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Production and Characterization of Mycorrhizal Fungal Inoculum

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Iris Charvat, Peter Avis, Kari Eichstaedt, Hong-Way Lin	

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16. Abstract (Limit: 200 words)

The aim of this research is to produce a local, diverse mycorrhizal inoculum for commercial production for use in the Minneapolis/St. Paul area. In earlier studies, spores of several arbuscular mycorrhizal fungal (AMF) species were isolated from a Minnesota remnant prairie and identified by morpholo gical features. For each species, single spore cultures were established and stored in the cold for 6 to 7 years. Objectives of this project were to produce pot cultures from the single spore cultures and to recommend AMF species for commercial production. To check the identity of the fungi before and after inoculum production, genetic identification of fungi used to develop the inoculum should be performed. The extraction and preservation of DNA of AMF species were done. DNA analysis showed general agreement between the morphological and molecular identification of the spores and their placement in genera. However, results suggested that some species placement might not be consistent where these comparisons can be made. Further research may result in the re-naming of some species. Several AMF species are recommended as candidates for commercial inoculum production based on production of spores in pot cultures, on their longevity in cold storage, and in some species on molecular traits.

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Production and Characterization of Mycorrhizal Fungal Inoculum

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Prepared by:

Iris Charvat
Peter Avis
Kari Eichstaedt
Hong-Way Lin

Department of Plant Biology
University of Minnesota

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Executive Summary

The use of mycorrhizal fungal inoculants in restoration, horticulture and agriculture is increasing in popularity as it is recognized that most plants depend on mycorrhizal associations for optimal growth and development. Although commercial inoculum is available, a local Minneapolis/St. Paul area inoculum has not been produced, as far as we know. Because of the concerns about introducing non-native arbuscular mycorrhizal fungal (AMF) species that may not be able to survive in this environment or that may be invasive, production of a local inoculum from a native prairie source is the best option.

The goal of this project was to produce a local, diverse mycorrhizal inoculum for commercial production for use in the Minneapolis/St. Paul area. In earlier studies, spores of several AMF species were isolated from a Minnesota remnant prairie and identified by morphological features. In this study our objectives were (1) to determine the viability of the AMF spores produced in earlier studies, (2) to establish pot cultures of AMF species for which there were viable spores, (3) to characterized some of the newly produced spores of AMF species using molecular techniques and (4) to recommend AMF species as candidates for commercial production.

The original AMF inoculum source was obtained from a remnant prairie in the Twin Cities metropolitan area, which is located on land maintained by Minnesota Department of Transportation. This soil was used as inoculum to establish trap cultures using prairie plants as hosts. From these cultures, AMF spores were isolated, grown in single pot cultures, and the newly produced spores were partially characterized based on morphological traits. These spores, either isolated or in a soil/sand mixture, were utilized as starting material for this project.

After being in storage for several years, it was not known if the spores were viable. The formation of colored formazan products on the reduction of tetrazolium salts has been employed as an indicator of biological reducing systems for a long time and was used to test spore viability. The tetrazolium stain had been used successfully to determine viability of AMF spores stored for a year or less. However, only seven starting cultures of 6 or 7 years of age showed viable spores using tetrazolium staining, but several more cultures produced viable spores in new pot cultures. The discrepancy between the staining and germination results may have been due to the inability of the staining solution to react with the reducing systems in old spores and/or to the limited number of spores available for the staining test. If few spores are used, tetrazolium staining can

provide only an indication of spore viability. Germination of spores in pot cultures was the most reliable indicator of viability.

For all mycorrhizal species, cultures were established using the starting soil/sand inoculum and by placing single spores on the roots of big bluestem seedlings. In general, the new pot cultures in the first round produced more spores if their starting cultures had high or moderate spores/gram of dry soil. In the second round of culturing, some species showed a large increase in spores/gram of dry soil when compared to Round 1, but most species produced fewer spores in the second round. In general, species with starting cultures containing a low number of spores were not recommended for commercial production because these cultures produced low number of spores in both rounds of culturing.

Although plant growth benefits are advertised as the advantage of inoculants, the potential of introducing exotic mycorrhizal fungi into habitats in which they previously did not exist raises a central question: Can the species and isolates of mycorrhizal fungi used as an inoculum source be accurately identified after large-scale inoculum production? In order to check the identity of the fungi before and after inoculum production, genetic identification of AMF fungi used to develop the inoculum should be completed. The extraction and preservation of DNA of diverse prairie species has been done. DNA analysis showed general agreement between the morphological and molecular identification of the spores and their genera (e.g. *Glomus* with *Glomus, Acaulospora* with *Acaulospora, Paraglomus* with *Paraglomus*). However, results suggested that some species placement might not be consistent where these comparisons can be made. In other cases, species morphological and molecular analyses agree. Further research may result in the renaming of some species. However, use of the protocols outlined in this report should provide DNA usable for similar analyses in the future as long as the DNA is properly stored.

Eleven AMF species are recommended as candidates for commercial inoculum production based on creation of spores in pot cultures, on longevity in cold storage and in some species on molecular traits. The selected species are *Acaulospora spinosa, Archaeospora leptoticha, Glomus caledonium, Glomus claroideum, Glomus clarum, Glomus constrictum, Glomus coronatum, Glomus sp. (with some characteristics similar to Glomus diaphanum), Glomus intraradices, Glomus mosseas (1997) and Paraglomus occultum.*

Introduction

The establishment of native prairie plantings along Minnesota roadways is a goal of the Minnesota Department of Transportation (Mn/DOT) that supports the federally mandated "prairie passages," networks of prairie preserves connected by corridors of prairie along roadsides and other rights-of-way. Newly constructed roadside areas generally lack arbuscular mycorrhizal fungi (AMF). By adding AMF inoculum to these sites, vegetation managers will likely be able to plant prairie species sooner, and, thereby, establish the health and vigor of these communities. The aim of this study was to produce cultures of several AMF species originally obtained from a remnant local prairie, to characterize some of these species using molecular techniques and to make recommendations for commercial inoculum production.

Our original mycorrhizal inoculum source was Crosstown prairie, a remnant prairie in the Minneapolis metropolitan area, which is located on land maintained by Mn/DOT [1]. However, to minimize the disturbance of the prairie, soil samples were taken from an area that was dominated by prairie vegetation, but just outside the protected prairie. This soil was used as inoculum to establish trap cultures using prairie plants as hosts. From these cultures, AMF spores were isolated, which were grown in single pot cultures and partially characterized [2, 3]. It is these AMF spores of different species that we are using to begin this project.

The use of mycorrhizal fungal inoculants in restoration, horticulture and agriculture is increasing in popularity as it is recognized that most plants depend on mycorrhizal associations for optimal growth and development. The first step in performing an inoculation experiment in the field is to obtain a mycorrhizal inoculum. Although commercial inocula are available, no local inoculum from a Minnesota prairie has been produced, as far as we know. Because of the concerns about introducing non-native mycorrhizal species that may not be able to survive in this environment [3] or that may be invasive, production of a local inoculum from a native prairie source, which would be close to the native fungal community composition, is the best option.

The aim of this project was to generate for use in the Minneapolis/St. Paul area a highly diverse fungal inoculum for commercial production. Our objectives were (1) to determine the viability of the AMF spores produced in our earlier studies, (2) to establish pot cultures of AMF species for which there were viable spores, (3) to characterize the spores of different species using molecular techniques, and (4) to assess the AMF species for commercial production based on the results obtained in objectives 1, 2 and 3.

Chapter 1, concerning the production of arbuscular mycorrhizal fungal inoculum, addresses objectives 1 and 2. Chapter 2 is about the genetic characterization of AMF species. Although plant growth benefits are advertised as the advantage of inoculants, the potential of introducing exotic mycorrhizal fungi into habitats in which they previously did not exist raises a central question. Can the species and isolates of mycorrhizal fungi used as an inoculum source be accurately identified after large-scale inoculum production? In order to check the identity of the fungi before and after inoculum production, genetic identification of AMF fungi used to develop the inoculum should be completed on several species. Finally, in Chapter 2, recommendations are made of AMF species for commercial production.

Chapter 1
Production of Arbuscular Mycorrhizal Fungal Inoculum

1.1 Overview

The goal of this research is to produce a local, diverse mycorrhizal inoculum for commercial production for use in the Minneapolis/St. Paul area. The two objectives discussed in this chapter are as follows: (1) to determine the viability of AMF spores produced in our earlier studies and (2) to produce pot cultures of AMF species for which there were viable spores.

1.2 Materials and Methods

1.2.1 *Isolation of spores from starting cultures.* Twenty-three cultures of 18 AMF species were tested from spores produced in earlier studies and stored since 1996 or 1997 in a cold room (4-5 C) at the Biological Sciences Center, University of Minnesota [1, 2, 3]. Dr. Hamdy Agwa, visiting professor from Egypt, characterized and tentatively identified the spores given the facilities and limited time he had available. The initial naming of the AMF species was based on morphological characteristics of spores, not molecular traits. The AMF species names are according to the current classification system [4, 5]. The previously stored cultures, referred to as starting cultures, were available in small amounts (i.e. an average of 120 grams {g} from 1996 cultures). The two cultures from 1997, *Glomus constrictum* and *Glomus mosseae,* had 1113 g and 347 g of starting material, respectively. Spores were isolated from the starting cultures by sucrose centrifugation [6]. Some spores were used for viability tests and others for establishing new cultures.

1.2.2 *Spore viability test*. Due to the small quantities of the starting cultures and the limited number of spores for some AMF species, only ten spores were placed in a sterile petri dish with 0.1% MTT (3-[4,5-dimethylthiazol-yl]-2,5-diphenyl-2H-tetrazolim bromide) solution [7]. The small number of spores available precluded statistical analysis of the data. A change in spore color means the spore is viable. After 72 hours, spores were checked with a dissecting microscope for any color change. For species with many spores after isolations, 3 replicates with 25 spores per petri dish were established. The spore viability test was set up in the sterile room using sterile technique and then stored in the lab at room temperature until read. If very few

spores were isolated from the starting culture, spore viability test was not done; instead the spores were tested for germination.

1.2.3 *Pot culture inoculum production (Round 1).* For most starting cultures, even those that tested negative for viable spores, germination tests were done by establishing new cultures using soil/sand inoculum [1, 8]. The soil/sand inoculum was 25 g of soil/sand mixture obtained from bags of a single spore culture originally established by Dr. Hamdy Agwa [1, 2, 3]. The inoculum was mixed with approximately 15 g of sterile potting medium. The potting medium was 1/2 sterilized Crosstown Prairie soil and 1/2 autoclaved silica-sand minispheres (Unimin Corp, Le Suer, MN). The prairie soil was obtained from just outside the marked boundaries of the remnant prairie with permission of Mn/DOT. Black plastic pots, 14.5 cm in diameter, and saucers were soaked in a 10% bleach solution for 1 hour. They were rinsed for 1 hour each in water and de-ionized (DI) water, and a layer of fiber fill (0.5 g) was placed in the bottom of each of the dried pots. The pots were filled half full with about 230 g of potting medium. A layer of inoculum was spread evenly on top of the potting medium and the pot was filled the rest of the way with potting medium. Twenty sterilized seeds of *Andropogon gerardii* Vitman (big bluestem) were placed on the top of each pot and a layer of sterilized silica sand minispheres (ca. 1 cm) was added that covered the seeds. Because of limited starting inoculum, only two pots were created for each species as well as several control pots (3 or 4 pots/growth chamber) lacking inoculum.

All work using the starting culture inoculum to establish pot cultures was done in a sterile room using sterile technique. The room was sterilized with an UV light for 1/2 hour in between samples to avoid contamination. Each pot was placed in a separate saucer to avoid contamination from watering. The pots were watered initially with 30 ml DI water. All pots were maintained in growth chambers set at day-length 16 hr, 25% incandescent and 75% fluorescent lighting, 25 C, and 50% relative humidity. The pots were watered daily or every other day as needed and allowed to grow for 3 to 4 months. During the last weeks of growth, the plants were fertilized with Plantex 15-0-15 (Plantex Corp., Brampton, ON, Canada). After three to four months, spores/g of soil were isolated from the cultures to determine if the spores in the inoculum had germinated and reproduced new spores.

1.2.4 *Single spore inoculum production.* Cultures also were set-up by placing single spores on the seedling roots of *Andropogon gerardii* Vitman (big bluestem). The spores were isolated from 10 or 15 g of sand/soil mixture from the starting cultures. One spore was placed on a seedling root (approximately 3/4 of the way down) and planted in potting medium. Six inoculated seedlings were planted per pot and 3 pots were planted for each species if spore numbers would allow. For species with two different sizes or colors of spores, the two types were placed in different pots. A thin layer of sand was added to the top of the pots to prevent cross contamination. The pots were watered with 30 ml DI water and placed in the growth chamber. Single spore inoculation was also done in the sterile room using sterile technique. Culture maintenance was the same as in round 1. Establishment of single spore cultures with one or a limited number of spores permit the isolation and re-establishment of a pure AMF culture if inconsistency in species identification occurred.

1.2.5 *Establishment of pot cultures in Round 2 using inoculum from Round 1 or by reseeding.* If spore isolation on 10 grams of soil from 3 to 4 months old pots (round 1), produced more than 10 spores per gram of moist soil, then 25 grams of the pot soil from the first round were used as the inoculum to establish pots in the second round. If spore isolation on 10 grams from Round 1 pots produced few spores, the pots were reseeded [5]. About 1/2 of the pot cultures were reseeded, which was done in the sterile room on sterile newspaper. The top layer of sand was removed from the pot, and the shoots were cut off with sterile scissors to 1 cm below the soil line. The shoots and sand were discarded. The remaining pot contents were divided vertically into two parts, and 1/2 was placed back in the original pot and the other 1/2 placed into a new pot. Potting medium was added to fill the pots. Thirty imbibed seeds (sterilized then soaked in paper towel for 2 days) were added to each new pot and covered with a 1 cm layer of sterilized sand. Pot culture maintenance and harvesting was conducted as in Round 1.

1.2.6 *Isolation of spores from cultures in Rounds 1 and 2.* Spores were isolated from the cultures by sucrose centrifugation [4]. The number of spores/g of dry soil for each AMF species is based on one repetition of spore isolation per culture because of limited culturing material. For the control pots in round 1, at least 2 repetitions of spore isolations were completed per pot culture to determine the number of spores/g of dry soil, with some control pot having 3 repetitions of spore isolations. Control pots in round 2 had one repetition of spore isolation due to time limitation.

1.3 Results and Discussion

1.3.1 *Species of AMF spores isolated from starting cultures by sucrose centrifugation.* In order to determine the number of spores/g of dry soil in six and seven year-old cultures of AMF species, spores were isolated and examined (see Tables 1.1-1.3). If the number of spores/g of dry soil exceeded 15, the starting culture was defined as having a high number of spores (Table 1), if between 5 to 14, a moderate number of spores (Table 2), and if below 5, a low number of spores (Tables 3).

In addition to the AMF species listed in Tables 1.1-1.3, four cultures of unknown species of *Gigaspora* were checked for spores. No viable spores were found in these cultures.

Table 1.1 Number of arbuscular mycorrhizal fungal spores/gram of dry soil and spore viability from cultures stored for several years. Cultures contained a high number of spores.

Species from starting cultures	spores/g of dry soil	spore # tested for viability	# of viable spores	% viable spores
Glomus claroideum	51.7	10	8	80
Glomus coronatum	19.4	75	1	1.3
Glomus sp. (diaphanum?)	21.1	50	0	0
Glomus mosseae (1996)	35.9	64	46	71.9

Table 1.2 Number of arbuscular mycorrhizal fungal spores/gram of dry soil and spore viability from cultures stored for several years. Cultures contained a moderate number of spores.

Species from starting cultures	spores/g of dry soil	spore # tested for viability	# of viable spores	% viable spores
Acaulospora spinosa	9.7	75	2	2.7
*Glomus constrictum**	7.3	9	0	0
Scutellospora pellucida	5.3	20	0	0

*Due to the dark colored spores of *Glomus constrictum*, the test could not be read.

Table 1.3 Number of arbuscular mycorrhizal fungal spores/gram of dry soil and spore viability from cultures stored for several years. Cultures contained a low number of spores.

Species from starting cultures	spores/gram of dry soil	spore # tested for viability	# of viable spores	% viable spores
Acaulospora mellea	4.3	10	0	0
Acaulospora scrobiculata	0.9	4	0	0
Archaeospora gerdemannii	1.6	8	8	100
Archaeospora leptoticha	0.8	2	0	0
Entrophospora	1.1	4	1	25
Gigaspora margarita	0.3	*	*	*
Glomus mosseae (1997)	1.3	5	0	0
Glomus caledonium	0.7	3	0	0
Glomus clarum	0.8	5	0	0
Glomus intraradices	3.2	8	0	0
Paraglomus occultum	2.5	10	10	100
Scutellospora erythropa	3.2	10	0	0
Scutellospora persica	2.1	5	0	0

*Not tested due to very low spore number.

1.3.2 *Spore viability of starting cultures..* After being stored for several years, it was not known if the AMF spores were viable. The formation of colored formazan products on the reduction of tetrazolium salts has been employed as an indicator of biological reducing systems for a long time and was used to test spore viability [9]. Species were defined as having high viability if the percentage of viable spores/g of dry soil was greater than 70%. Only spores of *Glomus claroideum* and *Glomus mosseae* (1996) have high viability and a high number of spores (Table 1.1). The starting culture of *Glomus constrictum* had a moderate number of spores, but the viability test could not be read due to the dark colored spores (Table 1.2). For starting pot cultures with low number of spores, *Archaeospora gerdmannii* and *Paraglomus occultum* had spores with high viability (Table 1.3). For the other species, the viability tests were inconclusive. In summary, only seven starting cultures showed viable spores using tetrazolium staining. These seven species were *Archaeospora germannii, Acaulospora spinosa, Entrophothpora, Glomus claroideum, Glomus coronatum,* and *Glomus mosseae (1996),* and *Paraglomus occultum.*

Although only seven starting cultures showed viable spores using the staining solution, several more cultures produced viable spores in new pot cultures (see Tables 4 and5). Since tetrazolium staining had been used successfully to determine viability of AMF spores stored for a

year or less [7], the discrepancy between the staining and germination results may have been due to the inability of the staining solution to react with the reducing systems in old spores. Then too, the very limited number of spores available for the staining test could have influenced the results. If few spores are used, tetrazolium staining can provide only an indication of spore viability since a statistical significant study is required. Germination of spores in pot cultures proved to be the most reliable indicator of viability.

1.3.3 *Spore production in pot cultures.* In general, the new pot cultures in the first round produced more spores if their starting cultures had high or moderate spores/g of dry soil (see Tables 1.1 and 1.2). Cultures of nine AMF species created high spore numbers with at least 30 spores/g dry soil (Table 1.4). Four different genera (*Acaulospora, Archaeospora, Glomus, and Paraglomus)* showed high spore production with *Glomus* being the dominant genus with 6 different species. A *Glomus* species with some characteristics similar to *Glomus diaphanum* (see Chapter 2) had abundant small, white spores.

In the second round of culturing, some species showed a large increase in spores/g of dry soil (Table 1.5) when compared to Round 1 (Table 1.4). These species were *Glomus claroideum, Glomus constrictum* and *Paraglomus occultum*. However, most species produced less spores in the second round of culturing.

Most of the cultures listed in Table 1.3 produced low number of spores in both rounds, and so the data are not shown. Further testing is needed to determine if these AMF species are possible candidates for commercial production.

No viable spores were isolated from the control pots in either round. These results support the view that cross contamination of the pot cultures in the growth chambers did not occur.

Table 1.4. Species of arbuscular mycorrhizal fungi and the number of spores/gram dry soil they produced in pot culture in Round 1

Species	Spores/g dry soil
Acaulospora spinosa	30.3
Archaeospora leptoticha	37.5
Glomus caledonium	33.0
Glomus claroideum	7.5
Glomus clarum	53.0
Glomus constrictum	52.3
Glomus coronatum	3.2
Glomus sp. (diaphanum?)	301.8
Glomus intraradices	39.8
Glomus mosseae (1997)	113.9
Paraglomus occultum	67.7

Table 1.5. Species of arbuscular mycorrhizal fungi and their number of spores/gram dry soil they produced in pot culture in Round 2

Species	Spores/g dry soil
Acaulospora spinosa	13.5
Archaeospora leptoticha	3.2
Glomus caledonium	4.9
Glomus claroideum	21.0
Glomus clarum	6.0
Glomus constrictum	68.5
Glomus coronatum	8.3
Glomus sp. (diaphanum?)	307.8
Glomus intraradices	9.4
Glomus mosseae (1997)	70.8
Paraglomus occultum	204.7

Little information is available in the literature about the longevity of AMF spores stored at 4-5 C for long periods [10]. Hence, the positive results of high spore production summarized in Tables 1.4 and 1.5 provide evidence that Minnesota prairie spores of several AMF species can survive long-term storage at ca. 4-5 C. It remains to be seen if spores from warmer geographical areas have a similar ability to exist for an extended time in the cold. It has been suggested that moderate exposure to cold (ca. 5 C), as during the winter, may be required to release spores from

dormancy [11] that prevent spores from germinating in the fall and preserve them for the spring vegetative season. Despite the advances made in understanding the biology of AMF, little is known about mechanisms that permit AMF to function under stressful environmental conditions [12].

Commercially and locally produced mycorrhizal inocula may contain different types of AMF propagules besides spores [10, 3]. These propagules include root segments colonized by AMF. Spores and other AMF propagules will likely be stored at room temperature following commercial production. Experiments should be done to determine how long the spores of AMF species listed in Table 1.4 can survive at room temperature.

In summary, the eleven AMF species listed in Table 1.4 appear to be good candidates for commercial inoculum production based on their ability to produce spores in culture and to survive cold storage for long periods.

1.4 Conclusions

• Eleven AMF species are recommended as candidates for commercial inoculum production based on their ability to reproduce satisfactory number of spores in pot cultures and on their longevity in cold storage.

• The species recommended are *Acaulospora spinosa, Archaeospora leptoticha, Glomus caledonium, Glomus claroideum, Glomus clarum, Glomus constrictum, Glomus coronatum, Glomus sp. (with some characteristics similar to Glomus diaphanum), Glomus intraradices, Glomus mosseas (1997) and Paraglomus occultum.*

Chapter 2
Characterization of Arbuscular Mycorrhizal Fungal Species and Recommendation of Species for Commercial Production

2.1 Overview

The use of mycorrhizal fungal inoculants in restoration, horticulture and agriculture is increasing in popularity as it is recognized that most plants depend on mycorrhizal associations for optimal growth and development. Although plant growth benefits are advertised as the advantage of inoculants [13] and have convinced a variety of land managers of their value, the potential of introducing exotic mycorrhizal fungi into habitats in which they previously did not exist raises a central question: Can the species and isolates of mycorrhizal fungi used as an inoculum source be accurately identified after large-scale inoculum production?

The third objective of this project was to improve on a molecular protocol to identify species of AMF and to use this protocol to identify a set of AMF isolated from a Minnesota prairie [2, 3]. The final objective was to produce AMF as an inoculum source for large-scale production of AMF inoculum in Minneapolis, St. Paul and surrounding area (ca. 35 to 50 miles). The selected AMF are considered candidates for large-scale inoculum production. In order to check the identity of the fungi before and after inoculum production, genetic identification of the species and isolates used to develop the inoculum is necessary.

2.2 Materials and Methods

2.2.1 *A brief overview of the protocol.* New individual pot cultures were periodically harvested and AM fungal spores were isolated from each (see Chapter 1). From each of these pot cultures, individual AM fungal spores were selected from which DNA was extracted. This isolated DNA served as a template for subsequent polymerase chain reaction amplification, restriction fragment digests, cloning and sequencing [14]. Sequences were compared to GenBank with BLAST searches and were also used in phylogenetic analyses to determine their identity. In most cases where DNA from target AM fungus was recovered, the identification to genus was successful and in several cases species could also be recognized. Genetic information was also collected that may be useful in identifying isolates/individuals in the future.

2.2.2 *The protocol, spore selection.* A single spore was picked off the Millipore filters using a pair of reverse forceps (Ted Pella, Inc., Redding, CA) and placed in 10-20 ul of extraction solution (Sigma's RedExtract-N-Amp Kit) in a 1.5 ml microtube. Usually, three spores per isolate were collected individually with each representing some point in the range of variation in spore size and color.

The spore was crushed before entering the extraction solution in the microtube. The cracked spore was then immersed and placed into the extraction solution. Observation of the microtube under a dissecting microscope was used to verify that the spore was in the extraction solution and that it was cracked. The microtube was then closed and set aside until other samples had been collected. Between samples, forceps were cleaned with 95% ethanol and wiped dry with a Kimwipe. Under the microscope, the tips of the forceps were checked to make certain there was no debris attached from a previous spore.

Once all spores were selected, the spores were processed according to the Sigma's RedExtract-N-Amp Kit. Extractions were kept in a –20 C freezer or used immediately for PCR amplification.

2.2.3 *ITS amplification and QIA-cleansing.* Using the protocol suggested by Sigma, fungal internal transcribed spacer (ITS) regions were amplified using the primers ITS1-F and ITS4. This primer combination selectively amplifies fungal ITS regions and we found it to be effective for DNA collected from AM fungal spores. All procedures of PCR followed those normally practiced by those described in Avis et al. [15]. Success of amplification was determined by running a 2.0% SeaKem gel. Successful amplicons (= PCR products) were then cleaned using a QIAGEN QIAquick PCR clean up kit following the suggested protocol for a final elution volume of 30 ul.

2.2.4 *Cloning of the ITS.* Pawlowska and Taylor [16] showed that spores of AM fungi are multinucleated but homokaryotic such that each nucleus carries several different ITS variants. Because of this, identification by sequencing must include a method to separate and individually sequence the different ITS variants contained in the genomes of AM fungi. Therefore, the ITS amplicons were cloned using the Invitrogen TOPO cloning kit following the manufacturer's suggested protocols. In short, cloning entails the insertion of the target DNA (= insert) into a plasmid, which is then incorporated into a bacterium. The bacteria are then cultured on LB-

ampicillin plates and those carrying the insert grown. Each colony contains many individual bacteria with each carrying a single copy of only one ITS variant. These "clones" can then be selected and DNA extracted. This DNA can then serve as a template for another amplification of ITS by PCR (following the Dr. S. Gantt Lab protocol, University of Minnesota, using his free Taq polymerase). Usually, this second round of PCR produced amplicons that were roughly 600-700 bp long which is the approximate size of the ITS region in AM fungi (Note: there was usually also a very high (>>1000) bp band thought to be the residual bacterial genome from the extraction). Occasionally, much smaller (200-300 bp) or slightly larger (1000+ bp) products resulted that were not interpreted with certainty with the exception that they were not target DNA. Amplicons of the expected size were then cleaned with the QIAquick method.

2.2.5 *Cloned ITS sequencing.* Sequencing was set up followed the guidelines of the UMN Advanced Genetic Analysis Center (AGAC) sequencing facility and the samples were then taken to AGAC.

2.2.6 *Analysis of ITS sequences and phylogenetic analysis of the 5.8S.* Files of sequences were sent to Dr. Peter Avis from AGAC by email. Sequences were opened, examined and edited with Sequencher version 3.0 (GeneCoder Corp, Ann Arbor, MI, USA). Sequences were also BLAST searched for a preliminary identification. Sequences of Glomeromycotan origin were then also used in further phylogenetic analysis [17]. Only the regions of the 5.8S were compared to an alignment developed by D. Redecker and T. Bruns [18] since the ITS 1 and 2 were extremely variable. Very basic phylogenetic analyses with Fast Hueristic using PAUP version 3.0b4 (Swofford DL. 2001. PAUP. Phylogenetic Analysis Using Parsimony. Sunderland, MA, USA: Sinauer Associates) were conducted to provide a "rough" identification to species or species group.

2.2.7 *Restriction Fragment Analysis of ITS.* Sequences Fragment analysis of ITS sequences by using restriction enzymes or by virtual fragment digestion (e.g. WebCutter 3.0) can provide a pattern of DNA fragments (e.g."fingerprint") diagnostic in the identification of fungal species. Because of the ITS heterogeneity described by Pawloska and Taylor (2004), though, it is not clear how effective restriction pattern approaches can be without sequence information. Therefore, virtual restriction patterns were developed by submitting the glomeromycotan ITS sequences to WebCutter 3.0 and selecting several commonly used restriction enzymes.

Digestion by restriction enzymes was also done in a few select cases but could be conducted more broadly if necessary. For protocol, see Avis et al. [15].

2.2.8 *Utility of ITS variation for population level analysis.* Since variation in the ITS 1 and ITS 2 regions seems to be high within AM fungi, these regions may be useful in identifying relationships within species. Although much remains to be learned about the intrasporal ITS variation, population level genetic markers would be optimal in determining the pedigree of spores produced by large-scale inoculum production.

To this end, we examined three separate ITS sequences gathered from three separate spores identified as *Glomus caledonium* and compared them to ITS sequences available for the same species in GenBank. Two isolates of *Glomus caledonium* from Switzerland were found and compared.

2.3 Results and Discussion

2.3.1 *Amount and success of sampling.* In total, 66 spores from nine morphologically identified species were examined. From these spores, 32 total cloned isolates were submitted for sequencing. Of these, 13 sequences were from Glomeromycotan fungi and 12 were from non-glomeromycotan fungi (e.g. "spore contaminants"). Six did not produce reliable sequence information for adequate identification.

2.3.2 *Glomeromycotan fungi.* Species level identifications will be important when inocula will contain multiple, morphologically similar species. In this case, the set of molecular protocols used here should be an important complement to additional morphological analysis. Figure 2.1 (Fast Hueristic phylogeny) shows how the 5.8S regions of the13 Glomeromycotan sequences are related to the other Glomeromycotan fungi included in a very species limited alignment of Redecker, et al. [18]. There is general agreement between the morphological and molecular identification of the spores and their higher level taxa placement (e.g. *Glomus* with *Glomus, Acaulospora* with *Acaulospora, Paraglomus* with *Paraglomus*). However, Figure 2.1 suggests that lower level (species) taxa placement by morphological and molecular means may not be consistent where these comparisons can be made. In some cases, morphological and molecular analyses agree. For example, *Glomus coronatum* from the Minnesota prairie was morphologically identified by H. Agwa and the 5.8S region from one spore of this isolate was identical to that of another *Glomus coronatum* included in a published phylogeny [18]. The congruence between morphology and molecules suggests that *Glomus coronatum* is the proper

identity of these isolates. On the other hand, spores identified morphologically as *Glomus diaphanum* in several cases were more closely related to *Paraglomus spp.* when examined molecularly. *Glomus diaphanum* is often misidentified as *Paraglomus occultum* but it is unknown whether molecular analyses have been conducted on type specimens of *Glomus diaphanum*. Therefore, questions remain whether the tentative morphological identification by H. Agwa was correct and/or whether the taxonomy and nomenclature of *Glomus diaphanum* needs revision. The identity of the spore that was picked from Pot D9 ("*Glomus mosseae* 1996") suggests some inconsistency in identification as well. However, since *Glomus mosseae* is an easily identified species making morphological misidentification unlikely, a spore of some other species may have been selected at time of sampling. This may have been especially true when initial pot cultures were being extracted and the potential for some of the early cultures to be multi-species cultures was higher. Since the identify of *Glomus mosseae* (1996) spore is in question and since another *Glomus mosseae* culture is available, the 1996 culture is not endorsed for production.

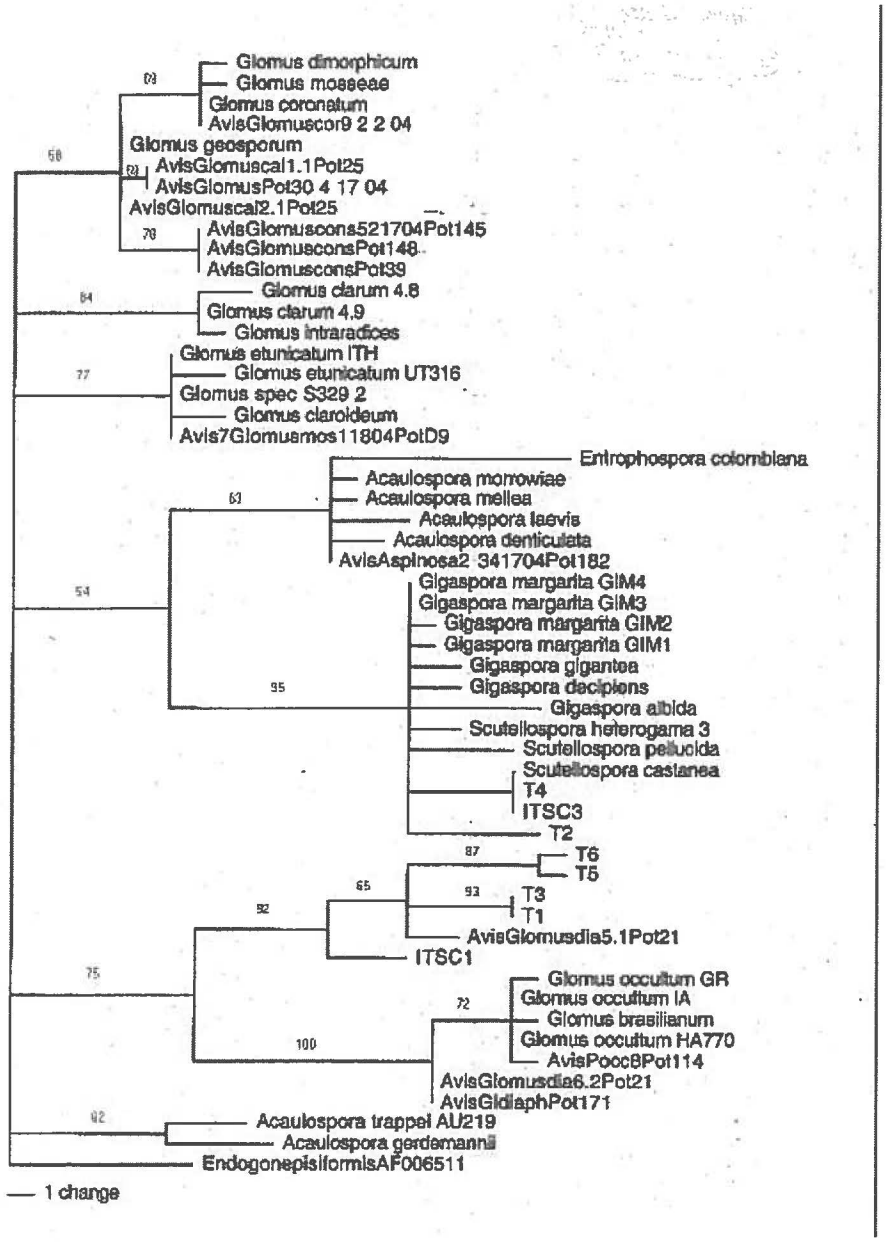

Figure 2.1. A phylogenetic tree diagram indicating how closely the AM fungal species from a Minnesota prairie (those preceded by "Avis") are related to other well known AM fungi from around the world. Numbers above the branches indicate how much statistical support there is for the groups of species on the branch. Higher numbers indicate a greater percent similarity.

2.3.3 Use of ITS 1 and ITS 2 for population discrimination. The identification of different isolates of the same AM fungus species will be important in making sure that only isolates originally from a particular location are included in the inoculum to be used in that location. Population level markers such as microsatellites have been used to examine the population structure in other fungi but the extremely variable ITS 1 and ITS 2 regions of AM fungi may also be of use.

We conducted a preliminary examination ITS variation among several individual spores from two geographically separate grasslands (one in Minnesota and one in Switzerland). Three spores from three different pot cultures identified as *Glomus caledonium* collected from the Minnesota site produced nearly identical 5.8S sequences [18]. These sequences were also very similar to the 5.8S regions of two spores isolated from the Swiss site (Swiss spore DNA information from GenBank). In comparing the ITS 1 and ITS 2 sequences of all five spores of *Glomus caledonium*, several polymorphisms were evident and the variation was large enough to segregate the isolates into their geographic groups. In fact, the level of variation observed is suggestive that these isolates could be considered different species (B. Dentinger and D. McLaughlin, University of Minnesota, personal communication). In addition, since only one set of ITS variants was recovered per spore, it is not clear whether there remain additional ITS variants that could further distinguish these isolates. Similar analyses attempted with *Glomus constrictum* (3 sequences isolated from these different spores each of a different pot) were inconclusive as other sequences of this species could not be located in GenBank or elsewhere. Nonetheless, this preliminary analysis suggests that the ITS regions may be useful in distinguishing geographically segregated isolates of the same species and/or in determining the pedigree of source and product inocula.

2.3.4 Considerations of DNA isolation and storage. For any spores considered for use in large-scale inoculum production, the extraction and preservation of DNA should be conducted. Use of the protocols outlined here should provide DNA usable for similar analyses in the future as long as they are properly stored. At the moment, storage as extractions in the Sigma solutions at –20 C appears to be reliable. However, we are unaware of research examining the longevity of viability of these extractions. Future technological innovations include using Whatman DNA

cards for dry storage and others. These may be important for consideration, when freezer space is limited. Expect that other technologies will improve the pursuit of these objectives.

2.3.5 *Species recommended for commercial production of a local inoculum.* A demand for large quantities of arbuscular mycorrhizal inocula has been created by the recent interest in mycorrhizal inoculum application at revegetation sites. However, indigenous inocula are not commercially available for specific ecological habitats or vegetation types in this area. This is especially important to restorationists and others concerned about potential problems resulting from application of non-native fungal species. Hence, they have resisted the use of non-local inocula ecotypes found in commercial inocula. As an alternative, some mycorrhizal researchers produce their own inoculum from regional AM species [13, 19].

Eleven AMF species are recommended as candidates for commercial inoculum production based on their ability to reproduce satisfactory number of spores in pot cultures, on their longevity in long-term cold storage and, for some species, on molecular traits. The selected species are *Acaulospora spinosa, Archaeospora leptoticha, Glomus caledonium, Glomus claroideum, Glomus clarum, Glomus constrictum, Glomus coronatum, Glomus sp. (with some characteristics similar to Glomus diaphanum), Glomus intraradices, Glomus mosseas (1997) and Paraglomus occultum.*

Conclusions

To check the identity of the fungi before and after inoculum production, genetic identification of AMF fungi used to develop the inoculum should be performed.

- The extraction and preservation of DNA of AMF species was done.
- DNA analysis showed general agreement between the morphological and molecular identification of the spores and their placement in genera.
- However, results suggested that some species placement might not be consistent where these comparisons can be made.
- Further research may result in the re-naming of some species.
- Use of the protocols outlined in this report should provide DNA usable for similar analyses as long as they are properly stored.

• Eleven AMF species are recommended as candidates for commercial inoculum production based on their ability to reproduce satisfactory number of spores in pot cultures, on their longevity in long-term cold storage and for some species on molecular traits.

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