#7	11.4	18.1	4.7	11.1
WV310#5	20.6	35.7	6.8	17.8
Average $\pm$ SE	12.6 $\pm$ 3.7 b	24.9 $\pm$ 3.7 a	8.6 $\pm$ 3.8 b	21.4 $\pm$ 3.8 a

Table 4. Genetic distance, geographic distance and percentage of contacts that resulted in compatible hyphal fusion (non-self anastomosis) in relation to the total number of hyphal contacts observed. Data from pairings where non-self anastomosis was evaluated in germinating spores (asymbiotic phase) and extraradical mycelium of *Glomus clarum* growing from mycorrhizal plants (symbiotic phase).

Pairing of isolates	Genetic distance (TrN+I)	Geographic distance (meters)	Anastomosis in asymbiotic phase (%)	Anastomosis in symbiotic phase (%)
6AmA#2 x 1UnC#7	0.013	15	0	0
6AmA#2 x CRwest#8	0.011	11000	0	0
1UnC#7 x CRwest#8	0.008	11000	0	0
1UnC#5 x 1UnC#7	0.011	0	5.8	0
1UnC#7 x WV310#5	0.014	190000	0	0
1UnC#7 x WV123A#6	0.014	190000	0	0
1UnC#7 x WV123A#7	0.014	190000	0	0
WV310#5 x WV123A#6	0.000	20	0.9	0
WV123A#7 x WV123A#6	0.000	0	1.6	0
WV123A#7 x WV310#5	0.000	20	2.2	0

Figure 1. Map of West Virginia state (USA) indicating sampled locations and respective distances. 6AmA, Valley, 1UnC, CRwest, CRcenter and CReast are abandoned coal stripmine sites near Morgantown. WV101 originated from a pasture near Cassville. WV123A and WV310 originated from sites in 4-6 year-old hydroseeded pasture mixture near Logan, WV. Two single spore cultures were obtained from each sampling population (except CReast, with one culture).

Figure 2. Neighbor-joining tree reconstructed from two concatenated sequences of microsatellite-flanking regions amplified from the *G. clarum* genome. Numbers at nodes indicate bootstrap values $\geq 50\%$. The model of nucleotide substitution is TrN+I, with a rate substitution matrix of A to G at 14.34 and C to T at 6.25 and proportion of invariable sites of 0.97.

Figure 3. Plant-fungal growth system (rhizohyphatron) designed to study anastomosis in isolates of *Glomus clarum*. a) Illustration of a single plant compartment unit. The cover slip compartment provides placement for three agar-coated cover slips. Nylon mesh with 41 μm openings (Sefar America Inc., Depew, NY) separates contents of the plant growth compartment from the cover slip compartment. Nylon mesh + cap tightly seal open end of the cover slip compartment. b) Assembled single unit containing sorghum seedlings preinoculated with spores. c) Illustration of a two plant compartment unit. Nylon mesh (blue lines) blocks roots so that only hyphae grow onto agar-coated cover slips in the cover slip compartment. d) Open rhizohyphatron showing mesh barrier, plastic supports for agar-coated cover slips spaced 1-cm apart in the center compartment, and sorghum seedlings preinoculated with spores.

Figure 4. Non-self anastomosis between asymbiotic hyphae of *Glomus clarum*. a) Fusion between hyphae of isolates WV123A#6 and WV123A#7, with insert showing complete wall fusion and protoplasm continuity, bar = 25 μm . b) Paired spores of the isolates 1UnC#5 and 1UnC#7, bar = 200 μm . c) Detail of region marked in (b) by a rectangle, bar = 25 μm .

Figure 5. Self anastomosis between symbiotic hyphae of *Glomus clarum* from paired plant compartments. a) A long bridge between two hyphae of isolate 1UnC#7 from opposite compartments (arrows), bar = 100 μm . b) Another long bridge from isolate 1UnC#7 forming between two hyphae from opposite compartments passing each other (arrows). Cytoplasmic flow into the bridge is evident, bar = 50 μm .

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CONCLUSIONS

The rhizohyphatron invented in this study is an experimental break-through because it permits the study of anastomosis in any culturable AMF species. This methodology allows a shift of focus from germinating spores to symbiotic hyphae, and both life cycle phases can be studied together. The rhizohyphatron can be used also as a comparative tool to study structure and behavior of mycelium in diverse AMF lineages.

Anastomosis mediates development of symbiotic hyphal networks in plants colonized by populations of *Glomus* species. Other taxonomic groups (*Ambispora*, *Gigaspora*, *Paraglomus*, *Scutellospora*) are unique in that symbiotic hyphae do not form linked networks via anastomosis when populations are used as source of inoculum. Such distinct behavior between *Glomus* and other AMF groups may indicate other unique symbiotic interactions/traits.

Self anastomosis occurs in both asymbiotic hyphae (germinating spores) and in symbiotic hyphae (mycorrhizae) of *G. clarum*. Non-self anastomosis only occurs in the asymbiotic phase at a low rate, so only during this short period is gene flow possible. Anastomosis of symbiotic hyphae from different plants can only occur when genotypes are identical (self anastomosis). The absence of non-self anastomosis in symbiotic hyphae likely extends to all other AMF lineages, since self anastomosis between paired colonies is absent in other groups such as *Gigaspora* and *Scutellospora*.

Distinct processes of non-self recognition in asymbiotic vs. symbiotic phases suggest that other physiological processes also may be unique to each phase of the life cycle and should be investigated. AMF seem to be unique amongst fungal groups because inter-individual interactions are limited to germinating spores. Once in an association, each individual grows and functions in isolation.

APPENDIX

Figure 1. Germinating spores of *Glomus clarum* isolates WV123A#6 (left) and 1UnC#7 (right) on a cellophane membrane over M medium. Note hyphal contacts (arrows) in regions where hyphae overlap. Bar = 100 µm.

Figure 2. Germinating spores of the *Glomus clarum* isolates WV123A#6 on a cellophane membrane over M medium. The main hypha from each spore fused to form one continuous hypha. Bar = 100 μ m.

Figure 3. Germinating spores of *Glomus clarum* isolates 6AmA#2 (left) and 1UnC#7 (right) on a cellophane membrane over M medium. The main hyphae from each spore are directed away from each other and thereby avoid any physical contact. Bar = 100 μm.

Figure 4. Germinating spore of *Glomus clarum* isolate WV123A#7 on a cellophane membrane over M medium. The main hypha branches immediately after emergence from the broken end of the subtending hypha. Two “spore-like” structures (arrows) are formed 14 days after germination. Bar = 25 μ m.

Figure 5. Symbiotic mycelium of *Glomus clarum* isolate CRwest#8 growing on a cover slip coated with 1.5% agar. Bar = 100 μm .

Figure 6. Symbiotic mycelia of *Glomus clarum* isolates 1UnC#7 (left) 6AmA#3 (right) growing on a cover slip coated with 1.5% agar. Hyphae from each isolate originated from mycorrhizal plant compartments in direct contact with the cover slip. Bar = 100 μ m.

Figure 7. Self anastomosis in symbiotic mycelium of *Glomus clarum* isolate 1UnC#7 growing on a cover slip coated with 1.5% agar. Two long bridges are formed (arrows) together with one spore. Bar = 50 μ m.

Figure 8. Symbiotic mycelium of *Glomus clarum* isolate 6AmA#2 growing on a cover slip coated with 1.5% agar. Intense sporulation occurs 6 weeks after inoculation of sorghum seedlings. Bar = 200 μm .

CURRICULUM VITAE

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Education

Ph.D. in Agricultural Sciences, with emphasis on Applied and Environmental Microbiology, Spring 2011 (expected), sponsored by Fulbright and CAPES
West Virginia University, Morgantown, WV, USA

M.Sc. in Soil Sciences, 2005
Universidade do Estado de Santa Catarina, Lages, SC, Brasil

B.S. in Biological Sciences, 2002
Universidade Regional de Blumenau, Blumenau, SC, Brasil

Research Experience

Research Assistantship, 2010 - 2011
West Virginia University, Morgantown, WV - Dr. Joseph Morton laboratory.
Maintenance of the INVAM culture collection and preparation of material supplied to other researchers. Establishment of *in vitro* root organ cultures of several isolates of arbuscular mycorrhizal fungi. Other responsibilities included ordering laboratory supplies, training and monitoring visitors in the laboratory.

Professional development

Workshop on Active Learning Techniques for the science, technology, engineering, and mathematics (STEM) disciplines
Office of Graduate Education and Life, West Virginia University, Morgantown, WV, 2010

Publications

Refereed Journal Articles

Purin S, Morton JB. 2011. *In situ* analysis of anastomosis in representative genera of arbuscular mycorrhizal fungi. *Mycorrhiza*. doi: 10.1007/s00572-010-0356-9.

Purin S, Rillig MC. 2008. Immuno-cytolocalization of glomalin in the mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. *Soil Biology and Biochemistry* 40: 1000–1003.

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Purin S, Klauberg Filho O, Sturmer SL. 2006. Mycorrhizae activity and diversity in conventional and organic apple orchards from Brazil. Soil Biology and Biochemistry 38: 1831–1839.

Book Chapters

Purin S, Klauberg Filho O. Glomalina: nova abordagem para entendermos a biologia dos fungos micorrízicos arbusculares. In: Siqueira, J.O.; Souza, F.A.; Cardoso, E.J.B.N.; Tsai, S.M. (Eds). Micorrizas: 30 anos de pesquisas no Brasil. Lavras: UFLA, 2010. 503-524.

Oral Presentations

In situ analysis of anastomosis in representative genera of Glomeromycotan fungi using a unique rhizohyphatron design.

14th Annual Davis College Graduate Research Day Conference, West Virginia University, Morgantown, WV, USA, 2010

In situ analysis of anastomosis in all genera of Glomeromycotan fungi using a unique rhizohyphatron design.

International Congress on Mycorrhizas (ICOM 6), Belo Horizonte, MG, Brasil, 2009

Teaching experience

College level

Guest Lecturer: “Beginner’s Guide to Genetics” (GEN 101), 2008

West Virginia University, Morgantown, WV

Topic: Meiosis, mitosis and errors in genetic recombination”

Teaching assistant: “Soil Microbiology”, 2004

Universidade do Estado de Santa Catarina, Lages, SC, Brasil

Topics: Concepts about microbial ecology, methods to assess diversity and activity of soil microbes, ecto and endomycorrhizal fungi.

Junior high school

Teacher: Science, chemistry and physics, 2006

Colégio Municipal de Indaial, Indaial, SC, Brasil

Awards

Ph.D. scholarship from Fulbright/CAPES, with complete funding for 4 years.

First place, Ph.D. paper presentation. 14th Annual Davis College Graduate Research Day Conference, West Virginia University, Morgantown, WV, USA, 2010

Second runner, oral presentation. International Congress on Mycorrhizas (ICOM 6), Belo Horizonte, MG, Brasil, 2009

Honorable mention, highest GPA in the graduating class, 2002
Universidade Regional de Blumenau, Blumenau, SC, Brasil

“Mérito Universitário Catarinense”, 2001. The State government of SC (Brasil) selected 3 students with outstanding academic performance to be awarded with an international trip. Full-time research activities were conducted at The University of Wisconsin Oshkosh, USA, for 30 days.

Service

Reviewer for the journals *Mycologia*, *Symbiosis* and *Scientia Agricola*

Reviewer of a proposal submitted to Fondecyt (Funding agency of the Chile government) – 2008

Member of the Search Committee for a faculty position in the Plant and Soil Sciences Division, West Virginia University, Morgantown, WV, 2010

Member of the Safety committee responsible for the South Agricultural Sciences Building Emergency Response Plan. West Virginia University, Morgantown, WV, since 2009

Part of the organizing committee of the National meeting on Soil Fertility, Plant Nutrition and Mycorrhizas (FERTBIO) in Brasil, 2004

Memberships

Gamma Sigma Delta Society, since 2009

International Mycorrhizal Society, since 2009