Université de Montréal

Extent Of Intra-isolate Genetic Polymorphism In *Glomus etunicatum* Using a Molecular Genetic Approach

par

Erin Zimmerman

Département de sciences biologiques

Faculté des Arts et des Sciences

Mémoire présenté à la Faculté des études supérieures

en vue de l'obtention du grade de

Maître ès sciences (M. Sc.)

en Sciences biologiques

avril 2009

Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé :

Extent of Intra-isolate Genetic Polymorphism in *Glomus etunicatum* Using a Molecular Genetic Approach

présenté par :

Erin Zimmerman

a été évalué par un jury composé des personnes suivantes :

Anne Bruneau
président-rapporteur

Marc St-Arnaud
directeur de recherche

Mohamed Hijri
codirecteur

Bernard Angers
membre du jury

Résumé

Les champignons mycorhiziens à arbuscules (MA) sont un groupe de champignons symbiotiques colonisant les racines des plantes et pour lesquels un grand intérêt est apparu dans les dernières années en raison de leur structure génétique inhabituelle et très complexe. Les champignons MA sont des organismes asexués, potentiellement de longue date, qui se reproduisent par la formation de spores à partir d'une hyphe mère. Les champignons sont coenocytiques; lors de la sporulation, un très grand nombre de noyaux parentaux migrent directement dans la spore fille. L'absence de stade uninucléé dans le cycle de vie du champignon MA, jumelé à l'accumulation dans le temps de mutations, ont favorisé l'apparition de populations de noyaux dissemblables, co-existant au sein d'un même organisme. Ce phénomène unique et les conséquences s'y rapportant sont, jusqu'à présent, peu comprises et apportent plusieurs interrogations sur ces organismes d'une grande importance écologique.

Deux aspects de la génétique des champignons MA ont été étudiés. Premièrement, un gène marqueur à plusieurs allèles présent dans l'espèce *Glomus etunicatum* a été utilisé pour déterminer les limites de variabilité et, du même coup, pour estimer le nombre de génomes distincts au sein d'un seul isolat MA. Ceci a été accompli par amplification du gène *PLS1* à partir de l'ADN génomique d'un seul isolat de *G. etunicatum*. Les allèles du gène ont été ensuite clonés et séquencés à partir de plus de 300 colonies bactériennes. Les 182 séquences entières du *PLS1* obtenues ont par la suite été analysées en utilisant une simulation de Monte Carlo, laquelle détermine le maximum théorique du nombre d'allèles dans l'isolat. De plus, une quantification par PCR en temps réel (qPCR) a été utilisée pour déterminer le nombre de copies du gène *PLS1* dans le génome du *G. etunicatum*. Malgré que plus de 100 allèles différents de *PLS1* ont été découverts, les efforts de séquençage ont été insuffisants pour prédire le maximum théorique du nombre d'allèles, en raison d'une diversité inattendue très élevée. Les résultats de qPCR ont donné une estimation du nombre de copies du gène inférieure à un, suggérant que la diversité du gène *PLS1* est étendue sur au moins 100 génomes uniques.

En second lieu, le même marqueur génétique a été utilisé pour analyser des différences dans la ségrégation nucléaire au moment de la sporulation, tel que mis en évidence par la variabilité génétique entre des spores soeurs. Plusieurs cultures monosporales du *G.*

etunicatum ont été établies en pot sur des plants de tomates. Après sporulation, 3 spores soeurs ont été récoltées de chacune de 4 cultures différentes. Pour chaque spore, une amplification génomique complète, suivie d'une amplification spécifique du *PLS* ont été réalisées. Les fragments de *PLS* amplifiés ont été étiquetés avec une courte amorce de reconnaissance à chacune des extrémités, afin de faciliter le pyroséquençage de l'amplicon. Cette méthode permet le séquençage de chacun des fragments de l'amplicon contenant les allèles multiples. Les données produites fournissent une image de la diversité et de la fréquence de chacun des allèles présents dans l'échantillon. Puisque les résultats de pyroséquençage ont été infructueux jusqu'à maintenant, les essais sont toujours en cours. La réussite du pyroséquençage nous permettra de comparer la fréquence d'apparition des allèles de *PLS* entre spores soeurs, permettant d'analyser si les noyaux ségrègent différentiellement entre les spores d'un même mycélium.

Ces résultats répondent en partie à deux importantes questions sur la biologie du champignon MA: combien de génomes différents pourraient posséder un organisme multigénomique, et quel est le comportement de ces divers noyaux lorsqu'ils sont transférés à la génération suivante?

Mots clés : champignons mycorhiziens à arbuscules, hétérocaryose, polymorphismes de l'ADN, ségrégation des noyaux

Abstract

Arbuscular mycorrhizal (AM) fungi are a group of symbiotic, root-inhabiting fungi which have garnered a great deal of interest in recent years due to their highly unusual genetic structure. AM fungi are putative ancient asexuals which reproduce via spores borne on a mother hypha. The fungi are coenocytic; at sporulation, a large number of nuclei from the parent migrate directly into the daughter spore. The lack of a uni-nuclear stage in the AM fungal lifecycle, coupled with the buildup of mutations over time, has allowed the development of a population of dissimilar nuclei, co-existing within single organisms. This unique phenomenon and the consequences arising from it are, as yet, poorly understood, and pose many questions to those investigating these ecologically important organisms.

Two aspects of the genetics of AM fungi were studied. First, a multi-variant marker gene occurring in the species *Glomus etunicatum* was used to determine its limits of variability and, by extension, to estimate the number of distinct genomes co-existing within a single AM isolate. This was accomplished by amplifying the gene, *PLS1*, from the genomic DNA of a single isolate of *G. etunicatum*. The gene was then cloned and sequenced in over 300 bacterial colonies. The resulting 182 whole *PLS1* sequences obtained were then analysed using a Monte Carlo simulation, which determines the theoretical maximum number of unique variants in the isolate. Additionally, real-time quantitative PCR was used to determine the copy-number of *PLS1* in the *G. etunicatum* genome. While over 100 unique variants of *PLS1* were discovered, sequencing efforts were found to be insufficient to predict a maximum, due to the unexpectedly high diversity. Copy number determination gave a copy number of less than one, suggesting that diversity in the *PLS1* gene is spread over at least 100 unique genomes.

Second, the same marker gene was used to investigate differences in nuclear segregation at the time of sporulation, as evidenced by genetic variation among sister spores. A large number of single-spore pot cultures of *G. etunicatum* were established on tomato plants. After sporulation, three sister spores were collected from each of four cultures. For each spore, a whole genome amplification, followed by a specific amplification for *PLS* was carried out. Amplified *PLS* fragments were tagged with a short recognition primer at each end, which was then used to facilitate amplicon pyrosequencing. This method permits the sequencing of each fragment of a DNA sample containing multiple variants of the amplicon.

The resulting data gives a picture of which variants are present in a sample, and at what frequency. While initial attempts at pyrosequencing were unsuccessful, trials are ongoing. Successful sequencing will allow the comparison of *PLS* variant occurrence between sister spores, leading to a determination of whether nuclei segregate differently among spores on a single mycelium.

These results will begin to address two important and unanswered questions in AM fungal biology: How many different genomes could this multigenomic organism possess, and what is the behaviour of these varying nuclei as they are transferred to the next generation?

Keywords: arbuscular mycorrhizal fungi, heterokaryosis, DNA polymorphism, nuclear segregation

Table of Contents

List of Tables

Chapter 2: High Genetic Diversity of *PLS* in a Single Strain of *Glomus etunicatum*

Chapter 3: Differential Nuclear Segregation in Individual Spores of *Glomus etunicatum*

Chapter 4: Autotrophic and Transformed Root Cultures of *Glomus etunicatum*

List of Figures

Chapter 1: General Introduction

Chapter 2: High Genetic Diversity of *PLS* in a Single Strain of *Glomus etunicatum*

Chapter 3: Differential Nuclear Segregation in Individual Spores of *Glomus etunicatum*

Chapter 4: Autotrophic and Transformed Root Cultures of *Glomus etunicatum*

List of Abbreviations

AM	arbuscular mycorrhizal
AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
Ct	cycle threshold
DGGE	denaturing gradient gel electrophoresis
dsDNA	double-stranded DNA
FISH	fluorescent *in situ* hybridization
gDNA	genomic DNA
ITS	internal transcribed spacer
LB	Luria-Bertani (medium)
M	minimal (medium)
MES	2-(N-morpholin) ethane sulphonic acid
MGB	minor groove binder
mRNA	messenger RNA
MS	Murashige and Skoog (medium)
MSR	modified Strullu-Romand (medium)
NFQ	nonfluorescent quencher
NPI	Native Plants Incorporated
PCR	polymerase chain reaction
PLS	polymerase 1-like sequence
qPCR	quantitative polymerase chain reaction
Ri T-DNA	transferred DNA of the tumor-inducing (Ti) plasmid of *Agrobacterium rhizogenes*
rRNA	ribosomal RNA
SNP	single nucleotide polymorphism
SSR	simple sequence repeats
SSU	small subunit
T-RFLP	terminal restriction fragment length polymorphism
UDG	uracil DNA glycosylase

Acknowledgements

First and foremost, I would like to thank my advisors, Dr. Marc St-Arnaud and Dr. Mohamed Hijri. Some of the graduate students with whom I have spoken found that having two advisors meant contradictory instructions and twice as many people to answer to- in short, not worth the trouble. I, on the other hand, was provided with two caring personalities whose strengths seem to complement each other perfectly. Over the course of my research, Mohamed has been a source of endless optimism, technical know-how, and crazy ideas; Marc, on the other hand, has provided down-to-earth advice, praise, encouragement, and a wonderful ability to deal with bureaucracy. Where one leaves off, the other picks up. Thank you so much to you both.

Some of the other people who have leant a hand along the way, and whom I would like to thank are: Dr. Simon Joly, who, without even being asked, wrote the program that allowed me to do my Monte Carlo simulation; Dr. Anne Bruneau and Carole Sinou, for their help in all things phylogenetic; Lyne Dubois and Marie-Pierre Gauthier, for their assistance in the lab; Eric Chevalier, for his translation abilities and abiding good nature; and finally, to Julie Marleau, for making the Hijri lab a more entertaining place to be.

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka! I found it!', but 'That's funny...' "

~Isaac Asimov

"There is a theory which states that if ever anybody discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened."

~Douglas Adams

To Leslie Zimmerman and Dr. Larry Peterson, my mentors,
for their patience, generosity, and faith in my abilities.
Thank you.

Chapter 1

General Introduction

[Published as: Zimmerman, E., St-Arnaud, M., and Hijri, M. (in press) "Sustainable agriculture and the AMF multigenomic model: how advances in AMF genetics will change soil management practices." In: Plant Microorgaanism Interactions. Eds. Kamal Bouarab and Normand Brisson. CABI, Oxford, UK.]

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.

Arbuscular mycorrhizal 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 knowledgably with the existing strains of AM fungi in their fields, or select an appropriate commercial inoculum.

In this chapter, I 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.

1.1 – Background

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, Archeosporaceae, Glomeraceae, Acaulosporaceae, and Gigasporaceae, which together make up approximately 160 species (Sanders 2002). Arbuscular mycorrhizal 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 phosphorous. 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, Zn, K and Fe (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 defense 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 P-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 P-solubilizing bacteria, one study using radio-labelled phosphorous in the form of rock phosphate showed that inoculation with AM fungi allowed the uptake of sources of P 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 phosphorous (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 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 program 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% per hectare 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 tonnes 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- a 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 a 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 analyze 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.

1.2 – Genetic Structure & Heterokaryosis

Arbuscular mycorrhizal fungi are primarily made up of vast, branching networks of hyphae (Fig. 1.1a). Unlike most higher fungi, these hyphae are coenocytic, that is, lacking in discrete cellular divisions. Hyphal walls form long, tubelike structures through which cytoplasm and organelles can migrate freely (Fig. 1.1b). 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. 1.1c).

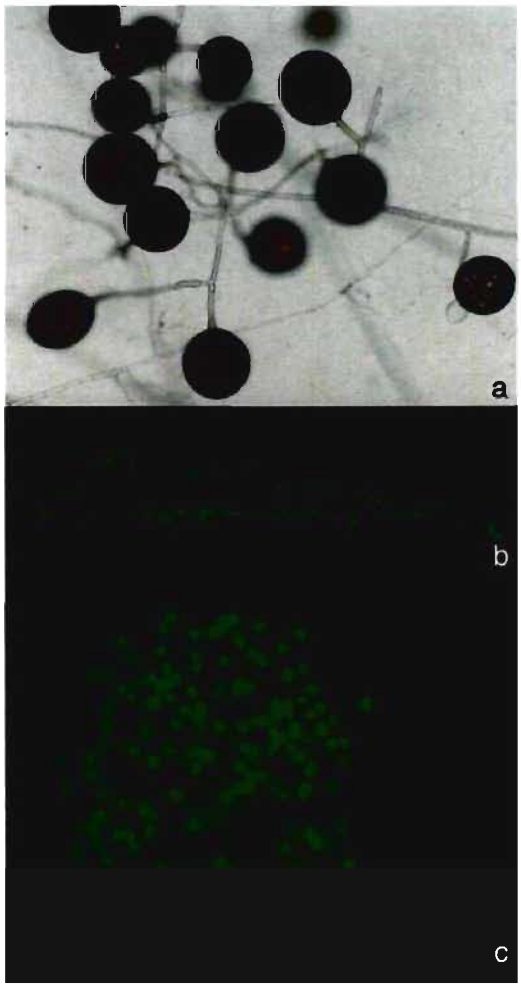


Figure 1.1)
a) Spores of *Glomus intraradices* grown in *in vitro* culture with transformed carrot roots and observed under a stereomicroscope.
b) Hypha of germinating spore of *Gigaspora rosea* observed under a confocal microscope. Nuclei were stained using SYTO Green fluorescent dye.
c) *Glomus diaphanum* spore showing typical multinucleate stage. Nuclei were stained using SYTO Green fluorescent dye and observed under a confocal microscope.

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 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 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 analyzed to reveal fifteen different variants in the genomic DNA of a single *Glomus intraradices* isolate. Collectively, all the research conducted on intra-specific polymorphism in AM fungi indicate 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 co-existence of these differing nuclei; first, that each nucleus does, in fact, possess a full quota of required genes (Fig. 1.2a). This

arrangement could then lead to competition amongst the genomes. Second, it is suggested that all the necessary genes coding for various functions are spread across numerous genomes, forcing their cooperation (Fig. 1.2b). 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. 1.3), or simply at random. 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.

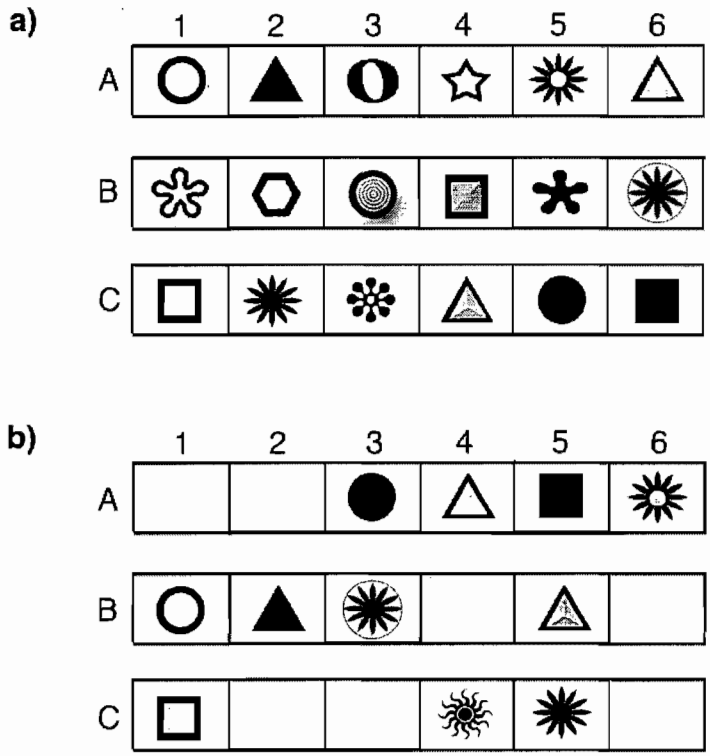


Figure 1.2) Diagram showing two possibilities for the arrangement of heterokaryotic structure among nuclei of AM fungi. Three different nuclei, represented by A,B,C, have six loci representing genes required for function. Shapes represent different possible variants of each of the six genes. Empty boxes represent nonfunctional alleles.
a) Each of the nuclei possess 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."

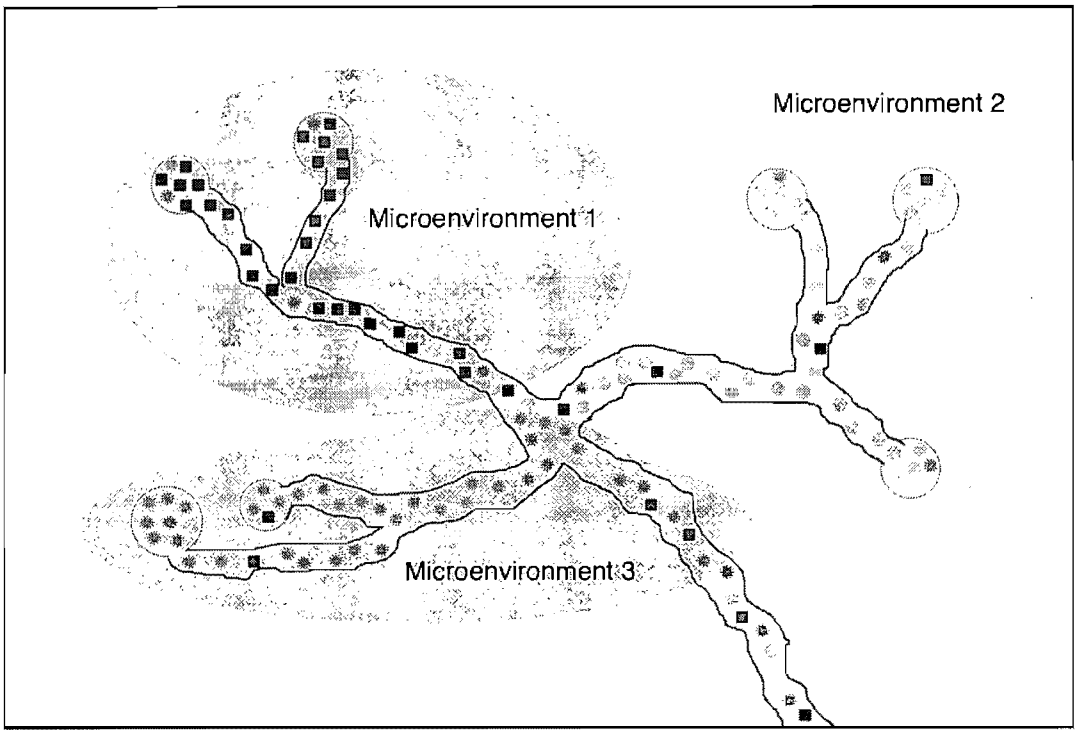


Figure 1.3) Diagram showing the possibility for genetically differing spores to arise on a single mycelium. Here, certain genotypes, represented by different shapes, are better suited to different microenvironments and are selected for within those regions, affecting the proportions of different nuclei which enter the developing spore.

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 amongst individuals of different species or geographical origins, it has been 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 germlings 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 *Scutellospora castanea*. Anastomoses allow damaged hyphae to re-establish a protoplasmic link as well as allowing nuclear exchange between mycelia (Fig. 1.4). 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.

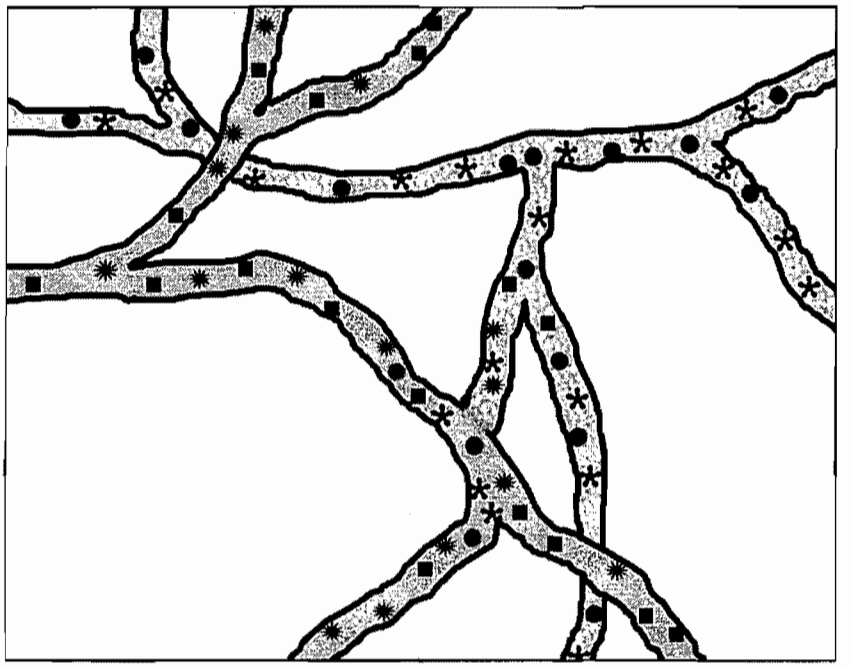


Figure 1.4) Diagram showing anastomosis of two genetically differing hyphae, allowing mixing and homogenization of different nuclear types (shapes) in the vicinity of the connection.

1.3 – Importance and Management of AM Fungi in Agriculture

While the abilities of arbuscular mycorrhizae 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 mycorrhizae 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 algae 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 P requirements is imprecise, and crop responses show a poor correlation with soil P 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 P 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 *Glomus 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 coworkers (Bever et al. 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 *Glomus 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 those 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 coworkers (2004) found five-fold differences in hyphal length amongst 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 overall abundance of these fungi in the field.

1.4 – What's 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 agro-business. 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 straight-forward 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 him or 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.

1.4.1 – 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 coworkers (Rodriguez et al. 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 *Glomus 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 amongst 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-alpha 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; Corradi et al. 2004b). 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, *Glomus 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.

1.4.2 – 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 amongst 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 SSU rRNA gene region in *Glomus 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 nonlinear relationship between spore concentration and amplified genomic DNA. It was suggested that this discrepancy may 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.

1.5 – 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 agro-ecosystem 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 native 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 P 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 lifecycles 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 intra-specific 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 *Glomus intraradices* phosphate dehydrogenase involved in P 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 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.

1.6 – Conclusion

Arbuscular mycorrhizal 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, arbuscular mycorrhizal fungi will come to represent a cornerstone in agricultural sustainability.

Introduction to the Experiments

While there exists extensive experimental evidence in favour of the multigenomic theory, many of the details of its function remain to be filled in. In particular, we have no real understanding of the diversity of dissimilar genomes in a given organism, or how the nuclei containing them are distributed through the mycelium. These are the two points which I set out to address in the following work.

The second chapter of this thesis uses a two-copy protein-coding gene, *PLS*, to make a preliminary estimate of the minimum number of distinct genomes existing in a single AM fungal strain via extensive cloning and sequencing. For those attempting to study populations of AM fungi, or to further characterize their genetic structure, the interpretation of experimental results requires at least a rough knowledge of the extent of intra-isolate diversity.

An ongoing, but not yet successful, experiment is described in chapter three. This experiment, also using the *PLS* gene as a marker, attempts to characterize the segregation among sister spores of differing nuclei. This will be done through the massive sequencing of the *PLS* complements of a number of sister spores to determine the presence and scale of differences therein. These differences can be used to make inferences regarding nuclear distribution in the mycelium. The nature of this distribution has implications for how genetic diversity is lost or maintained over long periods of time. If differing nuclei are distributed at random, each nuclear type which does not, by chance, find its way into a particular spore represents a loss of diversity. Conversely, nuclear types may be arranged such that each spore receives a full complement.

The fourth chapter of this thesis details three methods by which I attempted to produce large numbers of sterile or semi-sterile spores of the AM fungus *Glomus etunicatum* for use in the preceding experiments. Production of clean spores has long been an obstacle in AM fungal research, particularly for species which, like *G. etunicatum*, are less amenable to culture on transformed carrot roots. As such, two of the methods tested here use entire, autotrophic plants to better simulate the physiological conditions of symbiosis in the field. While none of the three culture types met with real success, the chapter contains useful information on alternative strategies and changes which could be made by those attempting this work in the future.

Chapter five summarizes the findings of this thesis as they relate to some of the more recent literature in AM fungal genetics, as well as making suggestions for future work building on these experiments.

Chapter 2
High Genetic Diversity of *PLS* in a Single Strain of *Glomus etunicatum*

Abstract

The variant diversity of the *PLS1* paralogue of the *PLS* gene in *Glomus etunicatum* was investigated. Using genomic DNA from a single strain of *G. etunicatum*, *PLS* was amplified, cloned, and sequenced. In 185 total sequences, 182 were found to belong to the *PLS1* group. Taking all polymorphic sites into account, 113 of these were distinct variants. A conservative estimate, in which only those sites that showed variation in multiple sequences were considered, produced 103 distinct variants based on 38 variable sites, 19 of which were located in the exons. Variation in the exons produced 62 unique amino acid sequences when translated. Monte Carlo simulations carried out for both real and conservative estimates produced nearly linear functions, showing that additional sampling is required in order to predict the maximum number of distinct variants of *PLS1* in this isolate. This survey uncovered a surprisingly large amount of genetic variation in a single AM fungal gene, and hints at a much larger number of distinct nuclei co-existing within a mycelium than had previously been suspected.

Keywords : variant diversity, Monte Carlo simulation, *Glomus etunicatum*, *PLS* gene

2.1 – Introduction

Arbuscular mycorrhizal (AM) fungi (Glomeromycota) are an ancient group of root-inhabiting, symbiotic fungi which form associations with over 80% of vascular plant species (Schüßler et al. 2001; Smith and Read 2008). In recent years, these organisms have received a great deal of attention due to their unique and poorly understood genetic structure: they have been found to possess multiple, divergent genomes within a single individual (Kuhn et al. 2001).

AM fungi reproduce via asexual spores which are populated by hundreds of nuclei received directly from the mother hypha. Due to this unusual reproductive mode, the AM fungal lifecycle is believed to lack a uninuclear stage (Sanders 2002). It is theorized that the absence of this genetic 'bottleneck' has allowed the buildup, over millions of years, of a population of dissimilar nuclei within a single fungal mycelium (Sanders 2002).

Experimental evidence for a multigenomic structure in AM fungi was first found in ribosomal genes; the internal transcribed spacer (ITS) regions flanking the 5.8S rRNA in *Glomus mosseae* were found to contain multiple distinct sequences within single spores (Sanders et al. 1995). An investigation of rRNA genes within the AM fungal species *Scutellospora castanea* also found high levels 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 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.

More recently, a protein-coding gene, *PLS* (*POL1*-like sequence) was found to exist in as many as sixteen variants in four isolates of the AM fungus *Glomus etunicatum*, despite having been shown to be two-copy in this species (Pawlowska and Taylor 2004; Hijri and Sanders 2005). *PLS* variants group into two separate phylogenetic groups, *PLS1* and *PLS2*. *PLS1* is more commonly found when working with genomic DNA and appears to be far more diversified, while *PLS2* is less variable, but is the predominant group found in the mRNA (Boon et al. Manuscript In Preparation). Hijri and Sanders (2005) have suggested that *PLS1* and *PLS2* are likely paralogues, such that each nucleus contains one copy of each type.

While strong evidence now exists for the presence of multiple, dissimilar genomes within individual AM fungal mycelia, no estimates have been made as to how many distinct genomes one isolate may contain. The objective of this study was to use the *PLS* gene to make such an estimate, as well as to investigate the variation in this gene and its arrangement. A large number of clones containing a *PLS* insert were sequenced, and these sequences were analysed to determine how many distinct variants of the *PLS1* group could be found. A simulation was also run to predict the theoretical maximum number of sequences occurring in the isolate. Finally, the copy number per genome of the putatively paralogous *PLS1* was verified using quantitative real-time PCR.

2.2 – Materials and methods

2.2.1 – Cloning and sequencing

Spores of *G. etunicatum* isolate NPI (Native Plants Incorporated, United Kingdom) grown in pot cultures were removed from soil via wet-sieving. Genomic DNA was obtained from approximately 150 spores using a DNeasy Plant Mini Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's directions. The DNA was then used as a template to amplify *PLS* (*POL1*-like sequence) with high fidelity *Pfu* DNA polymerase (Fermentas, Burlington, Ontario) and specific primers FwdPOL4 and RevPOL7 (Pawlowska and Taylor 2004). The reaction was performed in a 50 µl volume containing 1.25 units *Pfu*, 0.2 mM dNTPs, 0.5 µM of each primer and the PCR buffer. PCR was carried out for 34 cycles (94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min; preceded by an initial 2 min denaturation at 94°C and followed by a 10 min hold at 72°C) on a Mastercycler ep gradient S (Eppendorf, Mississauga, Ontario). After the PCR product was checked on an electrophoresis gel to ensure successful amplification, the amplified gene was cloned using a CloneJET PCR Cloning Kit (Fermentas, Burlington, Ontario), according to the manufacturer's instructions. Bacterial colonies containing the *PLS* insert were subcultured on Luria-Bertani (LB) medium (Bertani 1951) containing 100 mg/l ampicillin, and sent for sequencing at the Genome Quebec Innovation Centre in Montreal using pJET1.2 forward and reverse sequencing primers.

2.2.2 – Sequence analysis

Sequence chromatograms were visualized using the program Finch TV (version 1.4.0,

Geospiza Inc.); those which were low-quality, incomplete, or contained plasmid sequences were removed from the analysis. Sequences were aligned using MEGA4 (Tamura et al. 2007) and corrected by eye. Sequences were deposited in Genbank with accession numbers GQ325050-GQ325231.

In determining the number of distinct variants found, two estimates were made; a real estimate, in which all single nucleotide polymorphisms (SNPs) were assumed to have truly occurred in the sequences, and a conservative estimate in which all SNPs occurring in only one sequence were dismissed as artefactual. The online program DNAcollapser (Villesen 2007) was used to collapse the sequences to distinct variants, giving totals and abundance data for each estimate. After using MEGA4 to convert DNA sequences to translated amino acid sequences, the program Winclada (version 0.9.9, K.C. Nixon, 1999) was used to determine the number of unique amino acid sequences.

In order to predict the maximum number of distinct variants in the NPI strain, a Monte Carlo simulation was carried out. In this simulation, sequences are sampled additively, without replacement, and the number of distinct sequences is plotted against the total number sampled. For a sufficiently large sample size, the plot of distinct vs. total sequences produces an asymptotic function in which the asymptote is equal to the theoretical maximum number of distinct variants in the population (Fig. 2.1), which in this case is the total number of nuclei in the NPI strain of *G. etunicatum*.

One thousand replicates of the Monte Carlo simulation were carried out for each estimate using the R platform for statistical computing (www.r-project.org/, program compliments of Simon Joly). For each graph, 95% confidence intervals (mean value ± 1.96 x standard deviation) of the simulated values were also plotted.

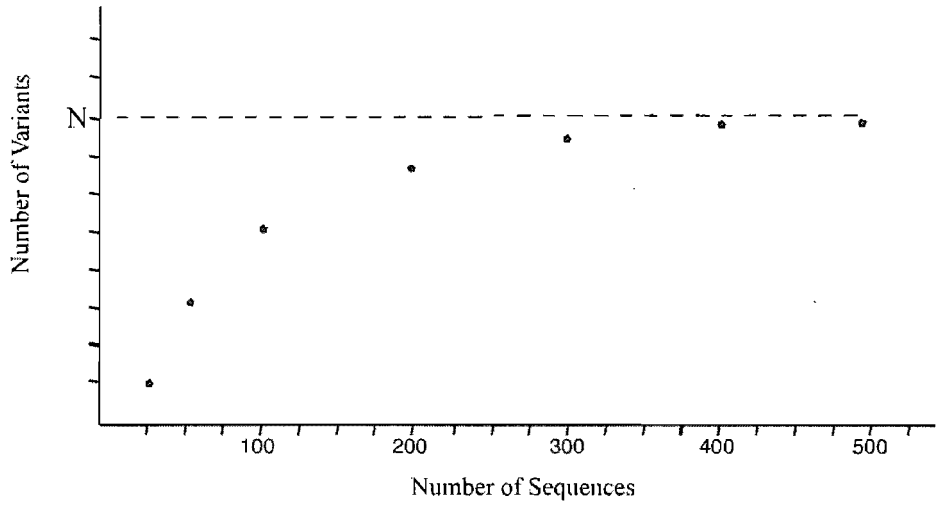


Figure 2.1) Example of a Monte Carlo simulation in which 500 sampled sequences produce an asymptote of value N. The theoretical maximum number of distinct sequences in this population is N.

2.2.3 – Copy number determination

In order to confirm paralogy, copy numbers for *PLS1* and *PLS2* were determined using real-time quantitative PCR. A genomic DNA sample extracted from multiple spores of *Glomus etunicatum* isolate NPI was tested, with plasmid extracts from transformed *E. coli* bacteria containing *PLS1* and *PLS2* variants serving as standards.

Plasmid extractions were made of two bacterial clones which had been previously sequenced- one containing a *PLS1* variant, and one containing a *PLS2* variant. The extractions were carried out using a GeneJET Plasmid Miniprep Kit (Fermentas, Burlington, Ontario) according to the manufacturer's directions. Genomic DNA of *G. etunicatum* isolate NPI was obtained from spores using a DNeasy Plant Mini Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's directions.

DNA concentration for both plasmids and the gDNA were determined using a SpectraMax M5 fluorescence microplate reader (Molecular Devices, California, USA) and a Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Oregon, USA) used according to the manufacturer's directions.

Probe and primer sequences were designed using File Builder version 3.1 (Applied Biosystems, 2005) and ordered as a Custom TaqMan SNP Genotyping Assay (Applied Biosystems, Toronto, Ontario): FwdPLS (5'-GCAAAACCATTGGCAGTTTTAATCAC-3'), RevPLS (5'-AATCTGTGTCACCATAAATAACCTACAAGTT-3'), PLS1probe (VIC-5'-CTCCATTGATAGTTTTTC-3'-NFQ-MGB), PLS2probe (FAM-5'-TCCATTGATACTTTTTC-3'-NFQ-MGB). Underlined bases in probes refer to variable sites. Primers were designed to amplify both *PLS* groups, while the *PLS1* probe and *PLS2* probe, labelled with VIC and FAM fluorophores respectively, differed at one site in order to

distinguish between the two groups. In the final experiments, only plasmids containing *PLS1* were used to create the standard, as the *PLS2* probe was found to be ineffective in preliminary testing.

In each of six replicate experiments, seven two-fold serial dilutions of gDNA (ranging from 103.1 to 1.61 ng) were amplified in a final volume of 20 µl, including 10 µl 2X Maxima Probe qPCR Master Mix (Fermentas, Burlington, Ontario), 9 µl diluted template DNA and 1 µl 20X Custom TaqMan SNP Genotyping Assay (Applied Biosystems; in 40X, primers = 36 µM each, probe = 8 µM). Also in each experiment, a sample of plasmid DNA containing a known amount of *PLS1* was used as a standard. Seven two-fold serial dilutions of the plasmid DNA (ranging from 1.12×10^9 to 1.75×10^7 plasmid molecules) were amplified as for the gDNA to generate a standard curve. Each experiment also included two negative control reactions without template DNA. After a uracil DNA glycosylase pre-treatment step (2 min at 50°C) and an initial denaturing step (10 min at 95°C), samples underwent 40 three-step amplification cycles (15 s at 95°C, 30 s at 60°C, 30 s at 72°C). Fluorescence was measured during the annealing step.

Amplification, detection, and data analysis were performed with a Mastercycler ep Realplex4 thermal cycler (Eppendorf, Mississauga, Ontario). The cycle threshold (Ct) values were given by the software as the fractional cycle number; these were then plotted against the log of the copy numbers of *PLS1*-containing plasmid molecules. This curve was then used to predict the copy number of *PLS1* for a given Ct value. A *PLS1* copy number was calculated for each sample of genomic DNA, assuming a genome size of 37.45Mb of DNA per nucleus (Hijri and Sanders 2005). The efficiency of the PCR reaction was calculated by converting the slope produced by the linear regression of the curve to a percentage efficiency as follows: Efficiency = $-1 + 10^{(-1/\text{slope})}$.

2.3 – Results

2.3.1 – Sequencing of PLS alleles

In total, 306 clones were sequenced, with 185 giving full, usable sequences (Fig. 2.2). The remainder produced partial sequences or fragments of the plasmid. One *PLS2* and two recombinant sequences were removed, leaving 182 *PLS1* sequences. Using the more conservative criteria, 103 distinct sequences containing 38 variable sites were found, while the real estimate gave 113 sequences containing 66 variable sites (Table 2.1).

For both real and conservative estimates, the three most abundant variants make up 11.5% of all sequences, while singletons make up 69.9% and 64.1% of the distinct sequences in real and conservative estimates, respectively. Twelve of the sequences contain an identical 5 bp insertion in the non-coding region following the third exon.

Based on the mutations which were considered real under the conservative estimate, only two sequences contained premature stop codons in their amino acid sequences, both caused by a single frameshift mutation; all others were putatively functional. Of the 180 translated sequences lacking premature stop codons, 49 were found to be unique. The variation in amino acid sequences were based on 19 variable sites (out of the total 38) located in the exons.

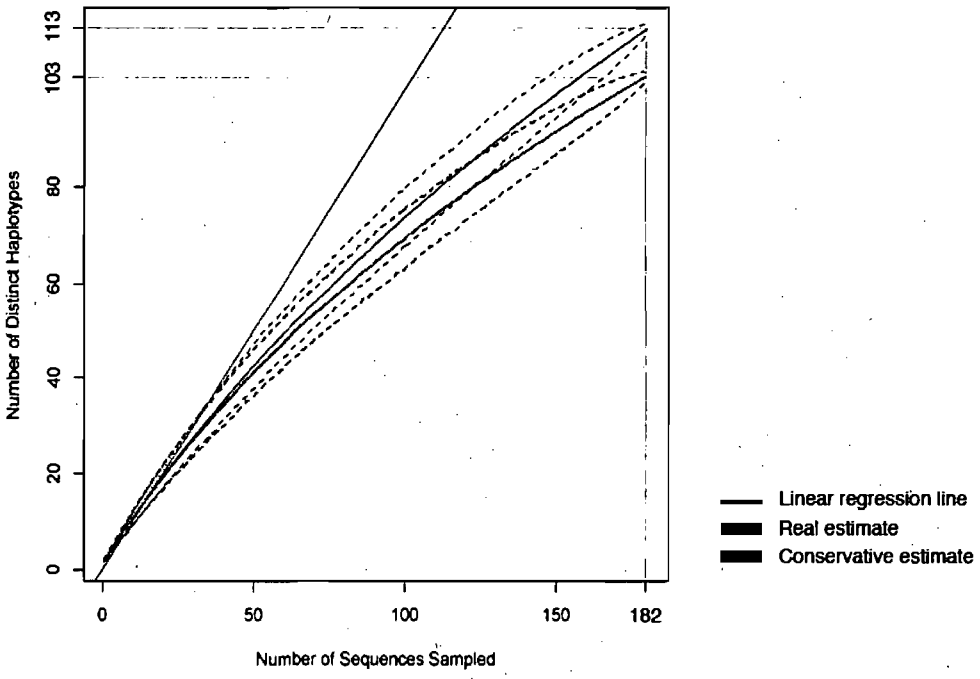


Figure 2.2) Monte Carlo simulations (1000 rep.) showing distinct variant numbers of *PLS1* sequences, for both real and conservative estimates. Dashed lines show 95% confidence intervals of the mean of the simulated values.

Table 2.1) Summary of results from real and conservative analyses of *PLS1* sequencing data.

Estimate:	**Real**	**Conservative**
Total sequences	182	182
Length (bp)	610	602
Distinct sequences	113	103
Variable sites (incl. 5bp indel)	66	38
Highest Frequency	7 (3.85%)	7 (3.85%)
Singletons as a % of Distinct Seq.	69.9%	64.1%
No. variants occurring:		
1 time	79	66
2 times	17	18
3 times	9	10
4 times	4	2
5 times	1	3
6 times	0	1
7 times	3	3

2.3.2 – Copy number determination

In preliminary tests of real-time PCR, both plasmid DNA containing *PLS2* and genomic DNA failed to cause fluorescence in the FAM-labelled probe; as such, only the copy number of *PLS1* was determined. Based on six replicate experiments, the copy number of *PLS1* was found to be 0.21 ± 0.07 (SE, n= 6) per nucleus (Table 2.2). All six replicates produced R^2 values of greater than 0.98.

Table 2.2) Summary of results from real-time quantitative PCR determination of copy number in *PLS1*

Run Number	**Avg. Copy#**	**St. Dev.**	**Standard Curve:**			
			R^2	Slope	Intercept	Efficiency
1	0.387	0.092	0.9810	-3.2570	40.735	2.028
2	0.241	0.042	0.9918	-3.0467	38.792	2.129
3	0.044	0.012	0.9995	-2.5166	34.846	2.497
4	0.112	0.026	0.9832	-2.9684	38.018	2.172
5	0.431	0.064	0.9899	-3.1508	40.885	2.077
6	0.041	0.021	0.9977	-2.6165	34.383	2.411

2.4 – Discussion

While it was expected based on previous findings (Pawlowska and Taylor 2004; Hijri and Sanders 2005) that a number of *PLS* variants greater than two would be found, indicating that alleles are segregated in differing nuclei, the enormous amount of diversity uncovered in this gene was unprecedented. The sequences in this experiment were amplified from an isolate which had originally come from a single spore culture. If we accept Pawlowska and Taylor's (2004) assumption that a *G. etunicatum* spore contains around 750 nuclei, our diversity estimate represents a high proportion of the original nuclear complement being dissimilar.

The bulk of distinct variants sequenced occurred only once, suggesting a high rate of divergence. This is due to the fact that in intraspecific gene evolution, older variants are expected to have a higher abundance, while more recently diverged sequences are more likely to appear as singletons (Posada and Crandall 2001). While the sheer number of singletons – over 60% of the distinct variants – might resemble the unrestrained neutral evolution of a pseudogene, the fact that only two of 182 sequences showed a premature stop codon suggests some level of positive selective pressure may in fact be acting on the gene. Furthermore, the lower, but still considerable variation in the amino acid sequences shows that many of the substitutions found in the gDNA are non-synonymous at the expression level. While analysis of protein structure and function was beyond the scope of this thesis, a high proportion of non-synonymous substitutions at the amino acid level may indicate the co-optation over time of the *PLS1* gene copy for another function, as has been observed in duplicated plant genes (Hofer and Ellis 2002). Duplicated genes can also accumulate complementary deleterious

mutations, such that the two function jointly despite some individual sequence degradation (Hofer and Ellis 2002).

An experimental copy number of 0.21 (± 0.07 SE) indicates that *PLS1* is, at most, single copy, meaning that each of the 182 distinct variants found here resides within a distinct genome. This, conservatively, places the number of differing genomes within this isolate alone at over one hundred. Furthermore, the graph created by the Monte Carlo analysis had not yet begun to level off, indicating that further sequencing will likely yield many more unique *PLS1* variants. If we consider that *PLS* is one of thousands of potentially varying genes, this yields an astronomical number of possible genomic arrangements.

The variation among the copy number estimates was unusually large, varying between 0.431 and 0.041 copies per nucleus. While this may have been due to unknown problems with the quality of the samples, or simple human error, it may also indicate a state in which some nuclei contain a single, non-duplicated copy of the gene. In an organism lacking a strong genetic bottleneck, some nuclei may have persisted in which no duplication event took place. The *PLS2* variant has been found to be much more prevalent in the transcriptome (Boon et al. Manuscript In Preparation), suggesting that it may be the original, functional copy of the gene. If nuclei exist in which no duplication has taken place, it is reasonable to assume that there will be no *PLS1* variant present. Furthermore, if certain spores carry a greater proportion of these single-*PLS* nuclei, any heterogeneity in the aliquots used for real-time PCR could cause large variations in the resulting copy number. While perhaps not the most parsimonious explanation for the results obtained, it bears consideration.

While there may be concerns surrounding artificial variation created by polymerase errors during the PCR amplification of these sequences, two steps were taken to minimize the effects of such errors. First, a high-fidelity proofreading enzyme was used, which, with a

reported rate of 1.6×10^{-6} errors per nucleotide, per cycle (Lundberg et al. 1991), is expected to create, on average, only 0.03 errors per sequence in this experiment. Second, because polymerase errors and sequencing errors were not specifically controlled for in this experiment, the conservative estimate was made to take this into account. The conservative estimate assumes any single nucleotide polymorphism occurring in only one sequence to be an artefact; therefore, precisely the same nucleotide incorporation error would have to occur in multiple sequences to cause a false positive. The likelihood of this happening is very low. The conservative estimate will, if anything, underestimate the number of distinct sequences, while the 'real' estimate will overestimate in the presence of any artefactual polymorphisms. However, because the difference between the two estimates is only three sequences out of 182, I believe they give an accurate reflection of the true sequence diversity.

The failure of the *PLS2* probe is perhaps due to the fact that, because of the low number of variant sites in the gene, it was designed around a single base pair difference between the two sequence types. While the minor groove binder incorporated into the probe decreases the incidence of non-specific binding, which indeed did not occur– the *PLS1*-containing plasmids showed no FAM fluorescence– it may also have prevented binding of the *PLS2* probe to *bona fide PLS2* fragments if additional variable sites existed in the area of the probe.

The data presented here represents a first estimate of the number of dissimilar genomes residing in an AM fungal isolate based on a single-copy gene. Further investigation is needed: because a plateau was not reached in the Monte Carlo simulation, all that can be given here is a minimum. Additional sequencing efforts of *PLS* would give a theoretical maximum as well, at least so far as variation based on this gene is concerned. However, even

the minimum estimate uncovered by this research suggests a diversity of nuclei which is far greater than previously expected.

For those studying genetic structure and diversity in AM fungi, it is crucial to the interpretation of experimental data that we have some understanding of the way in which the characteristically high levels of variation are structured. The estimate of the number of different genomes present in a given isolate is an important piece of this puzzle.

Chapter 3
Differential Nuclear Segregation in Individual Spores of *Glomus etunicatum*

Abstract

Arbuscular mycorrhizal fungi are made up of vast, branching networks of coenocytic hyphae housing numerous, dissimilar nuclei. At sporulation, heterogeneity in the distribution of these nuclei may cause sister spores formed in different regions of the mycelium to receive differing complements of the various nuclear types. It is currently unknown if, and to what extent, differential nuclear segregation occurs; data on this phenomenon will provide important insights into the genetics and evolution of these poorly understood organisms.

The segregation of different nuclear types at the time of sporulation in the AM fungus *Glomus etunicatum* was investigated. Using a number of single spore pot cultures grown on tomato plants, sister spores were tested to determine differences in the proportions of *PLS* variants they contained. Sequencing of multiple variants was carried out by pyrosequencing using the GS FLX system (454 Life Science). PCR amplicons of a 450 bp fragment obtained from the whole genome amplification of a single spore were massively sequenced. Due to difficulties in obtaining full-length reads with this relatively new technology, results are still pending.

Keywords : arbuscular mycorrhizal (AM) fungi, nuclear segregation, gene sequence diversity, heterokaryosis, multigenomic, pyrosequencing

3.1 – Introduction

Arbuscular mycorrhizal (AM) fungi are symbiotic, root-inhabiting fungi which comprise the family Glomeromycota (Smith and Read 1997; Schüßler et al. 2001). These fungi are unique in that they have been found to possess multiple, dissimilar genomes within a single individual (Kuhn et al. 2001).

Arbuscular mycorrhizal fungi are primarily made up of vast, branching networks of hyphae. Unlike most higher fungi, these hyphae are coenocytic, that is, lacking in discrete cellular divisions. Hyphal walls form long, tubelike structures through which cytoplasm and organelles can migrate freely. 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.

Mature AM fungal spores contain hundreds or even thousands of nuclei, and it is believed that there never exists a time in the glomeralean life cycle during 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).

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 creating 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 are 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 co-existence of these differing nuclei; first, that each nucleus does, in fact, possess a full quota of required genes. This arrangement could then lead to competition amongst the genomes. Second, it is suggested that all the necessary genes coding for various functions are spread across numerous genomes, forcing their cooperation. Under this arrangement, the individual must be defined as the aggregate of the genomes required to form a fully functioning organism.

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 exist in a given region of the mycelium in order for it to perform properly, segregation according to selection will be heavily restrained, and a certain level of homogeneity can therefore be expected. This will lead to sister spores which possess very similar gene complements, regardless of the area of the mycelium on which they formed. Alternatively, fully-functional, differing nuclei may be unevenly distributed within the mycelium according to their fitness within a particular microenvironment, or simply by random chance. In this case, we would expect to see significant differences in the proportions of various gene variants from one sister spore to another, a phenomenon which I will refer to as 'differential segregation'.

In order to determine whether differential segregation occurs in the AM fungus *Glomus etunicatum*, a number of pot cultures were created in which a tomato seedling was inoculated with a single spore. All second generation spores which arose were therefore sister

spores and, according to the null hypothesis, should possess identical gene complements. Three spores were taken at random from each of four pot cultures (a greater sample size being cost-prohibitive), and sequenced to determine the proportions of each variant of a multi-variant marker gene present in each spore. The sequencing technique used to accomplish this is called pyrosequencing. Pyrosequencing uses an emulsion PCR in which adaptors enable huge numbers of single-stranded fragments to bind to a tiny bead, such that each bead possesses a unique fragment. These microbeads are then encapsulated in an oil droplet within an emulsion. Each fragment is then amplified in parallel, free of competing sequences. This PCR amplifies each fragment to a concentration of several million copies per bead. The beads are then loaded onto a PicoTiterPlate for sequencing. Using a technique known as sequencing-by-synthesis, nucleotides are washed over the plate in a fixed order, producing light when they are incorporated. In this way, all beads are sequenced in parallel, giving a complete picture of the gene variants present (RocheDiagnostics 2008).

The marker gene used in this experiment is called *PLS* (*POL1*-like sequence). It is a two-copy protein-coding gene which bears a 56% amino acid sequence identity to a *Saccharomyces cerevisiae* gene encoding DNA polymerase-α, *POL1* (Pawlowska and Taylor 2004; Hijri and Sanders 2005). *PLS* has been shown to exist as a large number of variants, even within a single isolate (see Chapter 2, this thesis). Some of these variants are more common than others, making this gene ideal for examining differences in variant proportions between spores.

These results will provide our first look at the segregation behaviour of AM fungal nuclei at sporulation. Understanding how different nuclei are arranged in the mycelium may also help us begin to understand how the varying genomes in these multigenomic organisms interact with one another. Furthermore, a complete survey of the number of *PLS* variants

present in a single spore will enable us to make informed estimates of the number of differing genomes which exist in an individual AM fungus.

3.2 – Materials and methods

3.2.1 – Establishment of single spore pot cultures

Two hundred tomato seeds, cv. Micro-Tom (courtesy of Dr. Barry Micallef; Meissner et al. 1997), were germinated in Petri dishes containing wet filter paper. After four days, 150 healthy seedlings were chosen for use in the experiment. Spores of *G. etunicatum* isolate NPI (Native Plants Incorporated, United Kingdom) were separated from soil through wet sieving (500 µm, 250 µm, 180 µm, 106 µm, 63 µm sieves), then rinsed in sterile water and examined under a dissecting microscope. Spores which were opaque and dark brown in colour were considered healthy and chosen preferentially for use in the experiment.

One hundred and fifty 115 ml cone-tainers (Stuewe & Sons, Inc., Corvallis, OR, USA) were filled with a sterilized (autoclaved twice for 60 min at 121°C) mixture of 1:1:1 (v/v) perlite, sand and field soil (Montreal Botanical Garden). In each cone-tainer, a 1000 µl micro-pipette tip with the smaller end trimmed off was pushed into the substrate such that the larger end was level with the surface (Fig. 3.1). A germinated tomato seedling with a single *G. etunicatum* spore placed upon the root was then inserted into each micro-pipette tip, helping to keep the spore in close proximity to the root, and topped with more sterile soil mixture. Cone-tainers were then maintained in a growth chamber (Table 3.1).

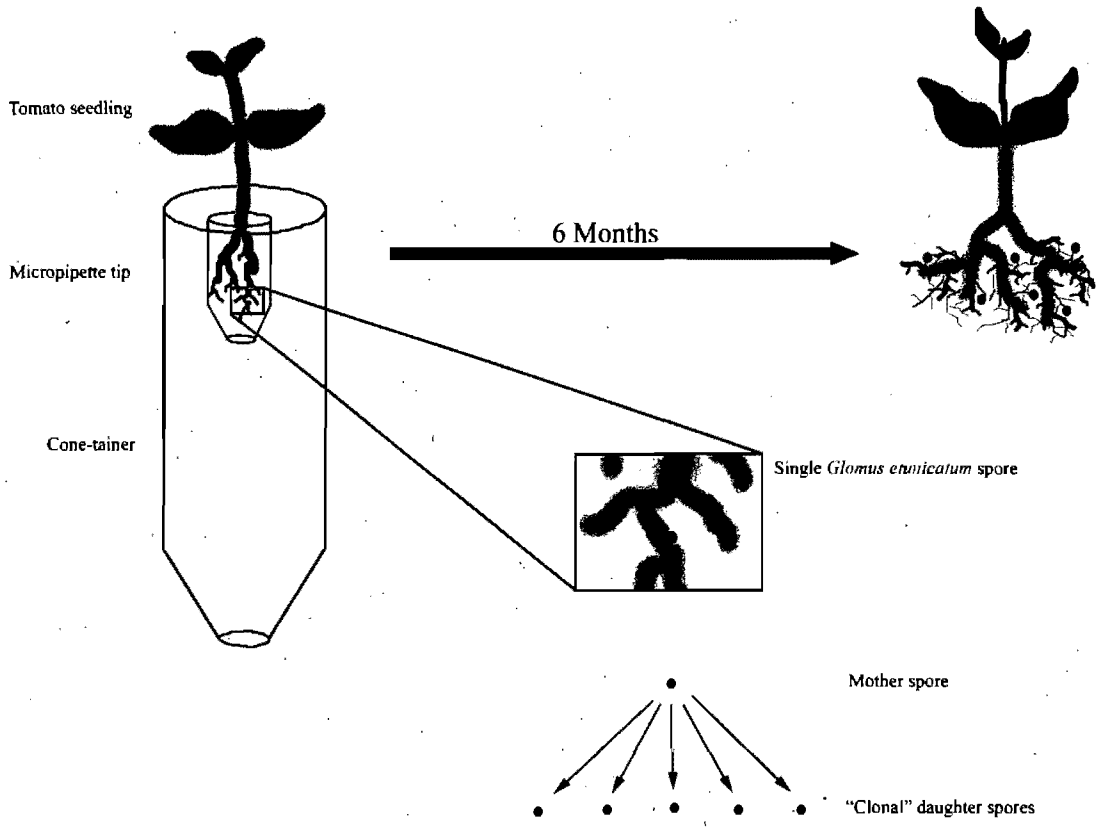


Figure 3.1) Schematic showing a single spore pot culture set up to produce numerous daughter spores. Tomato seedling is grown with roots placed inside a micro-pipette tip in order to keep spore in close proximity.

Table 3.1) Environmental conditions of the growth chamber in which tomato seedlings were grown.

Time	Temp. (°C)	Humidity (%)	No. Fluorescents	No. Incandescents
5h30	20	60	0	2
6h00	23	70	2	2
7h00	23	70	4	4
19h00	20	60	2	2
21h30	18	55	0	2
22h00	18	55	0	0

The cone-tainers were watered with distilled water each two days and fertilized once weekly with 100 ml of Miracle-Gro liquid plant food (Scotts Miracle-Gro Co., Marysville, Ohio, USA) at a concentration of ten drops per litre of water, beginning at four weeks after planting. Six months after planting, the tomato plants were cut off at the soil level, leaving only the roots and soil core. The roots and soil of 25 of the plants, chosen at random, were then torn into small pieces by hand and rinsed through a series of sieves (500 μm, 250 μm, 180 μm, 106 μm, 63 μm). Sieves and hands were thoroughly washed between each plant to prevent cross-contamination. Fractions from each of the smallest three sieves were retained and spores were separated from debris by centrifuging at 1620 g for 2 min in a discontinuous density gradient with a 60% (w/v) sucrose layer at the bottom. The spores were collected from the gradient interface with a Pasteur pipette and thoroughly washed with distilled water before being stored in distilled water at 4°C until needed.

3.2.2 – Gene amplification and sequencing of individual spores

Three spores from each of four replicate host plants were isolated for further analysis. These spores were picked individually, placed in 1 μl sterile water and crushed using fine forceps, which were flamed between each sample to prevent cross-contamination. The entire genome of each of the spores was amplified using the GenomiPhi Whole-Genome Amplification Kit (GE Healthcare, Amersham, United Kingdom) according to manufacturer instructions. A nested PCR was then carried out in which *PLS* variants were amplified using Dream*Taq* DNA polymerase (Fermentas, Burlington, Ontario) with primers containing adaptor sequences for pyrosequencing (underlined): PLS454-Fwd (5'-GCCTCCCTCGCGCCATCAGTGCTCRGGTAAAGAAATATG-3'), PLS454-Rev (5'-

GCCTTGCCAGCCCGCTCAGGTATTGATCATTATTGAATC-3'). The reaction was performed in 50 µl volumes containing 0.2 mM dNTPs, 0.5 µmol of each primer and the PCR buffer. PCR was carried out for 34 cycles (95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min; preceded by an initial 2 min denaturation at 90°C and followed by a 10 min hold at 72°C) on a Mastercycler epgradient S (Eppendorf, Mississauga, Ontario). The PCR product was checked on an electrophoresis gel to ensure successful amplification of the gene and purified using a MinElute PCR Purification Kit (Qiagen, Mississauga, Ontario) according to manufacturer instructions. These purified samples were then sent to the Genome Quebec Innovation Centre in Montreal for pyrosequencing using the GS FLX system (454 Life Science). An amplicon sequencing protocol was used in which the 450 bp fragments were sequenced in both directions and assembled at the Innovation Centre using bioinformatic software.

3.3 – Results and discussion

At harvest, nearly all cone-tainers from which I attempted to collect spores were at least lightly colonized, based on visual inspection of the material retrieved from sucrose gradients, with most showing moderate to heavy colonization. This demonstrates the feasibility of our method for producing large numbers of *G. etunicatum* spores from a single-spore culture.

Although both whole genome and specific amplifications were successful, attempts at pyrosequencing produced primarily only large numbers of short fragments- an average length of less than 90 bp compared to a full amplicon length of around 450 bp. Very few of these fragments could be assembled into full sequences (Table 3.2) and were judged not to be of use

in further analyses. Despite purification, electrophoresis gels of PCR products (Fig. 3.2) for each spore showed the presence of 'primer-dimer' fragments, in which primer molecules became bound to one another, rather than the template sequence. This contamination may have been responsible for the large quantity of short fragments produced during sequencing. Samples have been re-submitted for pyrosequencing and results are pending.

Table 3.2) Summary of data gathered from initial pyrosequencing attempt with spores A, B and C.

Spore	A	B	C
Total number of reads	8744	6013	7338
Total number of bases	1,051,088	1,015,950	1,349,889
Number assembled	6	4	3
Number partial	239	174	185
Number singleton	795	186	143
Number repeat	5119	4577	5799
Number outlier	2585	1072	1208

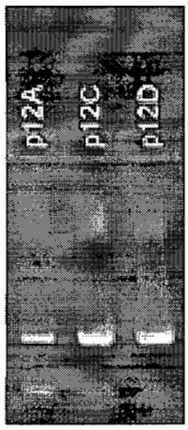


Figure 3.2) Electrophoresis gel showing amplicon of approx. 450 bp (upper bands), as well as small fragments (lower bands) caused by primer dimer, for three sister spores.

Chapter 4
Autotrophic and Transformed Root Cultures of *Glomus etunicatum* (or, Things I Tried that Didn't Work...)

Abstract

In order to produce large numbers of sterile or semi-sterile spores of *Glomus etunicatum* for experiments on genome segregation (see chapter 3), two autotrophic (whole plant) culture methods, as well as the standard transformed carrot root culture, were tested. Both types of autotrophic culture had severe methodological failings which prevented the maintenance of healthy plants, making colonization next to impossible. The transformed carrot root cultures did form several successful mycorrhizae, although at such a low percentage of the total number of cultures as to make them impractical for use in further experimental work.

Keywords: transformed root culture, autotrophic culture, *Glomus etunicatum*

4.1 – Introduction

One of the major limiting factors in our ability to study the transmission and segregation of AM fungal genetic material is the difficulty of acquiring large numbers of sterile spores which we know to have descended from a single parent spore (Sanders 2002). The most straight-forward and commonly used method of sterile spore production is that of a co-culture with Ri T-DNA transformed carrot roots (Bécard and Fortin 1988). While root organ cultures can indeed produce large numbers of spores for many AM fungal species, most belonging to the genus *Glomus*, there are several drawbacks. Because the plant host lacks photosynthetic tissues, normal source-sink relationships and hormone balances are affected (Fortin et al. 2002). Furthermore, sucrose must be added to the growth medium to replace photosynthates, causing both partners to be in direct contact with a sugar solution, potentially altering the biochemistry of the plant-fungus interaction (Fortin et al. 2002). Fortin and coworkers (2002) even speculate that this environment may change the morphology of the fungus, producing mycorrhizae with few arbuscules and vesicles, despite extensive intraradical growth. It is not unimaginable that these factors could perhaps have an effect on the neutral transmission of genetic material from parent to daughter spore in different parts of the mycelium. In an effort to circumvent the potential pitfalls of root organ cultures, two autotrophic culture systems for *in vitro* mycorrhization were tested.

In the system described by Voets *et al.* (2005), host plants are placed such that their roots lie across a layer of solidified gel medium in a Petri dish which is turned on its edge. The plant passes through a small hole in the upward-facing circumference of the dish, such that the shoot grows in the open air. The hole is sealed with grease to maintain sterility, and the roots are nourished via nutrients in the gel medium. The root compartment is wrapped in

aluminum foil to preserve darkness. With this arrangement, hosts are easily inoculated by simply placing spores onto the roots, while shoots are open to the air and free to carry out photosynthesis as usual.

The second system, developed by Fracchia *et al.* (2001), takes place under semi-sterile conditions in which the spore and the host are germinated separately in Petri dishes of the same size. Once both roots and germ tube are established, the host and its inorganic substrate are added to the surface of the spore's gel medium. The advantage of this method, according to the authors, is that, with a clear gel medium and a coarse, light coloured substrate, it is easy to observe the progression of colonization through the underside of the Petri dish.

Finally, as an accompaniment to the two autotrophic culture methods I attempted, I also set up a large number of single-spore, Ri T-DNA transformed carrot root cultures. Despite the drawbacks detailed above, this method has the advantage of being entirely sterile, which can be important when working with primers that may amplify non-Glomeralean DNA. The method also makes subculturing simple; unlike the other systems described here, no new plant material need be introduced.

Anecdotally, *Glomus etunicatum* has proven extremely difficult to maintain as a sterile root culture due to low germination and sporulation rates, as well as contamination issues (Mohamed Hijri, Yolande Dalpé, personal communication). The successful establishment of a collection of single-spore cultures of *G. etunicatum* would allow the analysis of genetic transfer between mother and daughter spores, and of differences amongst sister spores, under extremely well controlled conditions.

4.2 – Materials and methods

4.21 – Voets et al. (2005) method

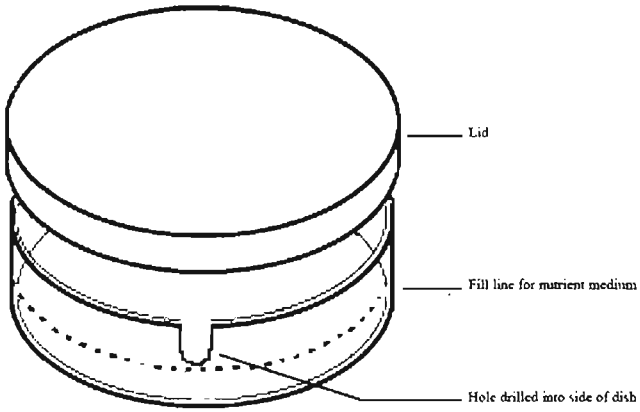
Under sterile conditions, U-shaped holes of approximately 0.75 cm were drilled into the sides of twelve 100x15 mm Petri dishes (Fig. 4.1a). The Petri dishes were then filled to just below the hole with MSR medium (Declerck et al. 1998) solidified with 4 g/l Gel-Gro (ICN Biochemicals, Ohio, USA), without sucrose or vitamins.

Spores of *Glomus etunicatum* isolate NPI (Native Plants Incorporated, United Kingdom) were separated from soil using a series of sieves and rinsed in distilled water, then disinfected as follows (modified from Hijri et al. 2002). The spores were folded into a moist No.2 filter paper (Whatman International Ltd., England) and placed inside a small, plastic sample cassette. The cassette was then soaked twice for 2 min in a 2% solution of Chloramine T with 2 drops Tween 20. Each treatment was followed by three 2 min rinses in sterile distilled water. Finally, the cassette was soaked for 5 min in a sterile solution of 0.02% streptomycin and 0.01% gentamycin. Once sterilized, the spores were stored in sterile distilled water at 4°C until needed.

Ten two-week-old plantlets of *Solanum chacoense*, grown from nodal cuttings in gelified (3 g/l Gel-Gro, ICN Biochemical, Cleveland, USA) MS medium (Murashige and Skoog 1962) with 20 g/l sucrose under sterile conditions were removed from their original medium and their roots were gently stripped of remaining gel using sterile forceps. Each plantlet was then laid on its side into a modified Petri dish such that its roots lay on the MSR medium, and its shoot projected through the drilled hole to the outside of the dish. A single *G. etunicatum* spore was placed onto the root of each plantlet. The lids were then replaced and

sealed with Parafilm (Pechiney Plastic Packaging, Chicago, Il., USA), except surrounding the drilled hole, where gaps were sealed with sterile silicone grease (Fig. 4.1b). Finally, the dishes were wrapped in aluminum foil to preserve darkness in the root chamber, and were set upright such that the plantlet projected upward. All plates were placed in a large, clear plastic box to help preserve humidity and kept in a growth chamber (Table 4.1).

After two and four weeks, all plates were opened under sterile conditions and a small amount of additional sterile growth medium (sufficient to cover the roots), cooled to 50°C to avoid root damage, was added to replenish nutrients. Plates were resealed and replaced in the growth chamber, and finally removed for observation after six weeks.



a) b)

Figure 4.1) a) Schematic showing Petri dish with modification for use in method of Voets *et al.* (2005). **b)** Assembled replicate showing two-week-old *Solanum chacoense* plant.

Table 4.1) Environmental conditions of the growth chamber in which plantlets were grown.

Time	Temp. (°C)	Humidity (%)	Fluorescents	Incandescents
5h30	20	60	0	2
6h00	23	70	2	2
7h00	23	70	4	4
19h00	20	60	2	2
21h30	18	55	0	2
22h00	18	55	0	0

4.2.2 – Fracchia et al. (2001) method

Forty tomato seeds, cv. Micro-Tom (Meissner et al. 1997), were sterilized for 2 min in 10% sodium hypochlorite, then rinsed with sterile distilled water. Two seeds were sown in each of twenty 50 mm Petri dishes containing 10 ml of an autoclaved (120°C, 20 min) 1:1 v/v mixture of perlite and vermiculite which had been previously washed in a 500 µm sieve to remove all small particles. The Petri dishes, without lids, were individually placed inside small, sterile, upside-down Magenta boxes with a small amount of sterile water in the bottom in order to avoid desiccation (Fig. 4.2). All Magenta boxes were then placed in a growth chamber (see above for conditions), watered regularly with distilled water, and given 1 ml of half-strength Long-Ashton nutrient solution (Hewitt 1966) weekly. After the seeds had germinated and grown to a height of several centimetres, half of the Petri dishes were removed from their Magenta boxes and all dishes were thinned to contain only the larger of the two seedlings.

Separately, forty 50 mm Petri dishes were filled with 10 ml of 10 mM 2-(N-morpholin) ethane sulphonic acid (MES) buffer (pH 7) plus 0.04 g of Gel-Gro. To each dish, a single spore of *G. etunicatum* isolate NPI (sieved and sterilized as described above), was added. The plates were then sealed with Parafilm and incubated at 25°C in darkness until germination.

Following the methodology of Fracchia *et al.* (2001), the twenty Petri dishes containing the spores with the most developed germination tubes would then have their lids removed. The contents of the twenty dishes containing a single tomato seedling in perlite-vermiculite mixture would then be placed on top, exposing the germinated spore to the roots in a semi-sterile environment (Fig. 4.2).

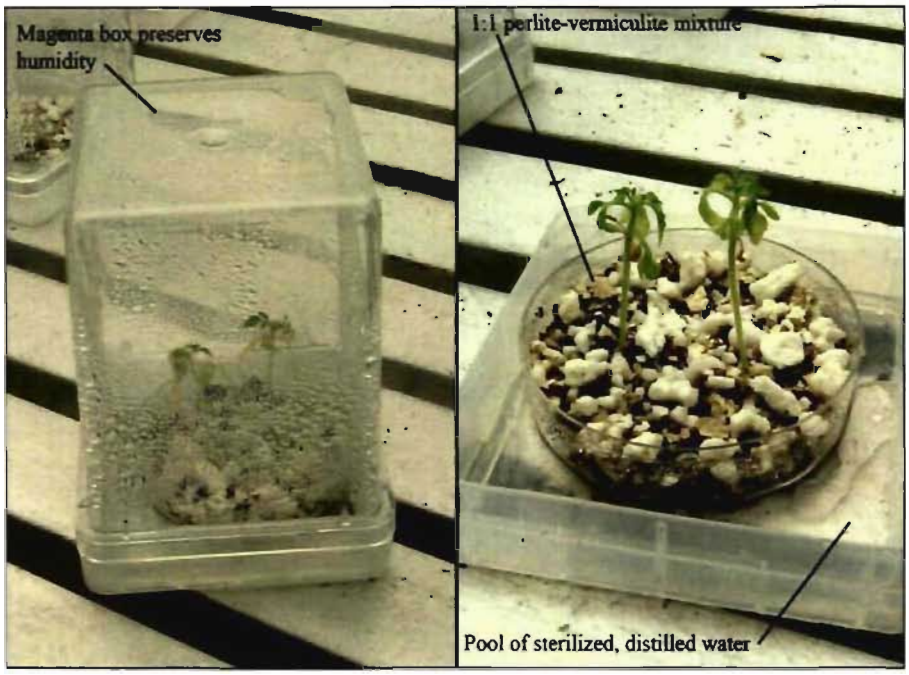


Figure 4.2) Micro-Tom tomato seedlings planted according to method of Fracchia *et al.* (2001).

4.23 – Transformed root cultures

Several thousand spores of *G. etunicatum* isolate NPI were sieved and sterilized as described above. Two hundred and fifty sterile spores were then placed, ten spores per plate, onto Petri dishes of distilled water gelled using 4 g/l Gel-Gro. These plates were incubated at 25°C in darkness and observed daily. Any spores showing contamination were cut out using a sterile scalpel. After three weeks, spores which had germinated were excised and placed individually on plates of M medium gelled with 4 g/l Gel-Gro (Bécard and Fortin 1988). Immediately next to each germinated spore was placed a small (approximately 2 cm) piece of *Agrobacterium rhizogenes*- transformed carrot root, including a terminal meristem. These plates were once again stored at 25°C in darkness and checked once weekly for colonization of the root by the fungus.

4.3 – Results

4.31 – Voets et al. (2005) method

All replicates of this method were successfully set up without contamination. While plantlets initially wilted severely when they were transferred to the new culture, all of them recovered fully after a day or two. Although the shoots still appeared healthy after two weeks, they had begun to take on a dark purple colouration on the upper stem and the undersides of the leaves. Leaves looked otherwise normal. When the plates were opened after the second week, the gel medium had retracted visibly and had a denser consistency than at the

beginning. The roots themselves showed marked growth, but were a darker shade of brown at their tips than they had been at the beginning. None of the spores had germinated at this point.

At four weeks of growth, the leaves of the plants appeared somewhat wilted, and the stems were deep purple along much of their length. The gel inside the plates had retracted further and seemed barely moist. Small white specks which appeared to be salt crystals dotted the surface. The roots had continued to deepen in colour and showed necrosis in some areas. At six weeks after transplanting, all plants were either severely wilted or dead, and the gel around their roots had thickened to a rubbery consistency. None of the fungal spores had germinated.

4.32 – Fracchia et al. (2001) method

Tomato seeds sown in the perlite/vermiculite mix initially germinated well and, the seedlings having reached a height of several centimetres after about one week, remained healthy-looking. Shortly thereafter, the two groups- those left in the Magenta boxes and those removed- both encountered problems. Despite attempts to adjust the watering schedule accordingly, the substrate was such that roots of both groups were either constantly bathed in water, or became completely dry. This was because the perlite-vermiculite mixture did not hold any significant amount of water. The seedlings which were removed from their Magenta boxes and exposed to the dry air of the growth chamber dried out more quickly and wilted despite daily watering. The other group showed areas of root necrosis and became etiolated due to the lower light levels in the Magenta boxes. None of the seedlings remained healthy long enough to be transferred to a plate containing a germinated spore. Furthermore, spore

germination for this experiment was very low, with only three of forty spores germinating. This may have been due to a poor spore lot or the use of MES buffer in the medium.

4.33 – Transformed root cultures

Of the 250 sterile spores which were sown on water Gel-Gro, close to one quarter were eventually lost to fungal contamination. Perhaps ten were lost to bacterial contamination. After the three week growth period, 89 spores had successfully germinated without contamination and were moved to co-cultures. Of these 89 co-cultures, seven eventually formed mycorrhizae following approximately eight months of incubation with the carrot roots (Fig. 4.3). Three of these mycorrhizae never formed spores, while the remaining four formed spores in the following numbers: 5, 6, 10, approx. 170.

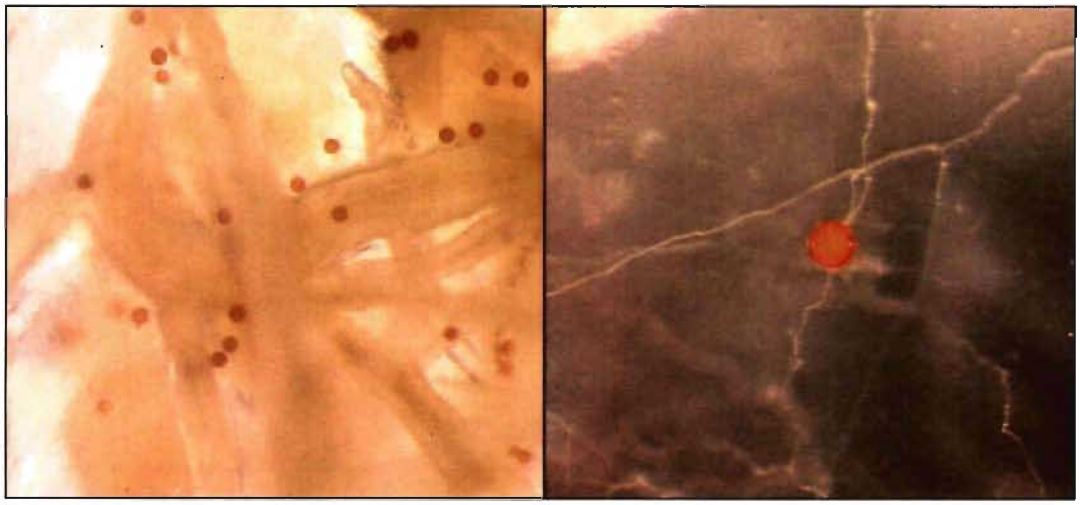


Figure 4.3) Spores of *Glomus etunicatum* produced *in vitro* with Ri T-DNA transformed carrot roots.

4.4 – Discussion

4.41 – Voets et al. (2005) method

Although this technique looked promising initially, its fatal flaw is that there is no way to sufficiently replenish the water used up by the plant. While the steps taken by the authors were followed as closely as possible, I was forced to change my methodology from that of the original experiment with respect to the addition of new medium. While the authors did not add more medium until four weeks after transplanting, the plants I used would not have survived that long; they required more moisture after only two weeks. Although I used a different species of *Solanum* than Voets *et al.* - *S. chacoense* versus *S. tuberosum*, it seems unlikely that this made such a large difference. Even with the plastic container helping to preserve humidity around the shoots, the water needs of the plants were too great for the small amount of medium used. The increasing salinity of the medium would likely also prevent the germination of the spores as well.

I considered adding a small amount of sterile water to the plates and allowing it to sit in the bottoms of the upturned plates, but having liquid flowing around the edge of the plate and onto the Parafilm presented too much of a risk in terms of maintaining the sterility of the plates. Although I am at a loss as to how the authors succeeded with this method as written, I believe it could be made to work with further adjustments. A plant with lower water requirements would likely fare better. I would also suggest lying the plates on their bottom rather than their edge, and letting the plant bend 90° as it grows. This way, a small amount of water, sufficient to be absorbed by the gel medium, could regularly be added to the plate without sacrificing sterility.

4.42 – Fracchia et al. (2001) method

Much like the previous method, the major flaw in the method of Fracchia *et al.* was the inability to maintain a proper moisture balance around the plant roots. Because the large-particle mixture of perlite and vermiculite did not hold any moisture, roots were either bathed in water, potentially suffering anoxia, or desiccated from lack of water. The seedlings kept in the Magenta boxes tended towards too much water due to lack of ventilation, while the unprotected seedlings tended toward too little. It was extremely difficult to find a 'happy medium'. While this same procedure could likely be carried out with sterilized soil, the attraction of this method lies in the ability to observe root colonization through the bottom of the plate; this would be next to impossible if soil was used. Instead, I would recommend the use of a commercial acrylamide-potassium acrylate polymer; these products absorb up to 500 times their volume in water to become gels and are largely resistant to microbial attack (Seybold 1994). As well as providing greater structural support to seedlings than a perlite-vermiculite mix, a polymer gel, perhaps mixed with some perlite to avoid becoming too dense, would maintain moisture about the roots of the plant while allowing enough light to pass through for viewing of the mycorrhiza from below.

Clover seedlings were used in the original paper, whereas tomato seedlings were used here. This could perhaps have made a difference in the water stress tolerance of the plants; however, it is difficult to imagine that even a clover seedling would grow to be healthy under such conditions. The size of the Petri dishes used is also problematic. Even the roots of Micro-Tom tomatoes, which are bred to remain small for use in experimental work (Meissner et al. 1997), quickly filled the 5cm dish. In the time required for the plant to grow and form a

mycorrhiza, the dish could become completely bound up in root mass. In further trials, I would suggest standard 10 cm Petri dishes.

4.43 – Transformed root cultures

A major difficulty in setting up transformed root cultures is the high rate of contamination. Sterilization with Chloramine T is fairly effective against bacteria, but does little to prevent fungal contamination. For obvious reasons, fungicides are typically not used. It was for this reason, as well as the low germination rate of *G. etunicatum*, that spores were first plated onto a gelled-water medium. This step saves the labour of individually plating dozens of spores which will never germinate, and provides an environment that is much less amenable to contaminant growth than M medium. Once the clean, germinated spores were transferred to plates containing nutritive medium and root segments, very few were lost to contamination.

Unfortunately, of the approximately 35% of original spores which germinated, only 8% *of these* actually colonized a root segment. While four of the cultures (1.6% of the original spores) did form sporulating mycorrhizae, this number was much too low for this method to be a practical form of spore-production for further experimental work.

It is unknown why so few of the germinated spores colonized successfully. In some cases, proximity to the root may have been a problem, although in many plates, the root segment was placed in direct contact with the germinated spore, and still no colonization took place.

4.44 – Conclusions

While none of the three culture methods tested here worked sufficiently well- as I set them up- to merit further use in my experiments, each showed enough promise to justify future trials with the adjustments I have recommended. However, for those requiring a fast, simple means of producing single spore cultures, and who, like myself, are using primers which are specific enough as to not require sterility, I feel that the pot culture method detailed in Chapter 3 of this thesis is the best choice.

Chapter 5

General Discussion & Conclusions

In studying AM fungi, researchers are confronted with an organism which apparently reproduces clonally, yet possesses remarkable intra-isolate genetic variation. Large numbers of dissimilar nuclei are moved *en masse* from one generation to the next, yet the seemingly inevitable loss of diversity with each passing generation is somehow avoided.

The work described in this thesis represents another small piece in the large puzzle that is AM fungal genetics. For each piece uncovered, it is a challenge not only to understand what we are seeing, but in what way it relates to the larger picture. We now have a clearer idea of the amount of diversification which can occur in a single coding gene within one AM fungal isolate, and some sense of the pattern that variation takes. However, several very recent discoveries will no doubt influence the way in which my results are understood in the greater context of the glomeralean lifecycle.

An obvious question arises when we see that, in *Glomus etunicatum*, a species with only around seven or eight hundred nuclei being passed from one generation to the next (Pawlowska and Taylor 2004), at least one hundred unique genomes can be found in one isolate- How is this diversity maintained?

5.0.1 – Anastomosis

While it is well known that AM fungi possess the ability to form cytoplasmic connections with hyphae originating from the same isolate (Giovannetti et al. 1999), it has now been shown via the use of molecular markers that the same process occurs between different isolates and, furthermore, that nuclear exchange between mycelia produces viable

hybrid spores (Croll et al. 2009). Based on analysis of the hybrid lines using AFLP markers, the authors found that nuclei originating from both parents were maintained via replication, as opposed to the genetic material of one parent 'out-competing' that of the other parent. These discoveries help to explain how it may be possible for the huge number of *PLS* variants to be preserved from one generation to the next. Any one daughter spore may not receive all, or even the majority of variants, but the larger group of many sister spores does, and the variants can be later retrieved via cytoplasmic connections and nuclear exchange between them. The fact that this phenomenon has been observed between hyphae of different isolates opens the door even wider- variants lost at random from an isolate are returned through anastomosis with an isolate in which it still resides. A natural population of an AM fungal species, comprised of many different isolates, becomes a vast cache of genetic diversity. From this perspective, one hundred variants of a single gene doesn't seem so staggering. But what if we went a step further? What if these putatively asexual organisms were capable of recombination as well?

5.0.2 – Cryptic recombination

A recent study of 11 polymorphic nuclear loci in 40 isolates of *Glomus intraradices* used phylogenetic and statistical tests to show that, while a number of the genotypes examined appeared to have evolved clonally, five isolates showed strong evidence for recombination (Croll and Sanders 2009). AM fungi have long been assumed to be ancient asexuals due to their lack of morphological differentiation compared to fossil records (Redecker et al. 2000) and no sexual structures having been observed (Normark et al. 2003). This belief has been somewhat controversial, as sexual recombination is believed to be necessary in order to avoid the accumulation of deleterious mutations over time (Normark et

al. 2003). The findings of Croll and Sanders (2009) suggest that in fact, AM fungi do use recombination as a means of purging mutations, albeit infrequently. While the authors intentionally chose loci in which there was no intra-isolate variation, there is no reason to believe that, if recombination occurs between isolates, it does not occur, and perhaps with greater frequency, within isolates. Intra-isolate recombination would help to explain the persistence of a high number of putatively functional *PLS* variants in a single, *in vitro* cultivated isolate of *G. etunicatum*. Furthermore, recombination provides a possible non-artefactual explanation for the *PLS1-PLS2* hybrid sequences discovered both here and by others (Mohamed Hijri, personal communication). This could be tested by using a methodology without PCR, such that there would be no possibility of *in vitro* recombination.

5.0.3 – Conclusion

When considering the potential importance of AM fungi to the future of sustainable agriculture, as discussed in the opening chapter, it becomes clear why improving our understanding of the genomic structure of these enigmatic organisms is crucial. Technology has reached a point where the manipulation of genetic material for specific tasks is not only possible, but commonplace. Commonplace, that is, for organisms in which our knowledge of the underlying mechanisms is extensive. In AM fungi, even such fundamentals as the structure of genetic variation and the way in which it is transmitted generationally are unknown.

The experiments described in this thesis set out to investigate both of these aspects of AM fungal biology. As is so often the case in research, neither experiment has provided a cut-and-dry answer to the question it addressed. Both experiments, however, provide a starting point for future study. The experiment described in chapter two on variant diversity paves the

way for further sequencing efforts, both with *PLS* and other low-copy genes, to develop a well-supported estimate of genome numbers in different species of AM fungi. While it is clear that there are many more distinct genomes, at least in *G. etunicatum*, than were originally expected, this diversity may be markedly different from one species to another, or depending on the type of marker gene used. We are far from a complete picture.

The experiment begun in chapter three on nuclear segregation provides a reliable method for producing large quantities of sister spores from a single-spore culture, as well as a means of dealing with the low quantity of template DNA present in each spore. Once this experiment returns some preliminary results on segregation, and with the ever-improving technology for massive sequencing, more ambitious trials could be carried out in an attempt to quantify the genetic drift which goes hand-in-hand with this unusual reproductive mode. Again, progress has been made, but we are far from a complete picture.

References

Barea, J.-M., R. Azcón and C. Azcón-Aguilar (2002). "Mycorrhizosphere interactions to improve plant fitness and soil quality." Antonie van Leeuwenhoek 81: 343-351.

Beauchemin, S. and R. R. Simard (1999). "Soil phosphorus saturation degree: Review of some indices and their suitability for P management in Quebec, Canada." Canadian Journal of Soil Science 79(4): 615-625.

Bécard, G. and J. A. Fortin (1988). "Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots." New Phytologist 108(2): 211-218.

Bertani, G. (1951). "Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli." Journal of Bacteriology 62: 293-300.

Bever, J. D. (2002). "Negative feedback within a mutualism: host-specific growth of mycorrhizal fungi reduces plant benefit." Proc. R. Soc. Lond. 269: 2595-2601.

Bever, J. D. and J. Morton (1999). "Heritable variation and mechanisms of inheritance of spore shape within a population of *Scutellospora pellucida*, an arbuscular mycorrhizal fungus." American Journal of Botany 86(9): 1209-1216.

Bever, J. D., J. B. Morton, J. Antonovics and P. A. Schultz (1996). "Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland." Journal of Ecology 84: 71-82.

Boon, E., E. Zimmerman, B. F. Lang and M. Hijri (Manuscript In Preparation). "Genome variation between nuclei of arbuscular mycorrhizal fungi persists at the transcript level."

Carlyle, W. J. (2004). "The rise of specialty crops in Saskatchewan, 1981-2001." The Canadian Geographer 48(2): 137-151.

Caron, M., J. A. Fortin and C. Richard (1986). "Effect of inoculation sequence on the interaction between *Glomus intraradices* and *Fusarium oxysporum* f.sp. *radicis-lycopersici* in tomatoes." Canadian Journal of Plant Pathology 8: 12-16.

Clapp, J. P., A. H. Fitter and J. P. W. Young (1999). "Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, *Scutellospora* sp." Molecular Ecology 8: 915-921.

Corradi, N., D. Croll, A. Colard, G. Kuhn, M. Ehinger and I. R. Sanders (2007). "Gene copy number polymorphisms in an arbuscular mycorrhizal fungal population." Applied and Environmental Microbiology 73(1): 366-369.

Corradi, N., M. Hijri, L. Fumagalli and I. R. Sanders (2004a). "Arbuscular mycorrhizal fungi (*Glomeromycota*) harbour ancient fungal tubulin genes that resemble those of the chytrids (*Chytridiomycota*)." Fungal Genetics and Biology 41: 1037-1045.

Corradi, N., G. Kuhn and I. R. Sanders (2004b). "Monophyly of *β-tubulin* and H^+*-ATPase* gene variants in *Glomus intraradices*: consequences for molecular evolutionary studies of AM fungal genes." Fungal Genetics and Biology 41: 262-273.

Croll, D., M. Giovannetti, A. Koch, C. Sbrana, M. Ehinger, P. J. Lammers and I. R. Sanders (2009). "Nonself vegetative fusion and genetic exchange in the arbuscular mycorrhizal fungus *Glomus intraradices*." New Phytologist 181(4): 924-937.

Croll, D. and I. R. Sanders (2009). "Recombination in *Glomus intraradices*, a supposed ancient asexual arbuscular mycorrhizal fungus." BMC Evolutionary Biology 9: in press.

Croll, D., L. Wille, H. A. Gamper, N. Mathimaran, P. J. Lammers, N. Corradi and I. R. Sanders (2008). "Genetic diversity and host plant preferences revealed by simple

sequence repeat and mitochondrial markers in a population of the arbuscular mycorrhizal fungus *Glomus intraradices*." New Phytologist 178(3): 672-687.

de Souza, F. A., G. A. Kowalchuk, P. Leeflang, J. A. van Veen and E. Smit (2004). "PCR-denaturing gradient gel electrophoresis profiling of inter- and intraspecies 18S rRNA gene sequence heterogeneity is an accurate and sensitive method to assess species diversity of arbuscular mycorrhizal fungi of the genus *Gigaspora*." Applied and Environmental Microbiology 70(3): 1413-1424.

Declerck, S., D. G. Strullu and C. Plenchette (1998). "Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection." Mycologia 90(4): 579-585.

Dickie, I. A. and R. G. FitzJohn (2007). "Using terminal restriction fragment length polymorphism (T-RFLP) to identify mycorrhizal fungi: a methods review." Mycorrhiza 17: 259-270.

Filion, M., M. St-Arnaud and S. H. Jabaji-Hare (2003a). "Direct quantification of fungal DNA from soil substrate using real-time PCR." Journal of Microbiological Methods 53(1): 67-76.

Filion, M., M. St-Arnaud and S. H. Jabaji-Hare (2003b). "Quantification of *Fusarium solani* f. sp. *phaseoli* in mycorrhizal bean plants and surrounding mycorrhizosphere soil using real-time polymerase chain reaction and direct isolations on selective media." Phytopathology 93(2): 229-235.

Fortin, J. A., G. Bécard, S. Declerck, Y. Dalpé, M. St-Arnaud, A. P. Coughlan and Y. Piché (2002). "Arbuscular mycorrhiza on root-organ cultures." Canadian Journal of Botany 80: 1-20.

Fracchia, S., A. Menendez, A. Godeas and J. A. Ocampo (2001). "A method to obtain monosporic cultures of arbuscular mycorrhizal fungi." Soil Biology & Biochemistry 33: 1283-1285.

Gamper, H. A., J. P. W. Young, D. L. Jones and A. Hodge (2008). "Real-time PCR and microscopy: are the two methods measuring the same unit of arbuscular mycorrhizal fungal abundance?" Fungal Genetics and Biology 45(5): 581-596.

Gavito, M. E. (2007). Mycorrhizae and Crop Production in a World of Rapid Climate Change: A Warning Call. Mycorrhizae in Crop Production. C. Hamel and C. Plenchette. New York, Haworth Food & Agricultural Products Press: 293-310.

Giovannetti, M., D. Azzolini and A. S. Citernesi (1999). "Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi." Applied and Environmental Microbiology 65(12): 5571-5575.

Graham, J. H., N. C. Hodge and J. B. Morton (1995). "Fatty acid methyl ester profiles for characterization of Glomalean fungi and their endomycorrhizae." Applied and Environmental Microbiology 61(1): 58-64.

Hamel, C. (2004). "Impact of arbuscular mycorrhizal fungi on N and P cycling in the root zone." Canadian Journal of Soil Science 84: 383-395.

Hawkins, H.-J., A. Johansen and E. George (2000). "Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi." Plant and Soil 226: 275-285.

Helgason, T. and A. Fitter (2005). "The ecology and evolution of the arbuscular mycorrhizal fungi." Mycologist 19(3): 96-101.

Helgason, T., I. J. Watson and J. P. W. Young (2003). "Phylogeny of the Glomerales and Diversisporales (Fungi: Glomeromycota) from actin and elongation factor 1-alpha sequences." FEMS Microbiology Letters 229: 127-132.

Hepper, C. M., R. Sen, C. Azconaguilar and C. Grace (1988). "Variation in certain isozymes amongst different geographical isolates of the vesicular arbuscular mycorrhizal fungi *Glomus clarum, Glomus monosporum* and *Glomus mosseae*." Soil Biology & Biochemistry 20(1): 51-59.

Hewitt, E. J. (1966). Sand and water culture methods used in the study of plant nutrition. L. Commonwealth Agricultural Bureau, England: Technical Communication No.22.

Hijri, I., Z. Sykorova, F. Oehl, K. Ineichen, P. Mäder, A. Wiemken and D. Redecker (2006). "Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity." Molecular Ecology 15: 2277-2289.

Hijri, M., M. Hosny, D. van Tuinen and H. Dulieu (1999). "Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores." Fungal Genetics and Biology 26: 141-151.

Hijri, M., D. Redecker, J. A. M.-C. Petetot, K. Voigt, J. Wöstemeyer and I. R. Sanders (2002). "Identification and isolation of two ascomycete fungi from spores of the arbuscular mycorrhizal fungus *Scutellospora castanea*." Applied and Environmental Microbiology 68(9): 4567-4573.

Hijri, M. and I. R. Sanders (2005). "Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei." Nature 433: 160-163.

Hofer, J. and N. Ellis (2002). "Conservation and diversification of gene function in plant development." Current Opinion in Plant Biology 5(1): 56-61.

Hosny, M., M. Hijri, E. Passerieux and H. Dulieu (1999). "rDNA units are highly polymorphic in *Scutellospora castanea* (Glomales, Zygomycetes)." Gene 226: 61-71.

James, T. Y., F. Kauff, C. L. Schoch, P. B. Matheny and V. Hofstetter (2006). "Reconstructing the early evolution of Fungi using a six-gene phylogeny." Nature 443: 818-822.

Jansa, J., F. A. Smith and S. E. Smith (2008). "Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi?" New Phytologist 177: 779-789.

Kistner, C. and M. Parniske (2002). "Evolution of signal transduction in intracellular symbiosis." Trends in Plant Science 7(11): 511-518.

Koch, A. M., D. Croll and I. R. Sanders (2006). "Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth." Ecology Letters 9: 103-110.

Koch, A. M., G. Kuhn, P. Fontanillas, L. Fumagalli, J. Goudet and I. R. Sanders (2004). "High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi." PNAS 101(8): 2369-2374.

Kuhn, G., M. Hijri and I. R. Sanders (2001). "Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi." Nature 414: 745-748.

Küster, H., A. Becker, C. Firnhaber, N. Honjec, K. Manthey, A. M. Perlick, T. Bekel and M. Dondrup (2007). "Development of bioinformatic tools to support EST-sequencing, *in silico*- and microarray-based transcriptome profiling in mycorrhizal symbioses." Phytochemistry 68: 19-32.

Li, H., S. E. Smith, R. E. Holloway, Y. Zhu and F. A. Smith (2006). "Arbuscular mycorrhizal fungi contribute to phosphorus uptake by wheat grown in a phosphorus-fixing soil even in the absence of positive growth responses." New Phytologist 172: 536-543.

Liu, A., C. Hamel, R. I. Hamilton, B. L. Ma and D. L. Smith (2000). "Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels." Mycorrhiza 9: 331-336.

Lloyd-MacGilp, S. A., S. M. Chambers, J. C. Dodd, A. H. Fitter, C. Walker and J. P. W. Young (1996). "Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi." New Phytologist 133: 103-111.

Lundberg, K. S., D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge and E. J. Mathur (1991). "High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*." Gene 108(1): 1-6.

McKenzie, R. H. and E. Bremer (2003). "Relationship of soil phosphorus fractions to phosphorus soil tests and fertilizer response." Canadian Journal of Soil Science 83(4): 443-449.

Meissner, R., Y. Jacobson, S. Melamed, S. Levyatuv, G. Shalev, A. Ashri, Y. Elkind and A. Levy (1997). "A new model system for tomato genetics." The Plant Journal 12(6): 1465-1472.

Murashige, T. and F. Skoog (1962). "A revised medium for rapid growth and bio assays with tobacco tissue cultures." Physiol. Plant 15: 473-497.

Normark, B. B., O. P. Judson and N. A. Moran (2003). "Genomic signatures of ancient asexual lineages." Biological Journal of the Linnean Society 79: 69-84.

Pawlowska, T. E. and J. W. Taylor (2004). "Organization of genetic variation in individuals of arbuscular mycorrhizal fungi." Nature 427: 733-737.

Pirozynski, K. A. and D. W. Malloch (1975). "The origin of land plants: A matter of mycotropism." Biosystems 6(3): 153-164.

Plenchette, C., T. Fraser and C. Hamel (2007). Mycorrhizal soil infectivity: A new approach for its determination. pg. 164, Proceedings of the Annual Meeting of the Canadian Society of Agronomy, Plant Canada 2007, Saskatoon, Saskatchewan.

Posada, D. and K. A. Crandall (2001). "Intraspecific gene genealogies: trees grafting into networks." Trends in Ecology & Evolution 16(1): 37-45.

Pozo, M. J. and C. Azcón-Aguilar (2007). "Unraveling mycorrhiza-induced resistance." Current Opinion in Plant Biology 10: 393-398.

Redecker, D., R. Kodner and L. E. Graham (2000). "Glomalean fungi from the Ordovician." Science 289: 1920-1921.

Remy, W., T. N. Taylor, H. Hass and H. Kerp (1994). "Four hundred-million-year-old vesicular arbuscular mycorrhizae." Proc. Natl. Acad. Sci. USA 91: 11841-11843.

Rillig, M. C. and D. L. Mummey (2006). "Mycorrhizas and soil structure." New Phytologist 171: 41-53.

Rivera, R., F. Fernández, K. Fernández, L. Ruiz, C. Sánchez and M. Riera (2007). Advances in the Management of Effective Arbuscular Mycorrhizal Symbiosis in Tropical Ecosystems. Mycorrhizae in Crop Production. C. Hamel and C. Plenchette. New York, Haworth Food & Agricultural Products Press: 151-195.

RocheDiagnostics (2008). "Genome Sequencer FLX System Bibliography." www.454.com.

Rodriguez, A., J. P. Clapp and J. C. Dodd (2004). "Ribosomal RNA gene sequence diversity in arbuscular mycorrhizal fungi (Glomeromycota)." Journal of Ecology 92: 986-989.

Rodriguez, A., J. P. Clapp, L. Robinson and J. C. Dodd (2005). "Studies on the diversity of the distinct phylogenetic lineage encompassing *Glomus claroideum* and *Glomus etunicatum*." Mycorrhiza 15: 33-46.

Rosendahl, S. (2008). "Communitites, populations and individuals of arbuscular mycorrhizal fungi." New Phytologist 178(2): 253-266.

Rosendahl, S. and R. Sen (1992). "Isozyme analysis of mycorrhizal fungi and their mycorrhiza." Methods in Microbiology 24: 169-194.

Ruiz-Lozano, J. M., C. Collados, J.-M. Barea and R. Azcón (2001). "Arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants." New Phytologist 151: 493-502.

Sanders, I. R. (2002). "Ecology and evolution of multigenomic arbuscular mycorrhizal fungi." The American Naturalist 160(Supplement): S128-S141.

Sanders, I. R., M. Alt, K. Groppe, T. Boller and A. Wiemken (1995). "Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities." New Phytologist 130: 419-427.

Schüßler, A., D. Schwarzott and C. Walker (2001). "A new fungal phylum, the *Glomeromycota*: phylogeny and evolution." Mycol. Res. 105(12): 1413-1421.

Seybold, C. A. (1994). "Polyacrylamide review: Soil conditioning and environmental fate." Communications in Soil Science and Plant Analysis 25(12): 2171-2185.

Sharma, S., D. Pant, S. Singh, R. R. Sinha and A. Adholeya (2007). Mycorrhizae in Indian Agriculture. Mycorrhizae in Crop Production. C. Hamel and C. Plenchette. Binghampton, New York, Haworth Press: 239-291.

Simon, L., J. Bousquet, R. C. Lévesque and M. Lalonde (1993). "Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants." Nature 363: 67-69.

Smith, S. E. and D. J. Read (1997). Mycorrhizal Symbiosis. London, Academic Press.

Smith, S. E. and D. J. Read (2008). Mycorrhizal Symbiosis. Amsterdam, Academic Press.

St-Arnaud, M., C. Hamel, M. Caron and J. A. Fortin (1994). "Inhibition of *Pythium ultimum* in roots and growth substrate of mycorrhizal *Tagetes patula* colonized with *Glomus intraradices*." Canadian Journal of Plant Pathology 16: 187-194.

St-Arnaud, M. and V. Vujanovic (2007). Effects of the arbuscular mycorrhizal symbiosis on plant diseases and pests. Mycorrhizae in Crop Production. C. Hamel and C. Plenchette. Binghampton, New York, Haworth Food & Agricultural Products Press: 67-122.

Stewart, L. I., S. H. Jabaji-Hare and B. T. Driscoll (2006). "Effects of external phosphate concentration on glucose-6-phosphate dehydrogenase gene expression in the arbuscular mycorrhizal fungus *Glomus intraradices*." Canadian Journal of Microbiology 52: 823-830.

Subramanian, K. S. and C. Charest (1999). "Acquisition of N by external hyphae of an arbuscular mycorrhizal fungus and its impact on physiological responses in maize under drought-stressed and well-watered conditions." Mycorrhiza 9: 69-75.

Tamura, K., J. Dudley, M. Nei and S. Kumar (2007). "MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0." Molecular Biology and Evolution 24: 1596-1599.

Tanaka, Y. and K. Yano (2005). "Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied." Plant, Cell and Environment 28: 1247-1254.

Toro, M., R. Azcón and J.-M. Barea (1997). "Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling." Applied and Environmental Microbiology 63(11): 4408-4412.

Toussaint, J.-P., M. St-Arnaud and C. Charest (2004). "Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith and Ri T-DNA roots of *Daucus carota* L. in an in vitro compartmented system." Canadian Journal of Microbiology 50: 251-260.

van der Heijden, M. G. A., J. N. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken and I. R. Sanders (1998). "Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity." Nature 396: 69-72.

Villesen, P. (2007). "FaBox: an online toolbox for fasta sequences." Molecular Ecology Notes 7(6): 965-968.

Voets, L., H. Dupré de Boulois, L. Renard, D.-G. Strullu and S. Declerck (2005). "Development of an autotrophic culture system for the in vitro mycorrhization of potato plantlets." FEMS Microbiology Letters 248: 111-118.