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THE EFFECTS OF PLANT INVASION ON ARBUSCULAR MYCORRHIZAL FUNGI: A REVIEW OF HOW THESE COMMUNITY DYNAMICS ARE STUDIED

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THE EFFECTS OF PLANT INVASION ON ARBUSCULAR MYCORRHIZAL FUNGI: A REVIEW OF HOW THESE COMMUNITY DYNAMICS ARE STUDIED

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ABSTRACT

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Arbuscular mycorrhizal fungi (AMF) are vital components in most plant communities and therefore in almost all terrestrial ecosystems. Historically, AMF symbioses have been observed through microscopy and spore isolation. Given advancements in molecular technologies over the last couple of decades, ecologists are now able to observe AMF community dynamics in a more complex way, thereby broadening the understanding of the scope of ecological interactions concerning AMF. One area of interest are the effects exotic plant invasions have on native AM fungal communities and consequently on native plant communities. Over the last 20 years there have been some interesting studies published indicating that the introduction of invasive alien plants has significant ecological impacts on native AMF populations. In this paper, I will examine the ecology of AMF and exotic plant invasion. Specifically, I will consider study design, previous research, molecular methods, data interpretation, limitations of AMF ecology technology and the future of AMF invasive ecology.

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Introduction

Most invasive exotic plants have been brought to North America from Europe and Asia. Because the latitudes and environments of Eurasia are similar to those of North America, alien plants that are introduced from one continent to another are often able to thrive in their new environment. Some invasive plants were intentionally brought to North America for ornamental or commercial use. For example, the forb *Lythrum salicaria* (purple loosestrife) was brought to North America for landscaping purposes because it bears beautiful purple inflorescences. Unfortunately, its aggressive growth and resistance to native pests were not anticipated, and the plant has now quickly colonized many native North American wetlands, creating dense monocultures and disrupting native wildlife patterns (Thompson et al. 1987). Garlic mustard (*Alliaria petiolata*) was intentionally brought to North America for use in erosion control, as a food source, and for its medicinal properties. It is now a tremendous ecological problem due to its thick colonization of native woodland habitats and disruption of beneficial fungal symbioses in native seedlings via allelopathy (Stinson et al. 2006). Other invasive exotic plants have been accidentally introduced in ship ballast or as contaminants in imported materials such as seeds and lumber. The submerged aquatic plant *Myriophyllum spicatum* (Eurasian water milfoil) was accidentally introduced to North America through ship ballast. *Myriophyllum spicatum* grows densely in North American lakes and causes both ecological and recreational problems (Smith and Barko 1990). Leafy spurge (*Euphorbia esula*), a non-native forb that colonizes many North American grasslands,

was introduced in part as a contaminant in the importation of European grass seed (Dunn 1985). No matter the mode of introduction, these invasive plants are able to grow aggressively outside their native range and can spread rapidly, thereby coming to dominate foreign ecosystems.

Invasive plants contribute to a variety of problems in native ecosystems. Due to their aggressive growth, invasive exotics create dense monocultures and inhibit the growth of native plants, thereby causing a shift in the composition of vegetation. The change in vegetation can be problematic for animal wildlife that depends on specific native vegetation for food and shelter. Furthermore, sensitive species that are threatened or endangered may become extinct due to the ecological changes caused by plant invasion. Because invasive plants often grow in dense monocultures, an overall increase in plant biomass usually occurs in an area that is colonized by an invasive plant. The hefty addition of plant material can change the frequency and intensity of natural wildfires, thereby affecting the natural rejuvenation of a given ecosystem (Brooks et al. 2004). The change in vegetation caused by alien weeds can also disrupt community interactions, such as relationships between pollinators and their preferential flowers (Ghazoul 2004), or the relationships between plants and the soil microbial community (Callaway et al. 2004). Prime examples of important plant-microbe relationships that may be affected by exotic plant invasion are mycorrhizal associations.

Mycorrhizal Fungi

Plant roots have adapted to perform a variety of functions: they facilitate uptake of nutrients and water from the soil, secure the plant in the soil, and store

photosynthates. However, exploiting soil resources is energetically expensive both in terms of the amount of root growth necessary to obtain sufficient resources and with regard to the metabolic processes necessary to convert ambient soil nutrient compounds into forms that are usable for the plant. Mutualistic symbioses between fungi and plant roots provide a means for plants to reduce some of the energetic stress associated with root growth, root absorption, and nutrient assimilation. In addition, fungal-plant mutualisms allow a plant to tap into soil resources that it may not otherwise be able to find or utilize.

Fungi that form mutualistic symbioses with the roots of plants are termed mycorrhizae. In Greek, "myco" means fungus and "rhiza" means root. In a mycorrhizal relationship, both the plant and the fungus receive benefits by growing in tandem. The fungus increases the root's surface area by sending its hyphae into the soil to absorb water and nutrients, thereby saving the plant the energy required to increase root growth and also reaching soil nutrients that the plant may not otherwise encounter. The acquisition and transfer of usable phosphates is one of the major services that mycorrhizae provide for their hosts, since phosphorous in commonly a limiting or co-limiting nutrient for plants. In the case of ectomycorrhizae, a fungal sheath (mantle) formed on the surface of the root tip may provide protection for the plant from soil pathogens and pests. The fungus benefits from the mycorrhizal symbioses by receiving photosynthates from the host plant in the form of sugars. Additionally, the plant roots provide a sheltered environment for at least some parts of the fungus. Many mycorrhizal fungi are obligate symbionts meaning that they are unable to live in the absence of the host plant.

It is estimated that 90% of all plants form symbioses with mycorrhizae (Cairney 2000). There are two major groups of mycorrhizal associations: ectomycorrhizae (ECM) and endomycorrhizae, which include the arbuscular mycorrhizal fungi (AMF also known as vesicular arbuscular mycorrhizae [VAM]), orchid mycorrhizae, and ericoid mycorrhizae. Ectomycorrhizal fungi are most common in woody plant species, mainly in conifers and hardwoods. Ectomycorrhizal associated plant families include the Fagaceae (Oaks, Chestnuts and Beeches), Salicaceae (Willows), Betulaceae (Birches) and Pinaceae (many Conifers). Most ectomycorrhizal fungi are classified in the phyla Basidiomycota and Ascomycota (Smith and Read 1997). Ectomycorrhizae are characterized by the formation of a hyphal sheath (also known as the mantle) on the exterior of a plant's roots. Ectomycorrhizae also form a Hartig net, an intercellular web of hyphae extending between the host plant's root cortical cells. Ectomycorrhizal fungi typically form macroscopic sexually-produced fruiting bodies, many of which are considered delicious edible mushrooms. Because most ectomycorrhizal fungi form macroscopic fruiting bodies, they are relatively easy to collect, identify, and study (although above ground fruiting does not necessarily reflect the extent of below ground colonization of roots by specific species). The dense mantle on roots colonized by ectomycorrhizal fungi allows for easy visual confirmation of fungal association. Additionally, the large amount of pure fungal tissue both on the mantle and of the fungal fruiting body provides ample genetic material for molecular identification and analyses.

In contrast, endomycorrhizal fungi grow within the root cortical cells of a plant, lack a mantle, and do not produce fruiting bodies. Because of these features,

endomycorrhizal colonization in plant roots is not readily observed without hyphal staining and light microscopy. Moreover, molecular identification has proven difficult because of the dearth of hyphae. The orchid mycorrhizal fungi (typically Basidiomycetes) exclusively form symbioses with members of the Orchidaceae and the ericoid mycorrhizal fungi (typically Ascomycetes) associate with plant hosts order Ericales, such as blueberry, cranberry and heathers (Peterson et al. 2004). Arbuscular mycorrhizal symbioses are the most common type of mycorrhizae and exhibit a variety of unique traits that have prompted taxonomists to create a new phylum specific to AMF, the Glomeromycota (Schüßler et al. 2001).

Arbuscular Mycorrhizal Fungi (AMF)

Until recently, the AMF were grouped in the order Glomerales (sometimes incorrectly spelled Glomales) in the phylum Zygomycota. However, with the recent advent of molecular phylogenetic studies, AMF have been assigned their own phylum, Glomeromycota, which houses the single class Glomeromycetes. The class Glomeromycetes consists of about 200 species, which have been historically differentiated based on the morphology of their spore walls (Redecker and Raab 2006). The Glomeromycetes consists of four orders: Archaeosporales, Diversisiporales, Glomerales, and Paraglomales, which have been most recently updated and distinguished based on molecular evidence (Schüßler 2001). Ten genera of AMF are currently recognized: *Acaulospora, Archaeospora, Diversispora, Entrophospora, Geosiphon, Gigaspora, Glomus, Pacispora, Paraglomus,* and *Scutellospora* (Redecker and Raab 2006) (Figure 1).

It is estimated that AMF infect about two thirds of the mycorrhizal plants in the world (Fitter and Moyerson 1996). Fossil evidence suggests that the first AMF-like organism evolved about 400 million years ago in the late Ordovician/early Devonian era, making the AM symbiosis one of the earliest symbioses in the fossil record (Redecker et al. 2000). These primitive fungi exhibited associations with bryophytic land plants, even before true roots had evolved (Brundrett 2002). This evidence suggests that AMF may have played a co-evolutionary role in the development of land plants and their root systems. Without the assistance of AM fungi, early plants may not have been able to obtain enough resources from the terrestrial environment. Therefore, it may be that the AM fungal symbiosis is largely responsible for the transition of plants from water to land (Brundrett 2002).

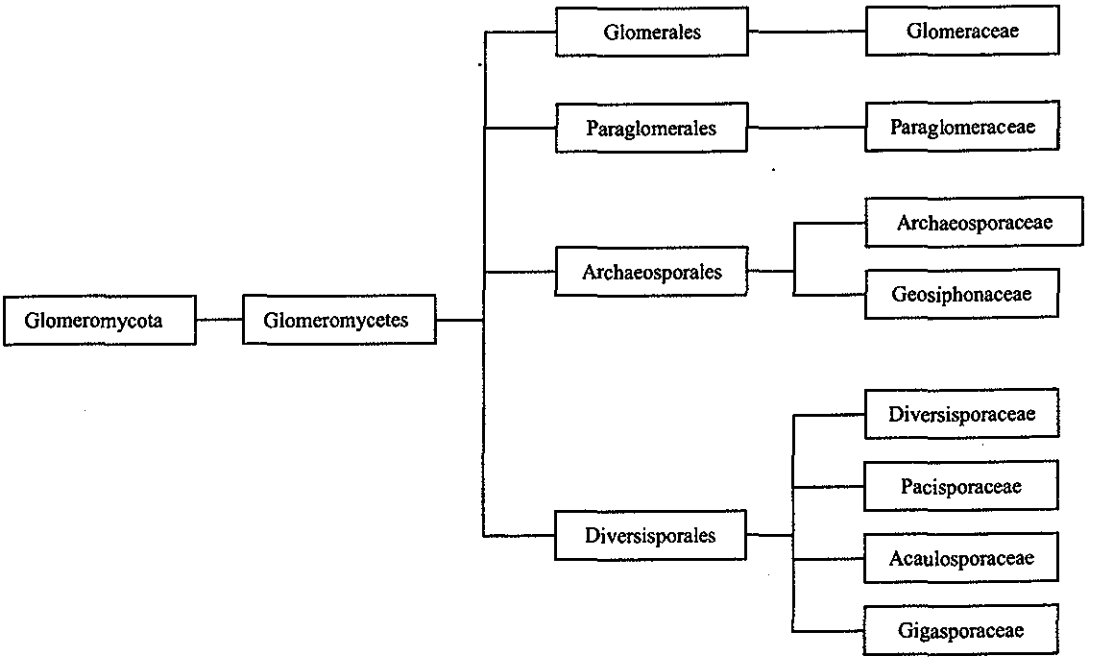

Figure 1. Classification of the Glomeromycota.

The Glomeromycota consist of eight families grouped into four orders that are all within the class Glomeromycetes.AMF commonly infect most herbaceous plants,

pteridophytes, bryophytes, and some woody plants. All of the AMF are considered obligate mutualists; a host is required for growth and reproduction. Unlike ectomycorrhizal fungi, AMF grow intracellularly in the plant roots. Modified haustoria (fungal infective structures) called arbuscules form within the cortical cells of an infected root to function in nutrient transfer between the fungal symbiont and the host. Some AMF also produce vesicles that probably function as storage structures for photosynthates acquired from the host plant (Smith and Read 1997) (Figure 2).

The AMF lifecycle is confined to the soil environment. Asexually produced spores serve as the primary method of AMF reproduction and dispersal. In fact, sexual reproduction has not been documented in any members of the Glomeromycota. Glomeromycotan sporulation most commonly occurs in the soil, where spores are released individually or tightly packed into sporocarps. Sporulation of AMF has been observed within infected root tissue, especially within the genus *Glomus*, but does not appear to be as common as sporulation within the soil environment (Smith and Read 1997). The main modes of AMF spore dispersal include water, air and small animals (Peterson et al. 2004). Upon germination, the spore produces infective hyphae that penetrate the root tissue of the host plant. Arbuscular mycorrhizal fungi may also spread to other roots or even a new host via a previously infected root. An extraradical hypha from a colonized root can penetrate into the root tissue of a new host, thereby manifesting a new infection. Host plants elicit chemical signals in the form of strigolactones, which attract AMF hyphae in the soil and cause them to increase their growth to reach the host root (Akiyama et al. 2005).

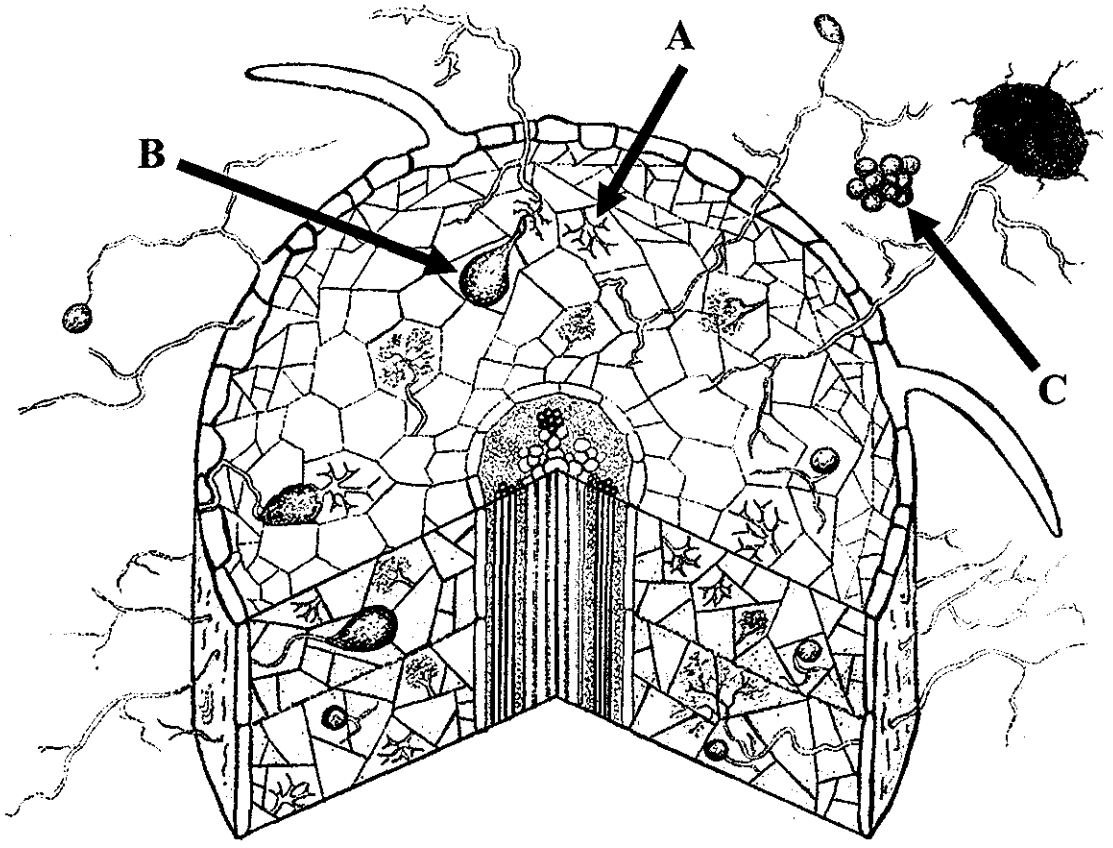

Figure 2. Cross- and radial sections of a root infected with AM fungi. The AM hyphae penetrate into the cortical cells of the root in addition to extending into the surrounding soil. **(A)** Highly branched arbuscules function as sites of nutrient exchange between the fungus and the host plant. **(B)** Some AMF form vesicles within the host cells. **(C)** Some AM fungi form asexual spores that arise from extraradical hyphae. (Drawing by Maria Lee)

Arbuscular mycorrhizal fungal hyphae have the unique ability to produce the glycoprotein glomalin, which is named after the AMF order Glomerales. Glomalin acts as a sort of glue, holding soil aggregates together. It is thought that AMF secrete glomalin to ensure that the soil aggregates in a manner that is conducive to the growth of host plants Additionally, Wright and Upadhyaya (1998) hypothesized that a function of glomalin is to protect the AMF hyphae that extend into the soil while carbon and other nutrient transfer occurs. Although each individual hypha is not

critical to an entire AMF mycelium, it may be worth the energetic expenditure for glomalin production to ensure safe transport of vital photosynthates to the fungus (Wright and Upadhyaya 1998).

Unlike ectomycorrhizal fungi, which are often host-specific, AMF are typically non-specific in their host associations. The assumption of non-specificity is derived in part from the fact that an estimated 200,000 species of plants form AM symbioses, while only approximately 200 species of AMF have been characterized (Fitter and Moyerson 1996). Although 200 is probably a gross under-representation of species, the low diversity of known AMF species compared to the large number of plants that form AM associations indicates that many AMF species must be generalists (Helgason et al. 2007). Additionally, lab and field studies have demonstrated that some AMF species are capable of infecting a wide variety of plants (Smith and Read 1997).

Mycorrhizal symbioses are an important component of any natural community. In fact, the mycorrhizal population in a given ecological community may influence the plant community composition and relative abundance of plant species, thereby producing ecological effects that cascade throughout the trophic levels. For example, Hartnett and Wilson (1999) showed that the presence of fungal symbionts was correlated to an increase in plant diversity in a tallgrass prairie. Arbuscular mycorrhizal fungi-influenced changes in the composition of a plant community are largely due to the variation in host response to colonization by various species of AMF (Watkinson 1998; Van der Heijden et al. 1998). It has been observed that a given plant species may derive a greater benefit from an association with one

AMF species over another. Furthermore, some AMF interactions may be parasitic on certain plant species and thereby prevent successful growth (Johnson et al. 1997). Klironomos (2003) studied 10 plant species, each species of plant individually inoculated with one of 11 AMF species, resulting in a total of 110 pairings in a greenhouse. Klironomos observed that no single species of AM fungi was consistently associated with positive or negative growth. In fact growth responses varied with all of the plant-fungus pairings, with an observed 53 negative growth responses and 47 positive growth responses. Klironomos' findings indicate that the AMF community should be considered as a driving factor when assessing and modeling plant community composition and changes.

Consideration of the role of AMF in vegetative community composition has implications that extend through many different aspects of plant ecology. For example, taking the AMF community into account may provide useful insights into the management of undesirable "weed" plants in agricultural systems. The inhibition of AMF in agricultural soils may prevent various AMF dependent weedy species from colonizing a given area. Alternately, it was observed that a variety of non-AM host species exhibited a significant decrease in biomass when exposed to multi-speciate AM inocula. Therefore, the addition of AMF inocula to agricultural soils may serve as a biological control for non-AMF dependent weed species that could potentially be parasitized by AMF associations (Jordan et al. 2000).

Root colonization by AM fungi may also confer disease resistance to the host plant. Newsham et al. (1995) experimented with grasses subjected to infection by the fungal root pathogen *Fusarium oxysporum*. Grasses that were colonized by AMF

from the genus *Glomus* exhibited less pathogenic damage and appeared to suppress pathogen development in the roots. Since undiseased plants are likely to exhibit faster growth, providing pathogen protection for plant roots is one example of how an AM fungus can bestow competitive advantages to its host.

Because the formation of AM symbioses likely contribute to the dynamics of plant competition, considering these interactions may prove useful in applied fields such as restoration biology. For example, Smith et al. (1998) conducted an experiment in which plots of grassland in a disturbed area undergoing restoration were inoculated with AM fungi. It was observed that plots that received AM inoculants initially had higher percentages of AM roots colonization than the uninoculated plots. Additionally, inoculated plots supported higher percent coverage of native grasses rather than weedy species. These findings suggest that the AM fungal community may play a vital role in supporting native vegetation and may be an important factor to consider when restoring grasslands that have been disturbed.

AMF and Exotic Plant Invasion

Currently the interactions between exotic invasive plants and native vegetation with respect to mycorrhizal symbioses are not well understood. However, recent studies have indicated that mycorrhizae play a role in the dynamics that occur when aggressive exotic plants are introduced to a new ecosystem. For example, the mycorrhizal interactions of the exotic grassland invader *Centaurea maculosa* (spotted knapweed) have been investigated in multiple studies. One study indicated that native vegetation in sites that were highly colonized by *C. maculosa* exhibited decreased AMF hyphal lengths and lower glomalin concentrations, as detected via

ELISA (Lutgen and Rillig 2004). As mentioned earlier, glomalin is produced in AM hyphae and contributes to soil aggregate stability. These results suggest that *C. maculosa* has a negative impact on soil quality via interference with AM fungal physiology.

Mummey et al. (2005) conducted a field experiment that indicated that AM fungal colonization in the roots of the common naturalized grass species *Dactylis glomerata* was affected by vicinal colonization of *C. maculosa*. Community analyses by T-RFLP revealed that *D. glomerata* that were growing in close proximity to *C. maculosa* hosted a more diverse assemblage of AMF species in comparison to *D. glomerata* growing in the absence of *C. maculosa*. The additional AMF species associated with *D. glomerata* in the presence of *C. maculosa* correspond to the AMF species found colonizing the roots of *C. maculosa*. The increase of AMF species diversity that correlates to the AMF species hosted by *C. maculosa* suggests that invasion by *C. maculosa* alters the AMF associations of native plants.

A further study by Mummey and Rillig (2006) used T-RFLP to compare grassland AMF communities in the presence vs. absence of *C. maculosa*. Results of the study indicated there was a significant difference in AMF community composition in the presence and absence of *C. maculosa*. Furthermore, it was observed that sites dominated by *C. maculosa* exhibited lower diversities of AMF communities than native grassland sites.

Although initially the findings that *C. maculosa* invasion leads to an increase of AMF diversity in specific roots yet an overall decrease in the AMF species diversity of the entire community may seem contradictory. However, a potential

explanation for these paradoxical findings is species transition. It may be that the increased diversity of AM species found in the roots of *D. glomerata* reflects the transition from the original AM symbionts present in the roots before *C. maculosa* invasion to the AM fungi that are preferentially hosted by *C. maculosa*. Therefore, the decrease in AM species diversity seen on the community level represents the overall effect of the shift of AMF associations occurring in the roots of the plants that were initially present in the study area.

Another study targeted *Alliaria petiolata* (garlic mustard), an exotic invasive member of the Brassicaceae that colonizes woodlands and savannas. Stinson et al. (1996) presented evidence that *A. petiolata* secretes anti-fungal compounds that disrupt the AMF of native tree seedlings, thereby suppressing their growth. A further study by Roberts and Anderson (2001) found that water leachates from *A. petiolata* inhibited AM spore germination and inhibited formation of AM fungi with sorghum and tomatoes under laboratory conditions. Additionally, a field study revealed a negative correlation between the presence of *A. petiolata* and the mycorrhizal inoculum potential of native plants, thereby indicating that the presence of *A. petiolata* may diminish native plant competitive ability (Roberts and Anderson 2001).

In field studies, Greipsson and DiTommaso (2006) observed high AM fungal colonization of the invasive plant species pale swallow-wort (*Vincetoxicum rossicum*), Chinese privet (*Ligustrum sinense*), and kudzu (*Pueraria lobata*). Ambient AMF spores in the soil of the swallow-wort invasion site were significantly higher than the adjacent control site. Furthermore, kudzu exhibited a high mycorrhizal dependency, growing poorly in forest soils that had been sterilized. The

results of Greipsson and DiTommaso's research demonstrate that alien invasive plants are able to form and benefit from associations with native AMF species. Assuming that some AM associations are more mutually beneficial than others (Vandenkoornhuyse et al. 2003), it can be hypothesized that invasion by exotic plant species alters the native AMF community in a way that contributes to the success of the exotic species. The authors also suggest that in the case of highly mycorrhizally dependent invasives, such as kudzu, the removal of AMF from an invasion site (i.e. soil sterilization) may help eliminate the invasive plant.

Hawkes et al. (2006) conducted a study in Utah and California that examined AMF in the roots of native grassland plants in the presence and absence of invasive exotic grasses. In California experimental plots were constructed for the study, whereas in Utah a research site that was undergoing invasion by *Bromus tectorum* (smooth brome grass) was sampled. Arbuscular mycorrhizal fungal species were identified through nested PCR followed by cloning, RFLP analysis and sequencing. The authors concluded that the presence of the exotic grasses was significantly correlated to a decrease in species richness of AMF in native plant roots. Additionally, they observed that the AMF community composition shifted in the presence of the exotic grasses. In samples retrieved from plots containing both native and exotic plants, the AMF species present in native plant roots reflected the AMF species present in the exotic plant roots. Hawke's results indicate that invasion by exotic plants can alter AMF species composition in a given ecosystem.

Ecological models suggest that the introduction of a new dominant plant into an ecosystem will affect both the AMF community and the native plant community.

Mycorrhizal community feedback models take into account the effects of symbioses on populations of both the fungal and botanical members of a given community. These feedback models attempt to discriminate host specificity within mycorrhizal interactions and predict how preferential colonization may give rise to changes in the composition of the plant and mycorrhizal community.

Mycorrhizal community feedback models can help ecologists describe the intricacies of symbioses within a plant-fungal community in terms of the magnitude of benefit received by each partner. Modeling mycorrhizal community dynamics allows exploration of the high variability that exists within the many facets of a symbiosis. Root colonization, host specificity, and symbiotic reciprocity should not be assumed to be equal among AMF species. Helgason et al. (2002) showed that some species of plants are preferentially colonized by certain species of AMF, whereas some plants are generalists with respect to their mycorrhizal partners. Additionally, some species of AMF are more compatible with certain plant species and may provide greater benefits for one plant versus another. The magnitude of benefits conferred between a host plant and a fungal symbiont can also be asymmetric.

Beyond that, not much is known regarding the specificity of the AM symbiosis. A few studies have investigated the host specificity and compatibility of AM fungal-host combinations. A wide variety of responses has been observed, and it seems that the mycorrhizal specificity and dependence of plants is particular to each species. For example, Moora et al. (2004) showed that two species of *Pulsatilla* (pasqueflower) showed differences in the amount of biomass produced by the host

plant, the concentration of phosphorous, and the degree of AM root colonization when grown in the presence of different AM inocula. In a greenhouse experiment, Bever (2002) found that certain AM fungal species showed variances in rates of sporulation when paired with different host plants. Bever et al. (2009) further demonstrated that plants preferentially allocated photosynthates to the more beneficial of two AM fungal species. The abovementioned studies provide a glimpse into the intricacy of specificity and reciprocity of AM symbioses, and there is potential for a myriad of studies that examine these dynamics.

Though there is a lack of information regarding the complexity of AM relationships, we can postulate on the degrees of benefit that may occur through the exchange of resources between a fungus and host plant. For example, a given species of mycorrhizal fungus may receive a benefit from a host plant that is not reciprocated in magnitude. In this way, some mycorrhizal symbioses may border on parasitism. Through the application of mycorrhizal community feedback models, such dynamics between plant and fungal community members can be predicted in a variety of situations.

Several different community feedback scenarios may occur when a native ecosystem is invaded by an invasive exotic plant (Figure 3). If an exotic invasive plant is preferentially colonized by a given species of AMF, then an increase in abundance of the alien plant will support an increase of the AMF species that it preferentially supports. This type of interaction causes a positive feedback response: an increase in the biomass of one organism as a result of the abundance of another organism. Alternately, the abundance of a given AMF may influence the

mycorrhizae that infect other native plants in the community. Plants that are not usually colonized by the newly abundant species of AMF may become infected and may not receive the same benefits or same degree of benefits from the new species of AMF as they do from their preferred species. These plants may become less abundant over time. In this way, the AMF species that preferentially colonizes the exotic plant is exerting negative feedback pressure on the native plant species in the community.

Klironomos (2002) conducted four experiments that demonstrated how soil feedbacks between plants and the soil biotic community influenced a plant's ability to invade a given ecosystem (Figure 4). In the first experiment, five 'highly invasive' plant species were grown alongside five 'rare and endangered' plant species in soil that was previously un-colonized by any of the aforementioned species. Klironomos determined feedback responses by comparing the growth of each plant species in the new soil relative to the soil in which the plants had already grown. All of the five 'rare and endangered' species exhibited negative feedback responses (decreased growth) to their own soil environment whereas four out of five 'highly invasive' species demonstrated overall positive feedback (increased growth) in the soil in which they had previously grown.

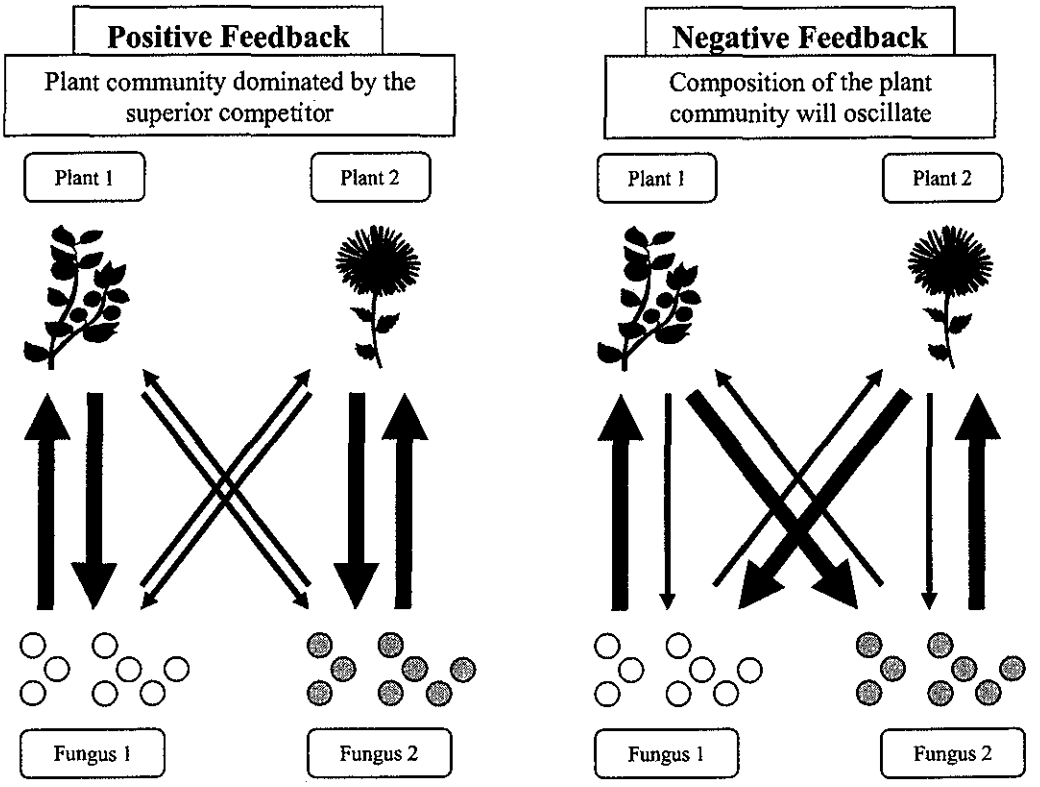

Figure 3. Relative magnitudes of benefits received by mutualists under positive and negative feedback scenarios. When a host plant and fungal symbiont both receive the same magnitude of benefit from the mutualism a positive feedback scenario occurs. The plant that was originally most abundant will have the competitive advantage and therefore dominate the community. However, when a host plant receives the highest benefit from a fungal symbiont that turn receives the highest benefit from a different host plant that in turn receives the highest benefit from a different species of fungus, negative feedback occurs. The plant that was originally the most dominant supports a symbiont that provides a competitive advantage for another plant in the community and the plant dominance in the community oscillates. Thickness of the arrows corresponds to the magnitude of benefit conferred by each partner. Figure adapted from Johnson *et al.* (2006).

Two subsequent experiments were then designed to determine which factors may have contributed to the results of the first study. Klironomos hypothesized that either pathogen resistance/susceptibility or ability to form/utilize mycorrhizal fungal associations contributes to the ability of a given plant species to experience positive and negative feedback with its soil microbial community. The same plant species from the first experiment were grown under three conditions: with the spores of AMF

that were extracted from the soil of the first experiment, with filtrate of the saprobes and pathogens present in the soil of the first experiment, and in sterile soil. Positive feedback was observed in plants inoculated with AMF species that had previously been found in association with that plant species, while neutral feedback occurred in AM host-fungal combinations that were not previously adapted. Negative feedback was most strongly observed in plants that were re-inoculated with root pathogens that had been previously associated with their roots. Klironomos' findings have several implications: 1) Adaptations/acclimations occur in the AM symbioses so that certain fungal-plant combinations may be more mutually beneficial than others 2) Plant-pathogen interactions have adapted so that in a native ecosystem, pathogens are deleterious to plant hosts in a magnitude that prevents the complete dominance of any one plant species, and this may contribute to maintenance of diversity in the plant community 3) Invasive plant species may exhibit increased success when colonizing a new environment due to the absence of soil pathogens that are well adapted to parasitize that species or strain of plant.

The third experiment was designed to determine if negative feedback responses were due to pathogenic/parasitic soil organisms. The three most abundant non-AMF species of fungi were isolated from the rhizosphere of each plant species and then individually paired with each plant species to test their specific impact on plant growth. All five rare and endangered plant species exhibited reduced growth when paired with inocula that had been retrieved from the roots of their own species. None of the invasive plants experienced reduced growth when paired with inocula that had been isolated from the roots of their same species. However, both the

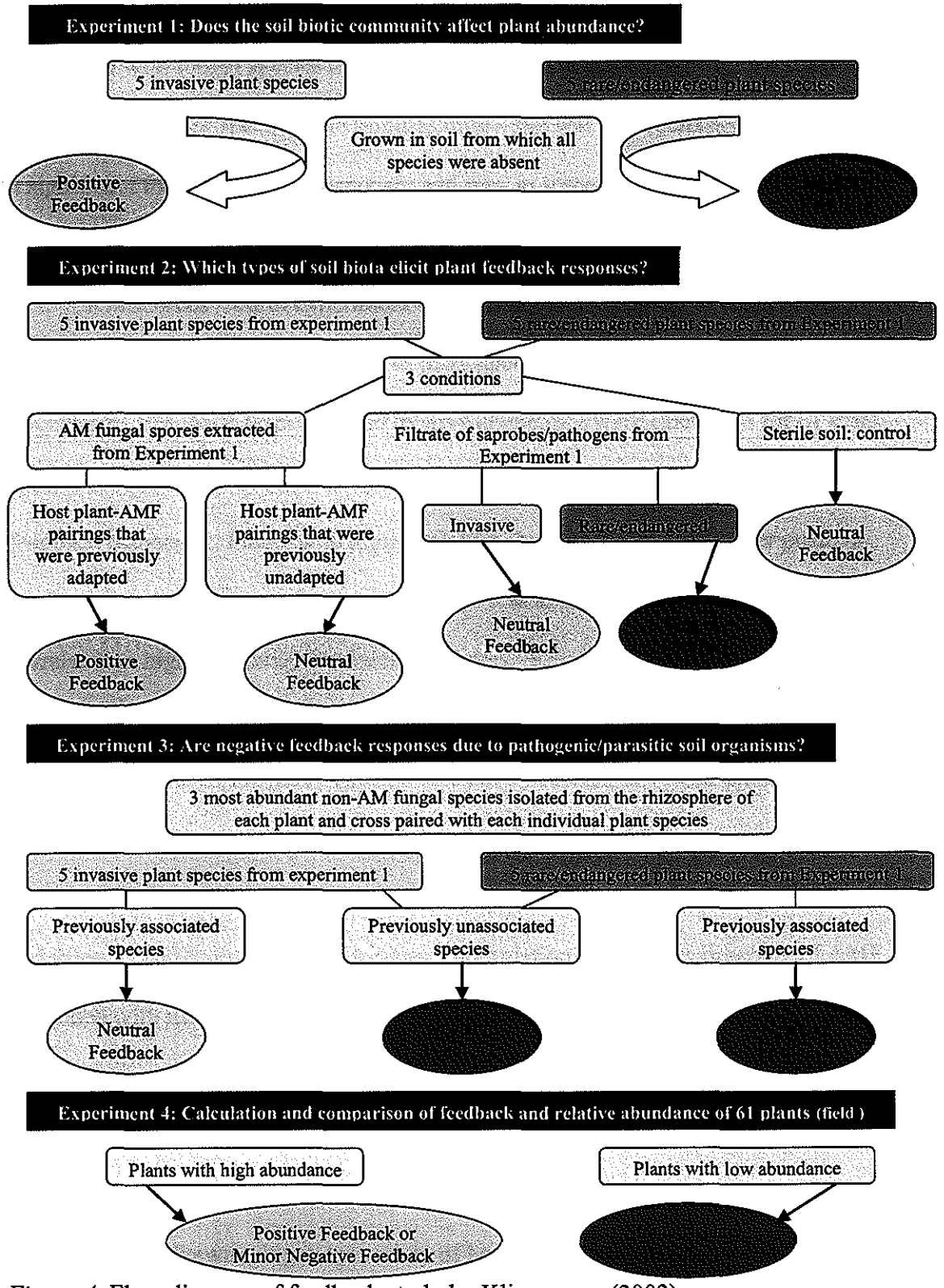

Figure 4. Flow diagram of feedback study by Klironomos (2002)

rare/endangered species and the invasive species experienced depressed growth when inoculated with fungal species that were not previously associated with the roots of their own particular species. The findings imply that soil pathogens and parasites play a significant role in feedback responses and may in some cases mask/counterbalance any positive feedback that results from AMF symbioses.

Klironomos' fourth experiment was a field study designed to observe the direction and magnitude of feedback of plant species in comparison to their relative abundance. Feedback responses and relative abundance were calculated for 61 plant species in an old growth field. Overall, most plant species displayed negative feedback responses. Plants with higher relative abundances showed either positive feedback or a low negative feedback, whereas less abundant plant species were correlated to higher negative feedback responses. Results of the fourth experiment suggest that soil feedback responses can contribute to plant diversity and abundance.

Given that exotic plant invasion leads to a decrease in overall plant diversity, AMF diversity studies can provide insight into some of the AMF community changes that occur as plant diversity decreases in response to alien plant invasion. Burrows and Pfleger (2002) observed that increased sporulation and species diversity of AMF coincided with increased plant diversity. The correlation between AMF sporulation and high plant diversity may be due to the presence of a wide variety of host plants. By interacting with a variety of plants, AM fungi may have the opportunity to form symbioses with hosts that provide greater benefits than others, thus maximizing their relationships. Likewise, a greater variety of host plants provide a more diverse environment that can support a greater variety of fungal symbionts.

Species diversity, specificity, and community feedback responses are just a few aspects of the countless areas of mycorrhizal community dynamics that can be studied. As with any scientific study, the researcher must carefully design an experiment that most specifically and accurately provides information about the hypothesis at hand. As questions regarding mycorrhizal community dynamics are inherently ecological, it is important for a researcher to create a study that can control enough variables to confidently draw conclusions, yet accurately reflects what occurs in nature.

Study Design

Two main types of studies can be applied to AM fungal research: greenhouse experiments and field studies. AM field studies can be further divided into ecological surveys (obtaining samples from pre-existing communities) and field plot construction (planting specific host plants in a field setting to simulate a desired community composition). The appropriate experimental design is dependent on the questions being asked and the type of data sought.

Greenhouse experiments permit the researcher to have greater control over environmental factors. Elements such as light, moisture, nutrient addition and soil composition can all be managed, thereby allowing for experiments that investigate the responses of AMF to alterations in specific environmental factors. Additionally, greenhouse experiments make it possible for individual AM fungal species to be studied, as specific inocula can be added to host plants in sterile soil.

Although greenhouse experiments can provide a researcher with the power to manipulate environmental and biotic variables, the sheer nature of the artificial

greenhouse environment may give rise to data that do not accurately reflect the manner in which AM fungi function in the field. Consequently, there are almost no invasive plant-AMF ecological studies that consist solely of greenhouse experiments. Field experiments can reveal how AMF interactions occur in a natural ecological setting, thereby producing results that reflect the influences of all variables present at the study site.

In a survey type of field study, AM fungi would be sampled either from the roots of plants that are pre-existing in their field environment or from spores that are obtained from field soil samples via centrifugation methods. For example, Greipsson and DiTomasso (2006) surveyed two sites of exotic plant invasions to observe AM root colonization and sporulation in native and exotic flora. Soil and root samples were brought back to a laboratory for analyses. Mummey et al. (2005) sampled colonized roots of a naturalized grass growing in the proximity of an exotic invasive plant and roots from the grass growing without the influence of the exotic plant. The roots were brought back to a laboratory where molecular methods were applied to assess the AM fungal community structure. Field survey study designs allow researchers to observe how AM symbioses occur and operate in their 'natural' state. Field surveys eliminate study design dilemmas concerning the accuracy of replicating a plausible field setting that truly accounts for all environmental variables. However, field surveys may, in some cases, be subject to too many influential environmental variables, so results may give rise to more questions rather than sound conclusions. Additionally, if several types of ecological settings need to be compared along with control sites, the researcher must seek out communities that already possess all the

characteristics necessary for the study. In contrast, construction of field plots can allow a researcher to create specific communities that include all of the variables and characteristics necessary for a study.

Constructing a field plot experiment for the study of AM fungal interactions might consist of manipulations to a field site such as nutrient addition, planting/removal of plant species, addition of AM fungal inocula, and implementation of management techniques (herbicides, fungicides, mowing, grazing, fire etc.). Field studies maintain the authenticity of the field environment while controlling and randomizing desired variables to achieve a more succinct experimental design than field surveys. To study the effects of plant invasion on AM community structure, Hawkes et al. (2006) created field plots using large steel drums set into the soil. The researchers were then able to control the plant community composition as well as the soil origin and composition while maintaining realistic environmental conditions. Helgason et al. (2007) performed a field experiment in which fungicide was added to a natural community to observe how the AM fungal community responded to disturbance. Helgason's experiment took advantage of the native community composition of both the host plants and symbiotic fungi in addition to the natural field environment, yet manipulated the density of the fungal community using fungicide to create the desired effect (empty niche).

As previously mentioned, a combination of greenhouse and field studies can often provide the most accurate representation of mycorrhizal community dynamics. For example, in Klironomos' 2002 study, three greenhouse experiments were used to evaluate feedback dynamics in specific pairings of plants with soil microbes. A fourth

study consisting of a field survey was performed to reveal how feedback was correlated with the abundance plants in the field. By conducting both field and greenhouse experiments, Klironomos was able to observe feedback phenomena in the field and elucidate the potential mechanisms through controlled greenhouse experiments.

The paucity of studies of AMF community composition may be due in part to past difficulties in species location and identification. Because AMF are confined to the soil community and do not produce fruiting bodies and mantles as do ectomycorrhizae, they are difficult to observe and study. Methods of AMF identification have evolved from microscopic identification to the use of molecular techniques such as PCR and DNA sequencing. Now that molecular techniques are becoming more specific and accessible, the study of AMF may become more accurate and common.

Methods for Identification of AMF

AM fungal colonization of roots can be visualized via hyphal staining procedures. A variety of stains are able to demonstrate the presence of hyphae and other AM fungal structures in root tissue. Most staining processes for AM fungi in root tissue samples require an initial clearing step. Root clearing removes cytoplasmic material and secondary metabolites that could otherwise absorb the stain and create background interference when viewing the samples using light microscopy (Vierheilig et al. 2005). Clearing can be achieved by heating the roots samples in a water bath or autoclave. Darkly pigmented roots can be cleared by heating in KOH followed by bleaching with alkaline hydrogen peroxide (Bevege 1968, Kormanik and

McGraw 1982, Vierheilig et al. 2005). Once roots have been sufficiently cleared, a staining dye such as Trypan Blue (Figure 5), acid fuchsin, cotton blue or CBE can be applied (Vierheilig et al. 2005). Such stains should illuminate all fungal structures present in the root sample. For an effective, low cost, safer alternative to the aforementioned dyes, root samples can be stained using writing ink diluted in vinegar (Vierheilig et al. 1998). Additional detection and staining techniques including non-destructive procedures for living roots and destructive and non-destructive methods for non-living roots are detailed by Vierheilig et al. (2005).

Figure 5. Trypan Blue staining AM hyphae in a colonized root.

Historically, most ecological studies on AMF have been conducted through identification of spores from the soil surrounding a given plant. Spores are extracted from the soil through sieving and centrifugation, with subsequent microscopic identification. Distinctive AM spore characteristics include shape, size, color, ornamentation, cell wall layer composition and the number of cell wall layers (Peterson et al. 2004). Microscopic spore identification for AMF is a difficult task, given that the spores within the same genera may appear strikingly similar (Figure 6).

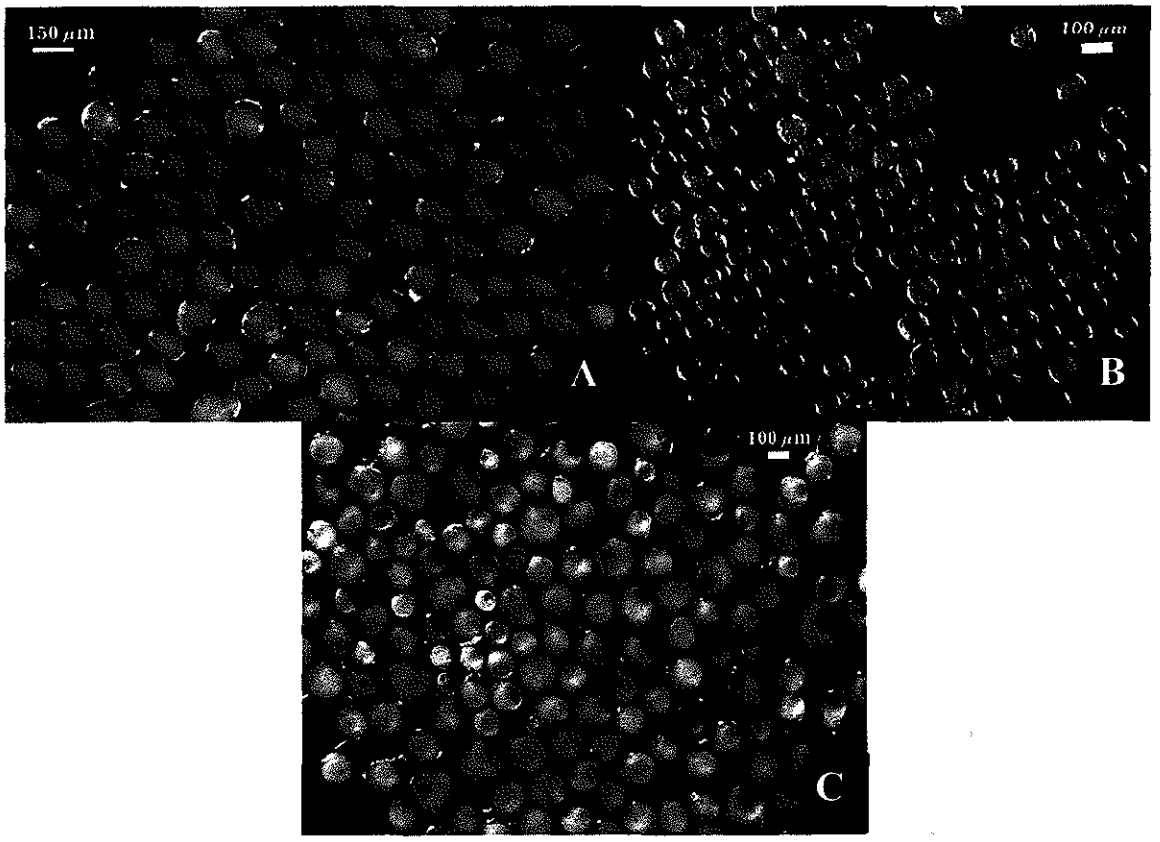

Figure 6. Spores of (**A**) *Acaulospora denticulata*, (**B**) *Acaulospora lacunosa*, and (**C**) *Glomus verruculosum*. Spores of AMF are often morphologically similar and therefore difficult to differentiate between genera (**A** and **B** vs. **C**), let alone species (**A** vs. **B**). Images: with permission: www.invam.caf.wvu.edu.

Aside from spore identification, hyphae, intraradical structures such as vesicles and arbuscules, and extraradical hyphal features have been used to

distinguish AMF (Figure 7). However, these vegetative intracellular root structures are poor identifiers; they can sometimes lead to identification at the family level, but may not even be that specific (Merryweather and Fitter 1998).

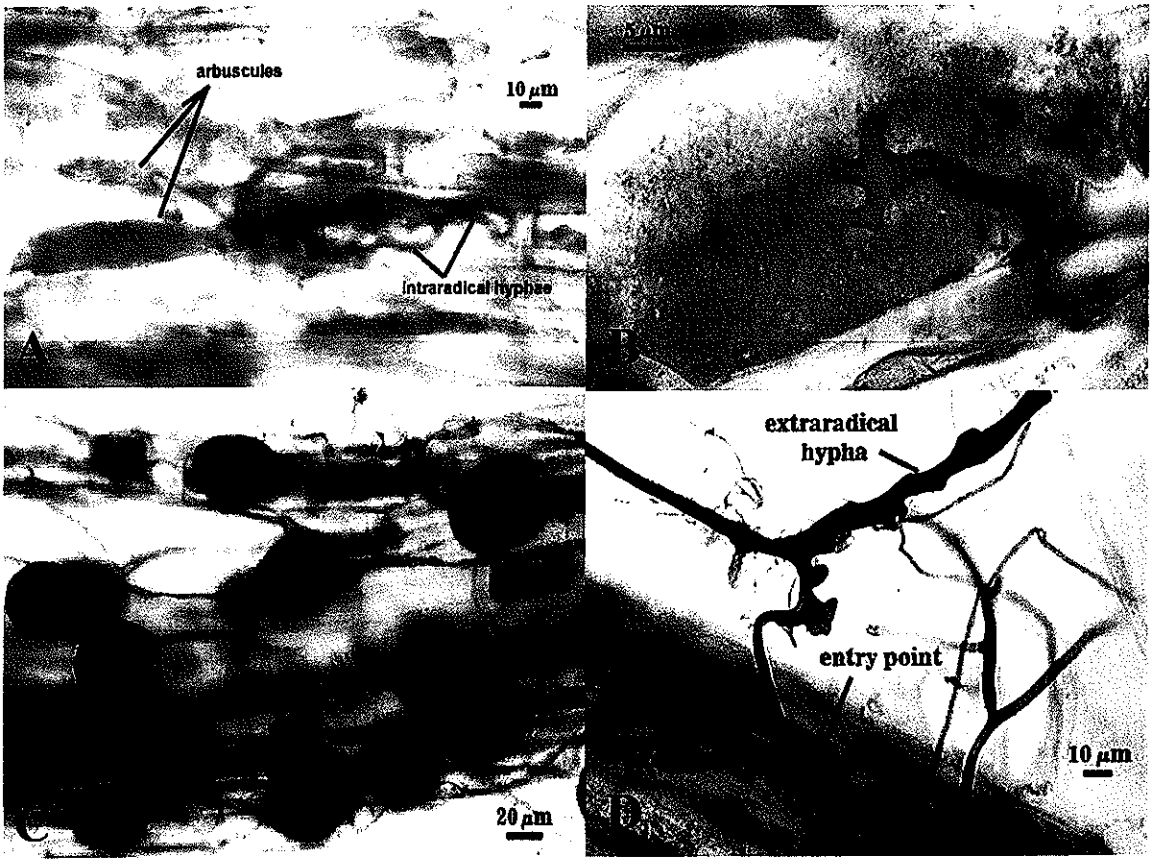

Figure 7. Intraradical hyphae **(A)** including structures such as arbuscules **(B)** and vesicles **(C)**, and extraradical hyphal features including the point at which a hypha penetrates **(D)** the host root can be used to differentiate AM fungal species. Images: with permission: www.invam.caf.wvu.edu.

With the recent popularization and accessibility of molecular techniques, AMF species identification through the use of PCR and DNA sequencing of AMF spores that have been centrifuged from soil samples has become widespread. Although molecular methods have allowed for more specific and positive identification of spores, AMF community characterization through the use of ambient soil spores is inherently flawed. Ecological studies that rely on the aforementioned

methods of species identification make the assumption that the spores extracted from the soil are representative of the species that are colonizing the plant roots in a given ecological community. In actuality, the studies may be characterizing only the AMF that have sporulated at the time of sampling, as sporulation is not necessarily indicative of root colonization and differs among AMF species (Bever et al. 1996). Sporulation of AMF species may vary with season, environmental conditions, and compatibility with the host plant. Additionally, some species of AM fungi in general may tend to sporulate more prolifically than others. Therefore, spores that are extracted from the soil around a plant may not reflect the species of AMF that are actually growing in the root system of that plant. More recently, procedures have been developed to isolate and amplify AMF DNA from colonized roots rather than from ambient soil spores. By characterizing the non-sporulating AMF that are present in plant roots, a more accurate depiction of AMF community composition can be acquired.

Polymerase Chain Reaction (PCR)

A variety of primers have been designed to amplify AMF DNA directly from colonized roots. Unfortunately, no one primer set seems to amplify solely AMF DNA without excluding at least one taxonomic group. Most molecular identification of AM fungal species from colonized root tissue requires nested PCR, a procedure that uses two consecutive PCR reactions to selectively isolate various portions of DNA from a sample that initially contains DNA from multiple species (in this case DNA from the host plant and the multiple fungal species inhabiting the root tissues). Methods of molecular identification for AM fungi often target the ITS (internal

transcribed spacer) regions of the fungal rDNA. The ITS sequences are variable length regions, usually between 600 to 800 base pairs. Individual ITS sequences are highly conserved within fungal species, yet are highly variable between fungal groups and are therefore an ideal portion of the fungal genome to use in PCR.

Table 1. Primer sequences used for PCR and sequencing

Primers	**DNA Sequence**	**Source**
NS5	AACTTAAAGGAATTGACGGAAG	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
ACAU1661	TGAGACTCTCGGATCGGG	Redecker (2000)
ARCH1311	TGCTAAATAGCCAGGCT-GY	Redecker (2000)
GIGA5.8R	ACTGACCCTCAAGCAKGTG	Redecker (2000)
GLOM1310	AGCTAGGYCTAACATTGTTA	Redecker (2000)
LETC1670	GATCGGCGATGGTGAGT	Redecker (2000)
LR1	GCATATCAATAAGCGGAGGA	Trouvelot et al. (1999)
FLR2	GTCGTTTAAAGCCATTACGTC	Trouvelot et al. (1999)
FLR3	TTGAAAGGAAACGATTGAAGT	Gollotte et al. (2004)
FLR4	TACGTCAACATCCTTAACGAA	Gollotte et al. (2004)
SSU-Glom1	ATTACGTCCCTGCCCTTTGTACA	Renker et al. (2003)
LSU-Glom1	CTTCAATCGTTTCCCTTTCA	Renker et al. (2003)

Redecker (2000) developed specific PCR primers to allow for identification of AMF species from colonized plant roots (Figure 8). The first set of PCR reactions uses the universal 18S rRNA fungal primers NS5 and ITS4 (White et al. 1990) to target the ITS (internal transcribed spacer) regions of the fungal rDNA. The second round of PCR reactions separates two different groups of AMF using primers designating small ribosomal subunit AMF sequences. The primers GLOM5.8R and

GIGA5.8R in combination with ITS1F select for *Glomus mossae/intaradices* and Gigasporaceae. The primers ARCH1311, LETC167, GLOM1310 and ACAU1660 paired with ITS4i select for the Acaulosporaceae *sensu stricto*, as well as *Acaulospora gerdemannii/trappei*, *Glomus occultum/brasilianicum* and *Glomus etunicatum/claroideum.* Although these primer pairings separate the PCR products into taxon specific groupings, cloning or RFLP is still required to obtain species identification. The primers may be useful when probing for specific groups of AM fungi; however, the nested PCR step requires at least five reactions (not accounting for dilutions) per sample. The high number of PCRs may lead to excessive use of expensive reagents and allow more opportunities for error when compared to AMF specific nested PCRs that require only one primer pairing for the second round of amplifications.

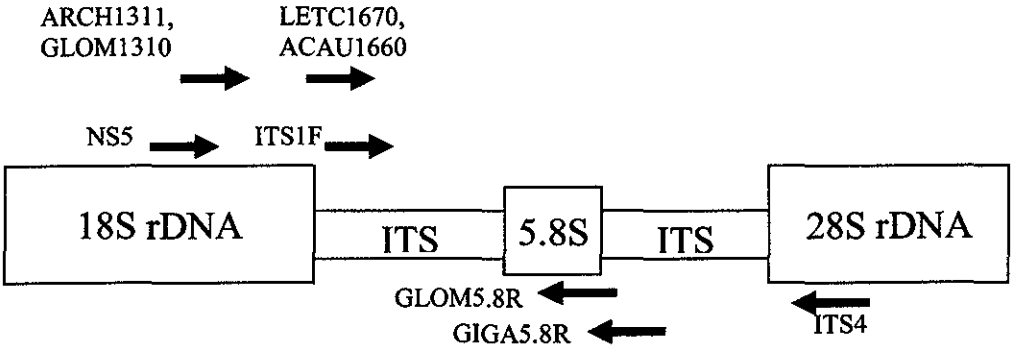

Figure 8. Locations of sites for primers developed by Redecker (2000). These primers target internal transcribed spacers (ITS) of ribosomal fungal DNA.

Renker et al. (2003) developed AMF primers with which the first PCR reaction is Glomeromycotan specific and uses the primers SSU-Glom1 and LSU-Glom1 to generate a ~1000-1200 bp fragment. However, some basidiomycete contaminants may be invariably amplified with these primers. Therefore, an intermediate restriction digest was performed with the enzyme *AluI*, which cuts at a

site conserved by the Basidiomycota but not present on most of the amplified fragments of Glomeromycotan fungi. Unfortunately, members of the Paraglomeraceae posses the *AluI* cut site and are therefore eliminated in the digest. Following the restriction digest, a second PCR was performed using universal fungal primers ITS4 and ITS5, thereby generating a fragment of ~600-800 bp (White et al. 1990). However, when employing the primers in my own research (Appendix A), I found that the *AluI* digest did not eliminate all basidiomycetous contaminants, and in fact my sequencing reactions yielded almost all basidiomycetous species, most within the yeast genera *Cryptococcus* and *Dioszegia*. In fact, SSU-Glom1 and LSU-Glom1 are so adept at detecting *Cryptococcus* and *Dioszegia* that Renker et al. (2004) published a study that used these primers to investigate the diversity of root inhabiting yeasts.

Gollotte et al. (2004) designed nested primers to target Glomeromycotan large subunit (LSU) rDNA obtained from colonized roots. The first PCR is a pan-fungal amplification that utilizes the primers LR1 and FLR2 (Trouvelot et al. 1999). The nested PCR employs the primers FLR3 and FLR4 to isolate AM fungal DNA. Although the authors showed that these primers may be capable of amplifying some Basidiomycete species, in their study only Glomeromycotan species were isolated via these primers.

Other AMF specific primers can be found within the body of primary literature, but a 'perfect' AM primer set has yet to emerge. Each primer set has its limitations and the specificity vs. sensitivity losses/gains must be assessed by each individual researcher. Selection of optimal primers for any given study is dependent

on the aim of the specific project and will vary according to the questions being investigated. Additionally, there are always opportunities for the creation of new primer sets. No matter which region of AMF DNA is amplified, further molecular techniques are required to retrieve AM fungal species identification from a colonized root sample.

Cloning

It is common that more than one species of AMF will infect the roots of one host plant. Therefore, in each PCR product more than one species of AMF may be present due to the fact that the nested primers target AMF sub-groups and not individual species. To separate individual species the nested PCR products may be cloned into bacterial plasmids, thereby creating bacterial colonies that contain only one PCR fragment from only one species of AMF.

The cloning process consists of 4 main steps: a restriction digest of the plasmid (also known as the vector), insertion of the desired DNA fragment into the vector, transformation of vector into bacterial hosts, and growth of bacterial colonies (Figure 9). Plasmids are circular portions of DNA that naturally occur in bacteria. Plasmids can be used as a vector to house desired portions of genetic information. In order to insert a DNA fragment into a vector, the circular DNA must be opened to create a site for the binding of the desired DNA fragment. Therefore, a digestion of the plasmid with restriction enzymes is required to open up the plasmid. Once the vector is opened, the DNA fragments (products of PCR) can be incorporated or ligated into the plasmids using the enzyme DNA ligase. Each vector is assumed to house one DNA fragment and therefore, in this case, the DNA from only one species

of AMF. Now that the vectors contain the desired DNA fragment, they can be incorporated into the bacterial hosts in a process called transformation. However, transformation results in a mixture of bacterial cells, some containing the plasmid with the insert, some containing just the plasmid, and some that did not undergo transformation. Therefore, the transformation product is plated on media that is selective for transformants. Agar containing the antibiotic ampicillin is most commonly used. Bacteria housing plasmids with inserts are unaffected by ampicillin whereas non-transformed bacteria are susceptible to ampicillin. Agar plates are incubated overnight until ample bacterial colonies grow. Each colony on the Petri plate should only contain one insert and therefore represent only one species of AM fungi. Following cloning, a number of bacterial colonies are selected for sequence analysis. By sampling from a statistically significant number of colonies, an accurate depiction of the AMF population in a single root may be formed.

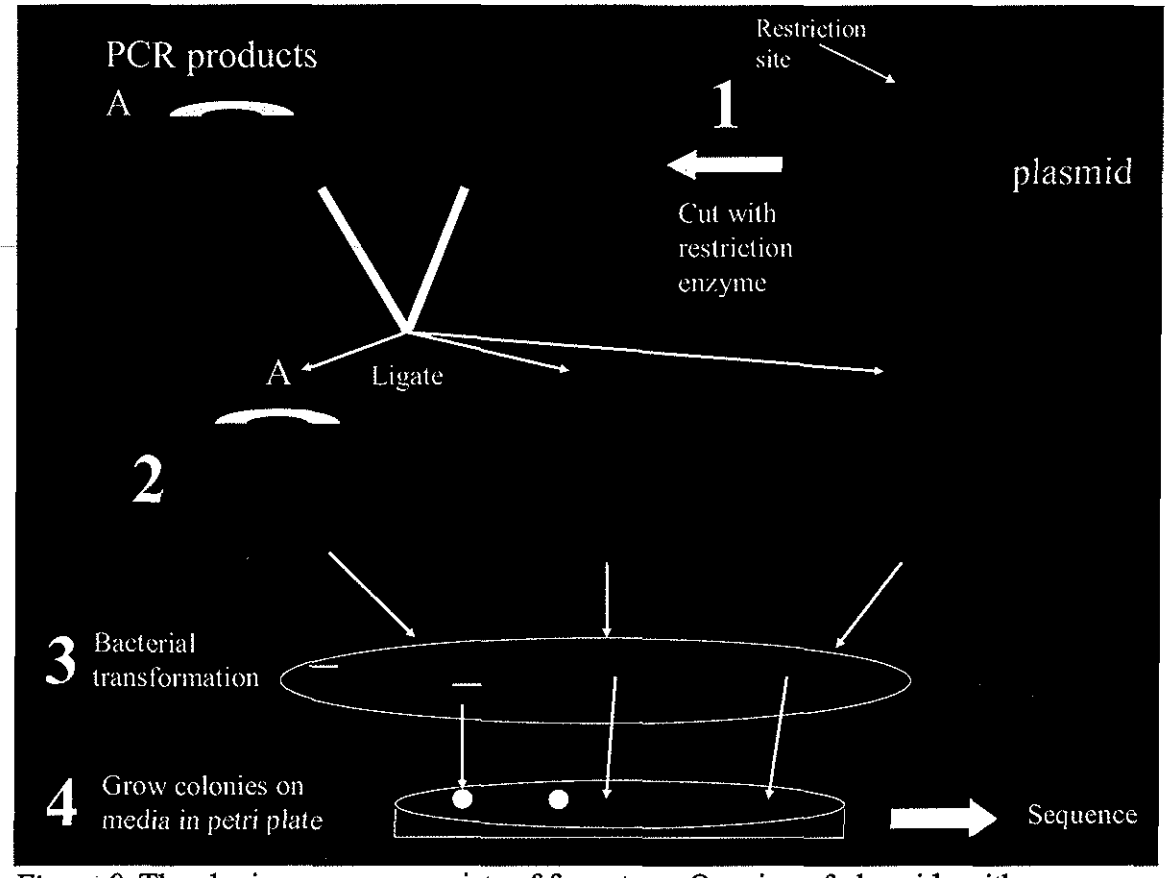

Figure 9. The cloning process consists of four steps: Opening of plasmids with restriction enzymes **(1)**, ligation of the desired insert into the plasmids **(2)**, bacterial transformation to incorporate the plasmid into *E. coli* cells **(3)**, and growth of bacteria on Petri plates containing selective media **(4)**.

However, cloning is time consuming and expensive. The number of clones that need to be sequenced in order to retrieve a statistically viable assessment of the AM fungi present in a sample is dependent on the study and even the sample. Consequently, a large number of sequencing reactions may need to be conducted in order to determine a species composition. For example if 50 roots are sampled, then maybe 15 colonies need to be sequenced from each plate to retrieve a statistically accurate representation of the species composition. Therefore, 750 colonies total would need to be sequenced for the entire study, and assuming that there are no repetitions due to technical errors. The cloning process can be complicated and offers

more opportunities for procedural errors. Biases in the cloning process may also be of concern; for example, an abundance of a given AMF species in a sample may mask some of the less abundant species. Additionally, the PCR products of some species may be more readily ligated into the plasmid, thereby producing an inaccurate representation of the species present in a sample. Some researchers may avoid the time, cost, and error associated with the cloning and sequencing processes by using restriction enzymes to differentiate among species.

RFLP and T-RFLP

Nested PCR products may be subjected to restriction digests and analyzed via RFLP (restriction fragment length polymorphism) or T-RFLP (terminal-RFLP). The RFLP analyses use restriction enzymes that cut at the sites of specific nucleotide sequences. The cut sites can be located on parts of the genome that are specific to a species. Therefore, when PCR products containing different species are cut with the same restriction enzymes, DNA fragments of different sizes are produced. The products of a restriction digest can be separated and viewed using gel electrophoresis. The digested DNA fragments may then be correlated with AMF species via comparison using a database of known fragment lengths for AM fungi. However, the use of RFLP is most effective when applied to PCR products that contain only one taxonomic species. If a PCR product contains multiple species, as is often the case with environmental samples of AM fungi, the multiple RF (restriction fragment) patterns may not be distinguishable from one another, thereby obscuring the identities of the species present. Therefore, when applied to environmental AM fungal research, RFLP is most usefully applied to PCR products that have been separated via

cloning. Although the cloning process is expensive and labor intensive, the use of RFLP can eliminate the costs and technical difficulties associated with sequencing.

T-RFLP is similar to RFLP, but uses terminally fluorescently labeled nucleotides on the PCR product to view the results of the restriction digest as peaks on a sequencing apparatus rather than bands on a gel. T-RFLP can be an especially useful tool for studying AMF ecology because it allows the researcher to analyze root samples that are colonized with multiple species without the need for separation of species, as is necessary for DNA sequencing. The differences in terminal restriction fragment (T-RF) patterns can inform the researcher of the diversity of species in an environmental sample without species identification. However, T-RFLP can be used to identify individual species, as long as the researcher has created a reliable and comprehensive database of T-RFs. By determining the T-RF patterns from a variety of PCR products from known species, the variation of peaks from an environmental sample should allow for the recognition of individual species within a multi-speciate sample. Database programs such as TRAMPR (Fitzjohn and Dickie 2007) can be used to construct an easily searchable library of experimentally generated T-RFs.

As with most molecular techniques, PCR, cloning, sequencing, RFLP and T-RFLP all have inherent difficulties and limitations. Obstacles are amplified when working with AMF due to the nature of their habitat and life history strategies. The soil and root environment pose many complications that can impede the extraction, isolation and amplification of AMF DNA. Primarily, soil and roots teem with millions of organisms: nematodes, insects, bacteria, fungal pathogens and saprobes, ectomycorrhizae, non-AMF root inhabiting fungi and other ambient soil microbes.

When an environmental sample undergoes an initial DNA extraction process, all DNA that is present in the sample will be retrieved including the DNA of the host plant, thereby yielding a product filled with a potentially low percentage of AMF DNA. During the first PCR amplification, the problem of non-fungal DNA should hypothetically be eliminated given that reliable pan-fungal primers are employed. When using PCR to isolate AMF DNA, an initial pan-fungal amplification is almost always performed. The pan-fungal amplification reduces the amount of primer binding competition from the DNA of other organisms and potentially replicates any AMF DNA to provide a higher amount of template DNA for AMF-specific primers. However, because pan-fungal primers amplify all fungal DNA within a given sample, the product of the first PCR amplification is a mixed sample of DNA from all root inhabiting fungi and ambient soil fungi that were present in the original root fragment. Consequently, the PCR product may still contain a low ratio of AMF to non-AMF DNA thereby making it difficult for primers to align with AMF templates and increasing the chance of non-specific binding errors.

Data Interpretation

As with any ecological study, it is important to be discerning as to what information is truly retrieved from the data collected. For instance, if a study aims to identify AMF species from a given plant species to determine the spectrum of AM fungi that associate with that plant, the researcher must be explicit about what conclusions are drawn concerning diversity. Issues such as sampling bias, primer bias, and taxonomic group exclusion due to non-comprehensive primers must be considered.

Furthermore, sampling replication within the root system of a single individual plant must be considered. Only a small amount of root tissue is necessary for a DNA extraction; is it possible that the root fragment being analyzed does not contain all AMF species associated with the individual plant? If so, sampling replication within the root system of an individual host plant may be required. Additionally, there may be a small amount of AMF DNA in relation to the amount of plant DNA. In this case, primer sensitivity becomes crucial as the AM fungal DNA content of a sample may be like "a needle in a haystack." Issues with primer sensitivity and the low amount of AMF DNA may lead to a lack of detection of rare and less abundant AMF species, thus creating an inaccurate depiction of community composition. Given the current state of molecular detection capabilities, it seems that broad conclusions regarding AMF community composition, especially in field studies, must be tempered by practical skepticism.

Future Studies

There is much that is yet unknown regarding the ecological dynamics of plant invasion and arbuscular mycorrhizal fungi. For example, it would be interesting to consider which AM fungi associate with alien plants in their native habitat versus the newly colonized habitat. Hierro et al. (2004) discuss the necessity of including a comparison of the native and introduced habitats when studying exotic plant invasions. There are a variety of hypotheses that seek to explain how a plant that exhibits 'balanced' growth patterns in its home range becomes an aggressive competitor when it is introduced to a new ecosystem. None of the hypotheses can be supported unless studies are crafted to contrast how the plant interacts in its native

community with how it interacts in its new range. For example, Reinhart and Callaway (2004) tested the Enemy Release Hypothesis on two reciprocally invasive species of *Acer*: *A. negundo* which is native to North America yet invasive in Europe and *A. platanoides*, a European native that has become invasive in North America. The Enemy Release Hypothesis supposes that invasive exotic species experience success in their introduced habitat because they are no longer subject to the adverse effects of the natural predators and pathogens that exerted selective pressures on the plants in their home range. Reinhart and Callaway explored the role of soil biota (soil pathogens and mutualists) in invasions by examining the success of each *Acer* species in sterile and non-sterile soils from different native and exotic ranges. The authors observed that *Acer* seedlings exhibited decreased height and biomass when grown in unsterilized soil from their native range in comparison to sterile controls. In contrast, seedlings grown in soil not associated with conspecifics from the non-native range exhibited an increase in biomass and height when compared to sterile controls. Reinhart and Callaway's findings not only support the Enemy Release Hypothesis, but also suggest that mutualists in the introduced range may provide an increased benefit that facilitates the success of invasive *Acer* species. The study represents only one of a few exotic plant studies that take into account the home versus introduced range of the plant in question. This sort of biogeographical approach to invasive plant studies could certainly be applied to AMF dynamics, and could provide useful information regarding the role of AM fungi in exotic plant invasions.

Much is still unknown regarding the mycorrhizal nature of many invasive exotic plants. It may be both useful and interesting to obtain information as to which

exotics form mycorrhizal symbioses, which ones disrupt native AM symbioses, and the specificity and degree of mycorrhizal dependence of specific exotic invaders. Questions must be addressed through individual studies that intensively focus on one invasive plant species. Therefore, a myriad of study possibilities exist in the realm of invasive plants and AM symbioses.

Many of the limitations that prevent the advancement of AM fungal studies, especially in field research, can be attributed to the lack of cost effective, time efficient and reliable detection methods. As molecular techniques continue to be developed and refined, environmental ecological studies will become easier and more accessible, allowing more questions to be answered regarding the soil microbial community. The information obtained about how exotic plant invasions affect and are influenced by mycorrhizal fungi may influence ecosystem restoration and management practices. By including the symbiotic soil community in management planning, we may be able to more efficiently and comprehensively eradicate exotic plant invaders and restore native ecosystems.

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APPENDIX A

PERSONAL ATTEMPTS AT AMF MOLECULAR IDENTIFICATION

The objective of the study was to observe the impact that invasion by *Euphorbia esula* has on the species and diversity of AMF present in the roots of native prairie plants, in particular, *Koeleria cristata* and *Euphorbia corollata*. Evidence from previous studies such as Hawkes et al. (2006) suggests that the presence of an invasive exotic plant is correlated with a decrease in diversity of AMF in native flora. Furthermore, community feedback models (Johnson et al. 2006) predict that the emergence of a dominant plant in an ecosystem will alter the composition of the AMF community. Therefore, it could be expected that invasion by *E. esula* at the Fort McCoy site would lead to a change in the AMF community and a potential decrease in the diversity of AMF species colonizing native plant roots.

Additionally, there is no formal documentation of the species of AMF that colonize the roots of *E. esula*. The study sought to illuminate the mycorrhizal nature of *E. esula*. For example, the number of species of AMF associated with *E. esula* would have been documented, and it would have been determined if *E. esula* appears to preferentially associate with certain species of AMF or if it is a mycorrhizal generalist relative to the other two species it co-occurred with. The hope was to provide more information and insight regarding the effects of *E. esula* as it colonizes a native ecosystem.

Materials and Methods

The study site is located on the south post of Fort McCoy (near Sparta, Wisconsin) and consists of a sand prairie that is undergoing invasion by *E. esula*. Samples were collected on June 6, 2007 when most of the vegetation, especially *E. esula*, was undergoing the energetically expensive process of flowering, and therefore

likely exploiting the mycorrhizal relationship to its fullest. The study was confined to one site so that differences in soil type, elevation, vegetation, moisture and temperature were avoided.

Three "patches" each of three different vegetational compositions were selected and sampled. Patch compositions were as follows: plots consisting of only native plant species, plots encompassing both native species and *E. esula*, and plots that were composed of *E. esula* in monoculture. Within each plot one plant of each species was sampled in duplicate. Sampling consisted of digging up the plant roots, shaking off the bulk excess of soil and placing each sample in large Ziploc bags for transportation and storage. Roots were washed and lyophilized within 24 h of collection. Lyophilized roots were stored in Ziploc bags at room temperature.

Roots were stained with trypan blue in an acidic glycerol solution to allow observation of intracellular hyphal colonization. The staining procedure was adapted from Koske and Gemma (1989). Lyophilized roots were ground in liquid nitrogen with a micropestle in 1.5 ml microfuge tubes. DNA extractions were performed using the DNeasy mini plant kit (Qiagen Inc.) following the manufacturer's instructions. Nested PCR was performed following procedures outlined by Renker et al. (2003). The first PCR reaction is Glomeromycotan specific and uses the primers SSU-Glom1 and LSU-Glom1 to generate a ~1000-1200 bp fragment. However, some Basidiomycete contaminants (particularly *Cryptococcus spp.*) may be amplified with these primers. Therefore, an intermediate restriction digest was performed with the enzyme *AluI*, which cuts at a site conserved by the Basidiomycota but not present on most of the amplified fragments of Glomeromycotan fungi. Unfortunately, members

of the Paraglomeraceae possess the *AluI* cut site and are therefore eliminated in this step. The elimination of the Paraglomeraceae was not of great concern, as the family consists of only one genus (*Paraglomus*) and only a few described species that seem to be somewhat rare. Following the restriction digest, a second PCR was performed using universal fungal primers ITS4 and ITS5, thereby generating a fragment of ~600-800 bp (White et al. 1990). Cloning and sequencing were used to obtain AM fungal species identification. Unfortunately, almost all the sequences obtained from cloned colonies aligned with basidiomycetous yeast contaminants despite the intermediate *AluI* digest.

Procedural Difficulties

Following DNA extractions with the Qiagen DNeasy kit, a variety of problems with the PCR reactions ensued. Primarily, PCR products were not consistently obtained, especially in the nested reactions. Several modifications to the PCR protocol were made to enhance the success of amplification. The most notable alteration was the replacement of "homemade" Taq polymerase that is produced by UWL's molecular biology lab with commercial Taq polymerase. The used of high grade Taq immediately produced PCR products from extractions that had previously shown no amplification.

Another modification that produced excellent results was the dilution of extractions and products of the first PCR amplification. Template DNA was amplified at full concentration, 100 and 1000 fold dilutions. Greatest amplification success with the clearest, strongest gel bands was generally observed with 100 fold dilutions. Increasing the $MgCl_2$ concentration led to gel bands that were brighter and

more concise than in previous attempts. Most PCR reaction protocols call for a 1.5 mM $MgCl_2$ concentration, but I found that a 2.5-3.0 mM concentration produced the best results. The amplification success with increased $MgCl_2$ concentrations may be due its role in the stabilization of the DNA backbone, therefore increasing primer binding. Unfortunately, after a year and a half of experimenting with various AM specific primers and PCR protocols, another procedural road block was encountered when attempting to clone and sequence PCR products. Often the sequences obtained did not align with any AM fungal related species in GenBank. Furthermore, cloning was not always successful, as more often than not plates inoculated with bacteria containing cloned plasmids would not produce any colonies. Eventually, due to the necessity to save time, funding and resources, the project had to be shelved until more feasible procedures become available.

There are a number of labs around the world working on the molecular identification of AM fungi from environmental sample. After communicating with a number of these labs in my attempts to refine and troubleshoot my procedures, the general consensus is that molecular AMF identification is extremely tricky and there is not yet a standard protocol that seems to be the most efficient and error free. Unfortunately, when AM studies that have successfully employed these methods are published, the failed attempts and troubleshooting are not included in the publication, nor are the published protocols complete. Therefore, a researcher attempting to utilize molecular AM identification methods based on the published literature may be largely unprepared for the myriad of pitfalls and intricacies that are inherent to this field of study.