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Sustainable Agriculture and the Multigenomic Model: How Advances in the Genetics of Arbuscular Mycorrhizal Fungi will Change Soil Management Practices

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Abstract

Arbuscular mycorrhizal (AM) fungi are important both in agriculture and in natural ecosystems due to their effects on the fitness of their plant hosts. As symbionts, AM fungi improve plant uptake of water and nutrients, and help to protect against pathogens. The study of these organisms has been obstructed in part by difficulty in identifying and quantifying them in the field, a problem springing from our poor understanding of their unusual genetic structure.

AM fungi have been shown to be multigenomic, possessing a large amount of genetic variation not only between individuals, but among nuclei within an individual. In order for simple, reliable identification and quantification techniques to be developed for large-scale use, this genetic diversity must be quantified and marker genes found for which the amount of variation is manageable. Once this has been accomplished, growers can work knowledgeably with the existing strains of AM fungi in their fields, or select an appropriate commercial inoculum.

In this chapter, we will discuss the current state of knowledge in AM fungal genetics and how it can be applied to develop molecular tools which permit the management of natural AM fungal populations in agricultural fields. Assessment of AM fungal biodiversity in natural and modified ecosystems, as well as the estimation of the mycorrhizal potential of agricultural soil, are bottlenecks that greatly limit our understanding of the ecology of these cornerstone organisms. The development of efficient and inexpensive diagnostic techniques will enable us to use them to their full potential in sustainable agriculture systems.

11.1 Introduction

The arbuscular mycorrhizal (AM) fungi are a group of asexual, root-inhabiting, symbiotic fungi which make up the phylum Glomeromycota (Schüßler *et al.*, 2001). This phylum is made up of five families: Paraglomaceae, Archaeosporaceae, Glomeraceae, Acaulosporaceae and Gigasporaceae, which together make up approximately 160 species (Sanders, 2002). AM fungi are among the fungi most frequently found in soil and are widely distributed geographically (Smith and Read, 1997). They form symbioses with the roots of more than 80% of all vascular plant species (Smith and Read, 1997). The AM symbiosis is believed to be over 400 million years old, and fossils show that the earliest land plants contained these endophytes (Simon *et al.*, 1993; Remy *et al.*, 1994; Redecker *et al.*, 2000). The AM symbiosis has, in fact, been proposed to be at the origin of land plants (Pirozynski and Malloch, 1975).

One of the principal host benefits of the AM symbiosis is the increased uptake of phosphorus. Phosphate ions in soil are largely unavailable to plants because they form insoluble complexes with naturally occurring metal cations. Fungal hyphae are able to extend beyond the root depletion zone, taking up bioavailable phosphate which is outside the reach of the plant (Helgason and Fitter, 2005). Plant hosts also experience improved uptake of water and nitrogen, both in the form of NH_4^+ released through mineralization (Subramanian and Charest, 1999; Hamel, 2004; Toussaint *et al.*, 2004; Tanaka and Yano, 2005) and as organic molecules (Hawkins *et al.*, 2000) of some other cations such as Cu^{2+} , Zn^{2+} , K^+ and Fe^{2+} (Liu *et al.*, 2000) via this extensive network of fungal hyphae, improving both nutrition and drought tolerance. Soil structure is improved in regions with well-developed AM fungal populations because of the ability of glomalin, a fungal protein, to bind soil particles, forming macroaggregates and helping to decrease soil erosion (Rillig and Mummey, 2006). Yet another benefit conferred upon a plant host by its AM symbiont is an improved level of resistance to pathogens, in particular root-infecting fungi and nematodes (Helgason and Fitter, 2005; St-Arnaud and Vujanovic, 2007). It has been variously suggested that AM fungi may protect their hosts through competition for colonization sites, through improved nutrition, stimulated plant defence responses, or through changes in the microbial community structure of the surrounding soil (Pozo and Azcón-Aguilar, 2007; St-Arnaud and Vujanovic, 2007). Finally, AM fungi are associated with several types of plant beneficial microorganisms, including nitrogen-fixing bacteria and phosphorus-solubilizing bacteria and fungi (Barea *et al.*, 2002). In fact, in the case of nitrogen-fixing bacteria, common signalling cascades suggest that the bacteria at one point simply modified recognition pathways which originally evolved to enable AM fungal colonization (Kistner and Parniske, 2002). Research into the tripartite symbiosis – composed of a legume, an AM fungus, and bacteria of the genus *Rhizobium* – has shown that the presence of the fungus improves both the nodulation and the nitrogen-fixing capabilities of the bacteria (Barea *et al.*, 2002). As with their plant hosts, AM fungi can also provide a measure of drought resistance to the bacteria, protecting nodules

from oxidative damage due to stress (Ruiz-Lozano *et al.*, 2001). With regard to phosphorus-solubilizing bacteria, one study using radio-labelled phosphorus in the form of rock phosphate showed that inoculation with AM fungi allowed the uptake of sources of phosphorus not available to the plant when only the bacteria are present. The dual inoculation significantly increased both the biomass of the plant and its accumulation of nitrogen and phosphorus (Toro *et al.*, 1997). With so many potential host benefits, it has long been recognized that managing the AM symbiosis could prove valuable in an agricultural setting.

While the use of AM fungi in agriculture has not yet been widely embraced by the intensive operations typical in Europe and North America, countries such as Cuba and India, where large amounts of chemical inputs are prohibitively expensive, have made impressive advances in this area. Following an intensive research programme in the 1990s, Cuban scientists developed an inoculum mix specific to soils in that region, as well as recommendations regarding the conditions under which it should be used. The researchers found that strains varied in their effectiveness depending on the type of soil in which they were used. Today the inoculum, called EcoMic, is recommended for a wide variety of regional crops and is used in many growing operations. Even in high-input systems, it has proven to be a very effective biofertilizer; used as a seedcoating, it produces yield increases of 10–80%/ha with a 6–10% application rate, by weight, depending on the crop species (Rivera *et al.*, 2007).

In India, where a great deal of applied mycorrhizal research has also taken place, commercial inocula are used on a large scale, with close to 2500 t produced in 2006 by four different Indian companies. Used with rice crops, researchers found that mycorrhizal inoculation could produce a modest yield increase of around 10%, but with a 25–50% reduction in the amount of fertilizer required – real savings in India's low-phosphorous soils (Sharma *et al.*, 2007).

For a prime example of how the large-scale development of a commercial symbiotic inoculum can produce economic change in North America, we need only look to the adoption of specialty legume crops, such as peas, lentils and chickpeas in the Canadian prairies, a region which is naturally poor in the *Rhizobium* bacteria required for the plants to fix atmospheric nitrogen. The development by the Saskatoon-based MicroBio RhizoGen Corporation in the late 1980s of *Rhizobium* inoculants appropriate to prairie soils made the production of these crops profitable through large savings in nitrogen fertilizers which dwarfed the cost of the inoculum itself. Over the past two decades, the area sown in Saskatchewan as specialty crops, the bulk of which are legumes, has increased from 1.2 to 16.1% of the total agricultural land in the province (Carlyle, 2004).

In order for the use of AM fungi to be widely adopted in North America, methods must be in place to quickly and cheaply analyse their status in fields so that producers can respond accordingly. We need a comprehensive picture of what taxa are present, how they are interacting with the host crop, and how they react to various soil treatments. One major roadblock in the development of these tests has been the peculiar and poorly understood genetic structure

the fungi seem to possess. In the following section, we will discuss the discoveries surrounding this unusual genetic organization and some of its implications.

11.2 Genetic Structure and Heterokaryosis

AM fungi are primarily made up of vast, branching networks of hyphae (Fig. 11.1a). Unlike most higher fungi, these hyphae are coenocytic, that is, lacking in discrete cellular divisions. Hyphal walls form long, tube-like structures through which cytoplasm and organelles can migrate freely (Fig. 11.1b). When an AM fungus sporulates, asexual spores are formed on the terminus of a mother hypha, and large numbers of nuclei simply migrate into the spores via that connection (Fig. 11.1c).

Because Glomeralean spores are formed containing hundreds or even thousands of nuclei from the mother hypha, it is thought that there never exists a time in the AM fungal life cycle at which the organism is uninucleate (Sanders, 2002). In most life forms, the uninucleate stage acts as a genetic bottleneck, ensuring that each somatic cell which follows will possess a nucleus that is an identical mitotic product of the original. In an organism lacking both sexual recombination and a uninucleate stage, mutations which occur in individual nuclei are allowed to pass on to the next generation, potentially allowing the development of individuals containing any number of varied genomes (Sanders, 2002). While a great deal of research has been done looking into the ecology and physiology of AM fungi, only relatively recently have inquiries begun to be made into their genetic structure.

Suspicions that AM fungi possess an atypical genetic structure began with the investigation of their enzymes; different isolates of the same species were found to possess different enzymatic isoforms (Hepper *et al.*, 1988; Rosendahl and Sen, 1992). Exploration then turned to ribosomal genes. These genes are present in multiple copies occurring in tandem arrays. The sequences within an array are normally kept very similar through the process of concerted evolution, a mechanism by which repeats within a gene family exchange sequence information, thereby maintaining a high level of homogeneity and allowing the family to evolve as a unit. Concerted evolution is thought to be driven primarily by gene conversion and unequal crossing-over during meiosis. Early investigations of the internal transcribed spacer (ITS) regions flanking the 5.8S ribosomal RNA (rRNA) in *Glomus mosseae*, however, showed that individual spores contained multiple distinct ITS sequences (Sanders *et al.*, 1995). Researchers investigating rRNA genes within the AM fungal species *Scutellospora castanea* also found a high level of polymorphism (Hosny *et al.*, 1999). A later study of the same species using specific fluorescent DNA-DNA *in situ* hybridization (FISH) investigated the frequency of two divergent sequences of the ITS2 region, referred to as T2 and T4 (Kuhn *et al.*, 2001). These sequences, which had been previously demonstrated to co-occur within individual spores (Hijri *et al.*, 1999), were shown to in fact occur in different frequencies from nucleus to nucleus within a spore. The researchers used a

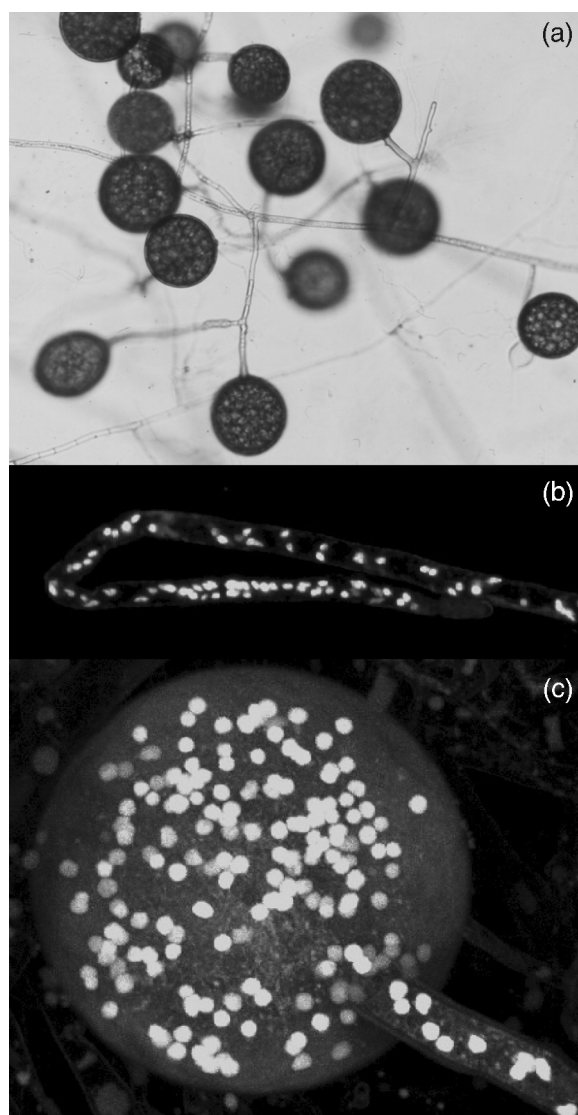


Fig. 11.1. (a) Spores of *Glomus intraradices* grown in *in vitro* culture with transformed carrot roots and observed under a stereomicroscope. (b) Hypha of germinating spore of *Gigaspora rosea*. (c) *Glomus diaphanum* spore showing typical multi-nucleate stage. In (b) and (c) nuclei were stained using SYTO Green fluorescent dye and observed under a confocal microscope.

phylogenetic approach to examine whether the differences between nuclei were likely to have been caused by recombination or by an accumulation of mutations in successive clonal generations. Calculated probabilities were significantly different than those expected in recombining organisms, leading the authors to believe that most of the observed variation was caused by mutation. In the same study, a binding protein-encoding gene, *BiP*, which is

single-copy in other fungi, low-copy in AM fungi, and highly conserved in eukaryotes, was analysed to reveal 15 different variants in the genomic DNA of a single *Glomus intraradices* isolate. Collectively, all the research conducted on intraspecific polymorphism in AM fungi indicates a unique and complicated genetic structure.

The implications of a multigenomic arrangement are many. The discovery of this heterokaryotic structure in AM fungi has necessitated a new way of thinking about what constitutes an individual, as well as many questions about our concepts of species and populations as they apply to these organisms (Rosendahl, 2008). Although, for the sake of simplicity, a single spore and the mycelium which grows from it is conventionally referred to as an individual, a single spore can also be thought of as containing a population of nuclei, each of which *may* be capable of functioning as an individual. That is to say, if each nucleus within a fungal spore contains a full and potentially differing complement of genetic material, then the nucleus itself, rather than the spore, could be considered an individual. Sanders (2002) advances two different possibilities for the coexistence of these differing nuclei. First, it is suggested that each nucleus does, in fact, possess a full quota of required genes (Fig. 11.2a). This arrangement could then lead to competition among the genomes. Second, it is suggested that all the necessary genes coding for various functions are spread across numerous genomes, forcing their cooperation (Fig. 11.2b). Under this arrangement, the individual must be defined as the aggregate of the genomes required to form a fully functioning organism.

There is also the question of how genetic diversity arises and is maintained. Because the nuclei which comprise a new spore simply migrate from the subtending hypha into the spore, any heterogeneity in the distribution of different nuclear types across the mycelium will cause spores to arise which differ genetically from others borne on the same mycelium (Koch *et al.*, 2004). This would suggest an ongoing loss of genotypes with each passing generation. These genetic differences could also translate into functional differences, affecting the mycorrhiza.

Here, the question of genome segregation within the hyphal network becomes of interest. If a full complement of functional genes, spread across numerous nuclei, must remain in a given region of the mycelium in order for it to perform properly, segregation according to selection will be heavily restrained within a microenvironment, and a certain level of homogeneity can therefore be expected. Alternatively, differing nuclei may be unevenly distributed within the mycelium according to their fitness within a particular microenvironment (Fig. 11.3), or simply by random chance. In either scenario, a heterogeneous arrangement of nuclei would quickly lead to the loss of all but one type of genome or all but one particular group of genomes, if nuclei are cooperating. Nuclear exchange through hyphal fusion, referred to as anastomosis, may be the means by which this is prevented.

Anastomosis, the fusion of two fungal hyphae of the same or different mycelia, has been shown to occur in some species of AM fungi, particularly those belonging to the genus *Glomus*. While this joining is not believed to occur among individuals of different species or geographical origins, it has been

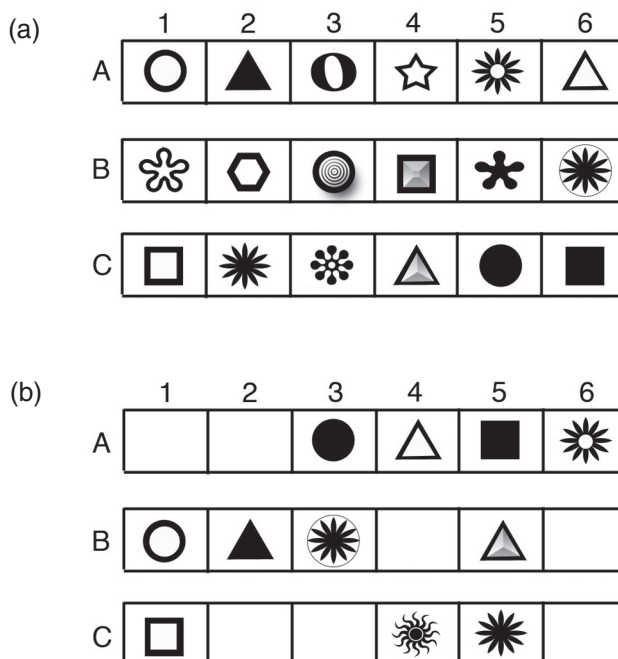


Fig. 11.2. Diagram showing two possibilities for the arrangement of heterokaryotic structure among nuclei of AM fungi. (a) Each nucleus possesses a full complement of required genes, with variants differing between nuclei. (b) No single nucleus possesses a full complement of functional genes, so nuclei are forced to 'cooperate'. Three different nuclei, represented by A, B, C, have six loci representing genes required for function. Shapes represent different possible variants of each of the six genes. Empty boxes represent non-functional alleles.

observed between hyphae originating from two different spores in the same isolate. One study found that in three *Glomus* species, between 34 and 90% of hyphal contacts between different germings of the same isolate resulted in anastomoses (Giovannetti *et al.*, 1999). Interestingly, in the same experiments, no anastomoses of any sort were observed in either *Gigaspora rosea* or *S. castanea*. Anastomoses allow damaged hyphae to re-establish a protoplasmic link as well as allowing nuclear exchange between mycelia (Fig. 11.4). This type of joining may also allow the re-homogenization of nuclei which have become heterogeneous across the mycelial network, thereby maintaining some level of genetic consistency throughout (Bever and Morton, 1999). Depending on the level of compatibility necessary for a given fungal species to anastomose, this phenomenon could also lead to the formation of a single, joined mycelium covering a large area and containing all available genotypes for that taxon.

Phenomena such as differential genetic segregation and homogenization via anastomosis may seem far removed from the business of managing AM fungi in the context of an agricultural operation, but as we will see, these unusual features have real implications for the efficacy of a given inoculum and our ongoing ability to control plant–fungus interactions in the field.

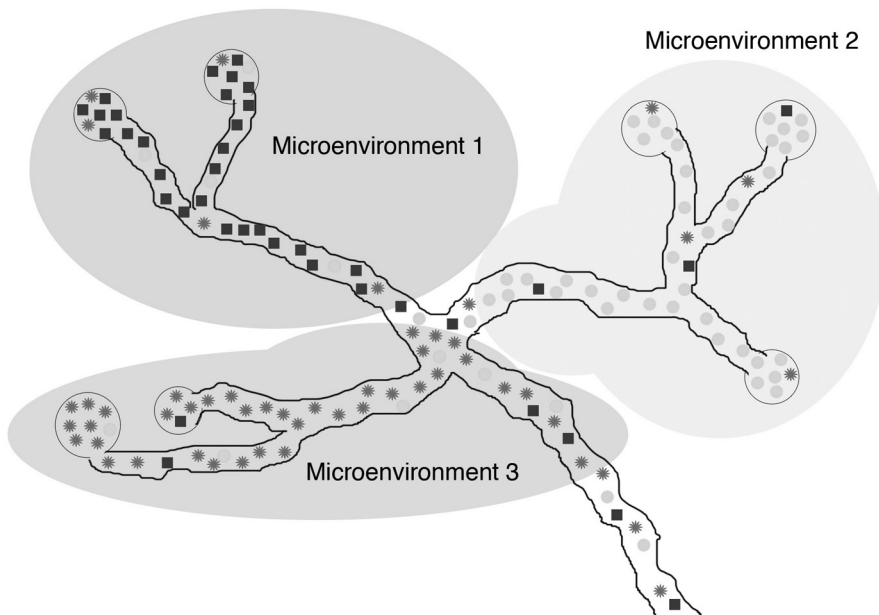


Fig. 11.3. Diagram showing the possibility for genetically differing spores to arise on a single mycelium. Certain genotypes, represented by different shapes, are better suited to different microenvironments and are selected for within those regions, affecting the proportions of different nuclei which enter the developing spore.

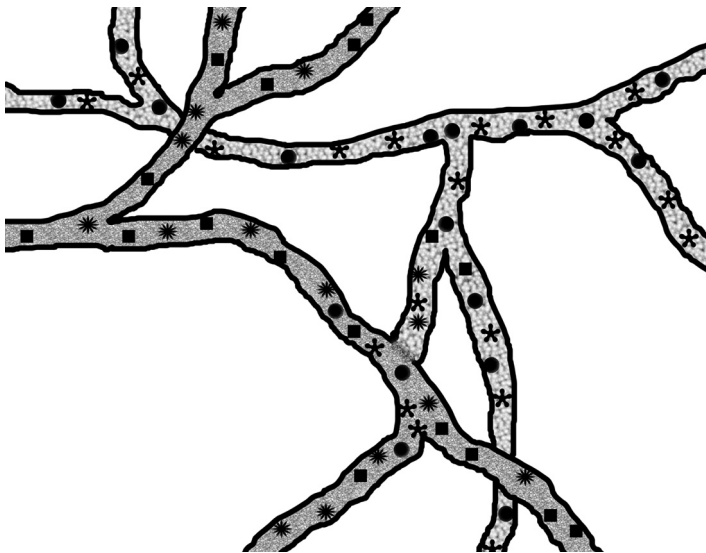


Fig. 11.4. Diagram showing anastomosis of two genetically differing hyphae, allowing mixing and homogenization of different nuclear types (shapes) in the vicinity of the connection.

11.3 Importance and Management of AM Fungi in Agriculture

While the abilities of arbuscular mycorrhizas to aid in plant fitness are not new discoveries, farming in the 21st century faces challenges which bring new importance to the effective use of AM fungi in agricultural operations. As awareness of the need for sustainable practices grows, efforts are being made to reduce our usage of chemical fertilizers and potentially harmful biocides. Furthermore, with the effects of climate change leading to increased plant stress in the form of drought, heat waves, pest problems and invasive species, the benefits of arbuscular mycorrhizas beyond that of plant nutrition also gain importance (Gavito, 2007). In the near future, the improved water uptake of mycorrhizal crops may prove more critical than any improvement in nutrition, particularly in western North America, where the most intensive grain production is carried out.

The managed use of AM fungi in agricultural operations has the potential to benefit not only the health of the crop plants, but the health of the soil itself. The long-term use of phosphorous fertilizer is associated with soil degradation and the pollution of nearby bodies of water, where runoff can cause algal blooms and disrupt plant and animal communities (Beauchemin and Simard, 1999). Soil phosphate saturation has reached problematic levels in many agricultural areas. Forecasting of crop phosphorous requirements is imprecise, and crop responses show a poor correlation with soil phosphorous test values. It is thought that this problem is in part due to functional variations in the naturally occurring AM fungi, which are not accounted for in forecasts (McKenzie and Bremer, 2003). Maintenance of a healthy and efficient AM fungal community in the field would allow a decrease in applied phosphate. This decrease would in turn benefit the AM symbiosis, as an overabundance of soil phosphorus is inhibitory to root colonization (Smith and Read, 1997). Furthermore, taking into account mycorrhizal activity, abundance and seasonality, more precise forecasts would be possible.

Current complications involved in managing mycorrhizal populations largely spring from our poor understanding of AM fungal biology. One important issue is the variability seen in the effect of a given fungus on different hosts. Studies have shown not only that AM fungal species are a factor in the derived benefit of the host plant (van der Heijden *et al.*, 1998), but that different isolates of the same species can have varying effects as well. Some host/symbiont combinations seem to result in an increase in fitness for only one partner, and it can be difficult to predict which partner this will be due to the occurrence of both positive and negative feedback in natural communities (Bever, 2002). One study was conducted in which different isolates of the AM fungus *G. intraradices* were grown under identical conditions for several generations to negate environmental maternal effects (Koch *et al.*, 2006). The authors then showed that a given isolate could in fact have a negative effect on one host while causing no harm in another, and that positive effects varied in intensity from isolate to isolate. A study by Bever and co-workers (1996) showed large amounts of variation in spore production by a given fungal isolate

from one plant host to another. These results highlight the importance of genetic variability in choosing appropriate fungi for large-scale inoculation, as well as assessing the efficiency of AM fungal strains in a natural community in order to favour useful strains.

It can be difficult to know whether a fungal species present in a field is helping or hindering a crop, especially since even those fungi which produce neither positive nor negative growth effects in their host may still make a significant contribution in terms of phosphate uptake, as well as having an associated carbon cost (Li *et al.*, 2006). Furthermore, benefits brought about by AM fungal colonization can take different forms: one AM fungal species may provide good pathogen protection, but offer no appreciable increase in growth or nutrient uptake (Caron *et al.*, 1986; St-Arnaud *et al.*, 1994). Another may have the opposite effect. The best way to avoid these problems, it seems, is to maintain high fungal biodiversity within the field, increasing the chance of including one very effective strain (van der Heijden *et al.*, 1998). A recent study (Jansa *et al.*, 2008) found that colonization by multiple AM fungi, in that case *G. intraradices* and *Glomus claroideum*, could have synergistic effects, providing greater host benefit than any one of the component taxa would singly, so long as no one taxon dominated the others. However, maintaining high fungal diversity in a high-input agricultural field may be easier said than done. Since some species of AM fungi function much better on a particular crop, monocultures and certain rotations, especially those including non-host crops, discourage diversity. Many common agricultural practices, such as tillage and the application of phosphates and fungicides, may kill off all but the most 'robust' species, and these are not necessarily the taxa which are most beneficial to their hosts.

Furthermore, due to the possibility of a heterogeneous distribution of multiple genomes in a fungal mycelium, spores may arise containing alleles of certain protein-coding genes which are completely absent in other spores borne on the same mycelium (Koch *et al.*, 2004). These genetic differences could translate into functional differences, affecting the efficacy of a commercially developed inoculum. Maintaining and encouraging fungi with desirable attributes could prove impossible if certain genomes are being lost with each new round of sporulation and not necessarily retrieved via anastomosis. This type of genetic drift could render carefully selected commercial inocula ineffective after just a few generations. Indeed, Koch and co-workers (2004) found fivefold differences in hyphal length among isolates originally grown from single spore cultures. The authors felt that the phenotypic variation observed was great enough to cause corresponding variation in plant host growth and nutrition. A better understanding of the way in which genomes segregate in the mycelium is therefore vital to those who would develop commercial inocula or manage the AM symbiosis in agriculture.

All of these complications could be improved upon with a better understanding of AM fungal populations in the field. What is needed, as a first step, is an efficient, inexpensive means of measuring both the diversity and the overall abundance of these fungi in the field.

11.4 What Has Been Done in Identification and Quantification

Well-meaning recommendations made by optimistic researchers working in laboratories and test plots are often rejected by growers because they do not take into account the pressures and uncertainty inherent in agrobusiness. To be worth the risk, new farming practices must be convenient, simple, cost effective, and come with a reasonable guarantee of improvement over established methods. The management and monitoring of AM fungi in Western intensive agriculture has failed on several of these counts. While increases in drought and pest problems, as well as potential crackdowns on phosphate use may well solve any issues of cost effectiveness, it will still be up to researchers to develop methods of managing AM fungi which are convenient and straightforward without being overly expensive. Ideally, a grower could send off soil and root samples and receive a profile of what AM fungal species are present and in what amount, allowing her to add inoculum, adjust fertilization regimes, or otherwise respond accordingly. In order for this to happen, testing protocols must be developed which are standardized, require only commonly used laboratory equipment, and do not require a great amount of expertise to interpret. Molecular PCR-based methods fit this description and will, in the coming years, be key to practical mycorrhizal analysis.

Identification

A wide variety of methods have been employed in attempting to accurately and conclusively identify different species of AM fungi. Traditionally, species were identified based on the morphological characteristics of spores. This required a great amount of expertise on the part of the researcher and often proved to be inexact due to an insufficient number of clear, informative characters. Molecular data, properly interpreted, would have the advantage of identification directly from host roots. This would provide a more accurate picture of the fungal community, since spore analysis does not necessarily predict future colonization. The first attempts at identification and phylogenetic placement based on genetic markers used variation in ribosomal DNA (Simon *et al.*, 1993; Sanders *et al.*, 1995; Lloyd-MacGilp *et al.*, 1996); however, the extremely high variation present in these sequences made this very difficult. Sequences were found which had no known species correlation, and sequences found in one species would frequently cluster with those of another species or even another genus (Clapp *et al.*, 1999; Rodriguez *et al.*, 2004). One early study found that ITS sequences amplified from within a single spore often showed more variation than those from different isolates (Lloyd-MacGilp *et al.*, 1996). Rodriguez and co-workers (2005) examined several isolates of two different AM fungal morphotypes and found them to contain extremely variable ribosomal gene sequences, as may have been expected. However, the two morphotypes were also found to contain several identical sequences. Finally, a study examining copy number in the ribosomal genes of *G. intraradices* found

that the number of genes could vary between two- and fourfold among isolates of just this one species (Corradi *et al.*, 2007), making ribosomal genes inappropriate for any attempt at quantification of AM fungi via the quantification of amplified genetic material.

More recently, several protein-coding genes have been investigated for possible use as markers. This type of gene has the advantage of typically possessing lower levels of variation than ribosomal sequences, potentially making for clearer partitions among species and isolates. The lower copy numbers found for coding genes, however, can make amplification more difficult.

One group searching for such markers examined actin and elongation factor 1- α genes and found that amino acid sequences were highly conserved across multiple spores within an isolate (Helgason *et al.*, 2003). Pending further investigation to determine the level at which the sequences vary, the authors felt these genes had potential for use as markers in identification. Alpha- and beta-tubulin, as well as the H⁺-ATPase gene were also evaluated for this purpose (Corradi *et al.*, 2004a, b). Beta-tubulins, in particular, may hold promise as useful markers. The authors were able to design primers which amplified only Glomeromycotan sequences from pot cultures, and found ample variation between species, but very little within, making them ideal for interspecific identification. Conversely, the H⁺-ATPase gene was found to contain high levels of variation and did not resolve fungi as monophyletic. Two RNA polymerase II subunits, *RPB1* and *RPB2*, were also used recently in the phylogenetic reconstruction of the early evolution of the fungal kingdom, and may prove useful for identification purposes within the Glomeromycota (James *et al.*, 2006).

Where the above genes fall short is in their ability to distinguish between different isolates of a single species. Given the large variation seen in the host-benefit from one isolate to another, making this distinction would prove valuable to those attempting to manage their AM fungal communities. This knowledge would be particularly useful in quantifying relative amounts of a commercial inoculum versus a native fungus of the same species, for example the ubiquitous species, *G. intraradices*. In the case of geographically close isolates, a mere change in the relative proportions of given sequences may be all that is available to separate a more desirable strain from a less desirable one. This type of determination cannot be made until we have gained a much better understanding of the amount, and arrangement of, intra- and inter-isolate variation in AM fungi. In some geographically well-separated isolates, however, enough variation has been found in the simple sequence repeats (SSR) and the introns of nuclear and mitochondrial genes for a combination of these markers to clearly distinguish between them (Croll *et al.*, 2008).

While several genes show potential for being able to consistently distinguish between AM fungal species, in most cases, larger sequence libraries need to be built up to ensure that variation is not undersampled. Furthermore, any marker gene will need to be sequenced in a wide variety of taxa in the fungal kingdom so that contaminant sequences can be easily recognized.

In terms of the actual methodology used in retrieving this genetic information, several different approaches have been successful, although there is often

a trade-off between cost and accuracy. Denaturing gradient gel electrophoresis (DGGE), wherein DNA run through a gel with a gradient of a denaturing compound can be separated based on single base-pair differences, is probably the most accurate and reliable method currently in use. This technique uses ribosomal sequence heterogeneity to its advantage, since a unique banding pattern is created as variants are separated, and as such has been able to consistently distinguish even between certain isolates of a given species, although multiple sequences are required to compensate for variant overlap (de Souza *et al.*, 2004). This trait may even circumvent problems of extreme variation when using ribosomal genes, so long as heterogeneity is consistent at different taxonomic levels. One drawback of this method, however, is that it requires costly equipment not commonly available in molecular biology laboratories, and each different type of test can require a long optimization period to establish protocols. Direct sequencing of PCR products has also been carried out successfully (Hijri *et al.*, 2006), but at prohibitively high cost for use on a large scale.

Perhaps the best compromise of cost, simplicity and effectiveness is an approach coupling PCR amplification and digestion using restriction enzymes. Terminal restriction fragment length polymorphism (T-RFLP), for example, uses amplification with fluorescently labelled primers followed by restriction digests to obtain fragments of variable lengths depending on the presence and location of cutting sites. These fragments appear as peaks on an electropherogram. Under conditions such as the analysis of field samples, where multiple species are likely to be amplified, databases of peak profiles, each corresponding to a known species, can be maintained in what is referred to as 'database T-RFLP' (Dickie and FitzJohn, 2007). Some potential difficulties with this technique are the need to have all AM fungal taxa which may be present in a sample already represented in the database, and the inability to distinguish peaks as being different taxa or simply different intra-isolate genotypes. Both of these problems could perhaps be circumvented through the extensive sampling of known species in order to build up a complete profile of frequently occurring peaks within that species. As with most other techniques, the effectiveness of T-RFLP as a means of identification depends heavily on the use of AM fungus-specific primers which can amplify a broad swath of the Glomeromycota.

Quantification

Another challenge for researchers working with AM fungi is the development of methods which will allow the overall quantification of AM fungal abundance. The ability to monitor fluctuations in the abundance of AM fungi in fields will give growers a good sense of the health of their soil and the effects of various treatments and practices, even if the exact taxa of AM fungi are not known.

The abundance of AM fungi in the soil is typically measured using fatty acid assays. Although currently the best technique available, this method is tedious and requires specialized equipment not found in most laboratories.

While fatty acids have been successfully used to determine the mycorrhizal potential of soils (Plenchette *et al.*, 2007), living biomass cannot be assessed as the lipids in question persist in the soil following the death of the fungi themselves. Accuracy is also an issue, as the diagnostic fatty acids vary in their abundance among different AM fungal species (Graham *et al.*, 1995) and also occur in some primitive organisms and Gram-negative soil bacteria.

Several studies have attempted to use real-time PCR to quantify fungal DNA in soil. The first (Filion *et al.*, 2003b) used specific primers targeting a fragment of the small subunit (SSU) rRNA gene region in *G. intraradices*. Results indicated that the technique was effective in comparing root colonization and spore numbers between soil samples of similar composition in a controlled experiment (Filion *et al.*, 2003a), but that absolute quantification was problematic due to a non-linear relationship between spore concentration and amplified genomic DNA. It was suggested that this discrepancy might have been due to the amount of polymorphism inherent in the ribosomal sequences.

A more recent study was done in which real-time PCR was used to quantify AM fungal DNA in soil using both actin and 18S ribosomal genes (Gamper *et al.*, 2008). Although the actin gene assays were less sensitive than expected, the authors found that, using the 18S gene, this method was sensitive and reliable in quantifying a specific taxon, even at low template concentrations. In fact, this study showed a good correlation between amplified genetic material and spore numbers. There was difficulty, however, in finding ribosomal gene regions which could amplify entire families or even genera of AM fungi. The authors stated that the limited availability of sequence information made using non-ribosomal genes impractical, and that future development of the method would rest largely on the expansion of sequence databases. This study highlights again the need for further research into appropriate AM fungal marker genes.

Furthermore, a major issue with this manner of quantification is that the spatial heterogeneity of nuclei within the hyphae makes the amount of DNA a poor predictor of the prevalence of hyphae within the soil. Gamper *et al.* (2008) tested a combination of nuclear and vital stains and found that living hyphal sections do not necessarily contain nuclei. Thus, test samples containing mostly hyphae or root fragments may underestimate the amount of AM fungal DNA, and conversely, samples with spores may lead to overestimation of AM fungal biomass. Our current inability to amplify entire groups of AM fungal species and the inapplicability of this method to taxa which sporulate infrequently make real-time PCR, though promising, ineffective for quantification in an agricultural setting.

11.5 How Management Practices Will Change and What Needs to Be Done

The development of a fast, cheap means of quantifying and identifying AM fungi in agricultural fields will lead to a better understanding of how the agroecosystem functions as a whole. At such time as efficient mycorrhizal

profiling becomes possible, producers will have a new level of control over their fields. New tillage or cropping regimes can be evaluated for their effects on AM fungi; fungal species and strains endemic to an area can be noted and, if sparse or ineffective, supplemented with a commercial inoculum. If they are aware of the level of AM fungal colonization in their crops, producers can adjust their fertilizer applications accordingly, saving money while encouraging the further development of the fungal community. The ability to correlate fungal abundance with crop phosphate uptake over the course of the growing season will enable more accurate phosphorous requirement forecasts to be made, accounting not only for the amount of AM fungi in a field, but for seasonal variations in their functionality. In the face of growing evidence of the importance of both host species and soil type on the mycorrhizal efficiency of a given fungus, easy, accurate field testing could lead to the development of 'designer' inoculum, produced for use under specific conditions. The practical experience of farmers in such endeavours will, in turn, lead to more advanced research on the life cycles and functioning of these organisms in the field.

Before these goals can be realized, much more needs to be learned about the extent and arrangement of variation in AM fungi. Appropriate marker genes need to be characterized which, ideally, would allow for some distinction even between intraspecific isolates. Sequence libraries will need to be built up so that even less common variants can be identified.

One exciting possibility for future research is the potential to go a step beyond identifying and quantifying fungi, and actually assay for their physiological activity in the soil. With different gene products produced in the pre-symbiotic and symbiotic stages, it could be possible to break down the proportions of the fungi in an area according to their stage in the life cycle, and to actually measure their nutrient-uptake efficiency once they have colonized the host. One recent study used real-time quantitative reverse transcriptase PCR to measure the expression of a *G. intraradices* phosphate dehydrogenase involved in phosphorous metabolism (Stewart *et al.*, 2006). While this experiment was conducted under laboratory conditions with a single known species, it may be possible one day to use the technique with more diverse field-collected samples. Other emerging techniques, such as untargeted expressed sequence tags (EST)-sequencing and microarrays are beginning to see use in profiling mycorrhiza-related genes in both plants and fungi (Küster *et al.*, 2007). In the future, these methods may elucidate many currently unknown pathways, bringing much more precision to enzyme-based assays for fungal activity in the soil.

11.6 Conclusion

AM fungi, with their many peculiarities, present us with both great possibilities for improving the health and stress tolerance of our crops, and with a number of unusual obstacles to our understanding of them. These fungi defy many of the rules we have come to think of as applying to all multicellular organisms, as

well as our usual means of classification. Heterokaryosis in AM fungi requires us to develop a more fluid concept of identification than is allowed for in the label of 'species'. The populations of these organisms at work in agricultural fields and elsewhere are more a large collection of interacting genotypes than a small collection of interacting species. Filing various specimens as similar aggregates of gene variants rather than under hard-and-fast phylogenetic labels will allow for a more flexible and dynamic understanding of the true nature of AM fungi, and may aid in our thinking as we develop the techniques necessary to fully comprehend their function. With today's myriad environmental crises and the need for meaningful change, AM fungi will come to represent a cornerstone in agricultural sustainability.

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