Diversity and efficacy of arbuscular mycorrhizal (AM) fungi isolated from soils of soybean fields

by

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi, members of Glomeromycota, establish symbiotic associations with up to 90% of vascular plants and generally enhance plant diversity, plant growth, crop yield, and crop quality and contribute to the stability of the ecosystem. The net gains of the host plants from these associations are determined principally by characteristics of the plant and fungus entering the relationship. Thus, determination of the fungal diversity is fundamental to understanding the ecological effects of mycorrhizae.

The first study in this project was a field survey that evaluated the population composition of AM fungal species in Clarion (a well drained fine-loamy, mixed, mesic Typic Hapludoll) and Webster (a poorly drained fine-loamy, mixed, mesic Typic Endoaquoll) soils of four Iowa soybean (*Glycine max*, L.) fields.

Soil tests for available P ranged from 14 (low) to 40 (high) mg P kg^{-1} soil in Clarion soils, of fields 1 and 3, but those from fields 2 and 4 tested much higher (130 to 143 mg P kg^{-1} soil). Tests for Webster soils ranged from 38 to 69 in fields 1 and 3 and from 178 to 203 mg P kg^{-1} soil in fields 2 and 4. Average AM fungal colonization of field-collected soybean roots varied from 53 to 72% for most soil locations. Spore counts were <1 spore g^{-1} soil and significantly decreased in very high P soils.

Spores from six species of *Glomus* and from the genera *Acaulospora*, *Gigaspora*, and *Paraglomus* were found in the original field soils. *G. claroideum*, *G. etunicatum*, *G. mosseae*, *G. viscosum*, and *Paraglomus occultum*-like spores were prevalent in both Clarion and Webster soils of all four fields. Trap cultures that involved BSR201 and Iowa2052 soybean plants grown in pots inoculated with Webster soil samples from fields 1 and 4 or BSR201, Iowa2052, Mandarin, and Peking soybean grown in pots inoculated with composite samples from Clarion or Webster soils of all four fields led to detection of several additional AM fungal species. The variability in the distribution of AM fungal species was larger among the fields than within the fields. The richness of AM fungal species varied from eight species in the Webster soil of field 2 with very high available P to twelve species in Webster soil of field 3 where the available P was not as high. Spores of *G. mosseae* constituted 90% of the spore population in the very high P Webster soil of field 4. Spore morphology-based identification of four *Glomus* species, *G. claroideum*, *G. etunicatum*, *G. intraradices*, and *G.*

mosseae, plus the *Gigaspora* genus found in our soils were confirmed using polymerase chain reaction (PCR)-based rDNA fingerprinting protocols. Their presence in some roots was also verified either directly from the field samples or from pot cultures. Additional studies are needed to develop methods for the identification of AM fungal species directly in the roots of the host plants.

In the second study, the selective ability for AM fungi of four soybean cultivars, BSR201, Iowa2052, Mandarin, and Peking, was assessed. The soybean cultivars were inoculated with composite soil samples collected from Clarion and Webster soils of the four soybean fields described in the preceding study. The soybean cultivars were harvested when they reached the reproductive stage R7 rather than all at the same time. A total of 12 morphotypes of AM fungal species were identified based on spores produced in the pots where the plants were grown. The distribution and richness of AM fungal species differed among the soybean cultivars. Pots of Iowa2052 soybean cultivar harbored all 12 AM fungal species. Spores of *E. infrequens* were found uniquely in Iowa2052 pots. Ten AM fungal species were identified in pots sown to BSR201 and Mandarin. Peking had only eight different types of AM fungi. The abundance of these species depended on the cultivar. For example, *G. claroideum* produced a high proportion of the spore population in pots of BSR201 (about 16 to 75%) and Iowa2052 (16 to 50%), medium in Mandarin (15 to 25%), and low in Peking (2 to 12%) when the inocula were derived from Webster soils. The rDNA fingerprinting using working species-specific primers confirmed the identity of spores and the presence in the roots of only four of twelve species. Subsequent work is needed to identify species that are still unknown or unconfirmed and to determine the distribution of additional species. Determination of the distribution and abundance of AM fungal species inside the roots is needed to better understand the AM fungal isolates actively colonizing roots.

The efficacy of strains of *G. claroideum*, *G. etunicatum*, and *G. mosseae* was evaluated on the growth of BSR201, Iowa2052, and Peking soybean. The fungal strains differed either by the soil of origin, Clarion or Webster from fields 1 and 4, or by the cultivar used to propagate them, BSR201, Mandarin, or Peking. The fungal strains used in this study included five strains of *G. claroideum*, two strains of *G. etunicatum*, and one strain of *G. mosseae*. The inoculated 10-wk-old plants produced significantly higher shoot dry weights and seed

numbers per pot than noninoculated plants, and the increase in these growth parameters depended on both the host cultivar and the infecting AM fungal strain. Isolates of *G. claroideum* and *G. etunicatum* originally from Clarion soils typically increased shoot dry weight more than did isolates of either *G. etunicatum* or *G. mosseae* derived from Webster soil. Plants produced similar or higher shoot dry weights when grown in the presence of *G. mosseae* than they did when grown with isolates of either *G. claroideum* or *G. etunicatum* when the isolates were derived from Webster soil. Isolates of *G. claroideum* and *G. etunicatum* generated higher dry weight in Peking plants than in the plants of the other two cultivars. BSR201 plants, on the other hand, reached their highest shoot dry weight when they were inoculated with *G. claroideum* strains obtained from Webster soil and isolated on Mandarin soybean. Mycorrizal colonization of the inoculated roots was confirmed. Large spore populations greater than 50 spores g^{-1} soil were found in the pots after harvest. The spores were identical to the introduced spores for all strains of *G. claroideum* and *G. mosseae*. Pots inoculated with *G. etunicatum*, however, had about 25% contaminating spores in the pots. The spore morphologlogy of the contaminating spores was similar to that of *G. vesiforme*. The identification of AM fungi was based solely on spore morphology that does not account for the nonsporulating AM fungal species. Thus, it is crucial to use more discriminating methods to be certain that AM fungal isolates other than the ones producing spores are not found in the roots. This could be achieved through PCR amplification of a region of rDNA using universal AM fungal primers followed by sequencing.

CHAPTER 1. GENERAL INTRODUCTION

Arbuscular-mycorrhizal (AM) fungi are known for their positive contributions in preserving plant diversity, promoting plant growth, enhancing crop production, and maintaining the stability of the ecosystem. Their effects on plant nutrition have received the most attention. In fact, enhanced phosphorus (P) uptake by plants under low P fertility has been the most documented effect of AM fungi. AM fungi can make P more available, and P is considered to be one of the most limiting essential plant nutrients in the soil (Bever et al., 2001; Daniell et al., 2001). In the presence of mycorrhizae, plants acquire greater resistance to both biotic and abiotic stresses, such as protection against soil pathogens and drought (Harrier, 2001).

Members of Glomeromycota, AM fungi, are soil-inhabiting organisms characterized by dichotomous branching of their arbuscules located inside the root cortical cells of the host plants. The hyphae are mostly aseptate, and the asexual chlamydospores are coenocytic. Single spores can have anywhere from hundreds to thousands of nuclei, each with different genomes. They form mutualistic symbiotic associations with up to 90% of vascular plants (Helgason et al., 1998) and are considered to be the most important and ubiquitous symbionts on Earth, for they have been found even under adverse environmental conditions (Bever et al., 2001; Dodd et al., 1996).

The distribution of AM fungi in time and space and their effectiveness in promoting plant growth vary under the influence of a myriad of factors. These include genetic makeup of the host plants and of the associated AM fungi (Lambais and Mehdy, 1996), abiotic and biotic factors such as climate, season, vegetation, chemical and physical soil properties, soil management practices, coexisting soil organisms, and their interactions. The effects of AM fungi are mostly beneficial but can be neutral or detrimental (Johnson et al., 1997).

Traditional identification, classification, and diversity studies of AM fungi have been based mainly on the morphological description of resting spores and the characteristics of their cell walls (Cornejo et al., 2004; Dodd et al., 1996). The current taxonomic classification of AM fungi was constructed by grouping the fungal strains based on similarities and differences in their morphological characteristics. Many limitations have been associated with these techniques. Often it is difficult to achieve sufficient resolution among similar

isolates that exhibit different functions, especially in samples collected from fields harboring mixed communities. More recently, molecular techniques that complement morphological techniques and overcome associated problems have been developed for identifying AM fungi in soils, roots, and spores.

The goal of this research was to acquire an understanding of the diversity, efficacy, and ecology of AM fungi associated with soybeans in field soils of Story County in central Iowa. Three objectives were established for this research. The first objective was to examine the composition of the AM fungal community in Iowa soybean field soils. Identification and classification of AM fungal isolates indigenous to Iowa soils were based on examination of both mycorrhizal roots and AM spores collected from the mycorrhizosphere at the study sites, using a combination of morphological and molecular techniques.

The second objective of the study was to investigate whether cultivars of the same plant species will select for different AM fungal species when grown in potted soils inoculated with the same Iowa field soil containing AM fungal propagules, including chopped roots. Four soybean cultivars were inoculated with soils collected from four soybean fields in Central Iowa and grown in a greenhouse. The population composition, distribution, and relative abundance of AM fungal species were determined in pots of each cultivar.

The third objective involved assessment of the effects of individual AM fungal strains native to Iowa field soils on the growth of three genetically different soybean cultivars. Single spore cultures failed to produce sufficient inocula of AM fungal strains, so individual strains of AM fungi were mass-produced from trap cultures. Soybean cultivars grown in potted soils in the presence of AM fungi from two Iowa soybeans fields were used to propagate spore populations used in the efficacy study. Three strains of AM fungal species, *G. mosseae*, *G. claroideum*, and *G. etunicatum*, were dominant in these pots and were evaluated for their effects on plant growth.

Dissertation Organization

This dissertation began with a general introduction in Chapter 1, followed by a literature review in Chapter 2 that provided background and up-to-date information relevant to the research material in the following chapters. The literature review first discussed the

ecological significance of AM fungi related to crop production and crop quality and the factors affecting the outcome of the plant-fungus mycorrhizal association.

The history of mycorrhizal research was covered in the literature review, and the rapid dynamic change in the classification of AM fungi was highlighted along with their up-to-date classification. Information about the guidelines and strategies for classifying was compiled through consultation of material published by expert classifiers. The dissertation also outlined the mechanisms related to C and P exchange between the host and the fungus.

The literature review continued with a discussion of topics pertaining to the research in this dissertation, including the distribution, diversity, and plant-fungus relationships of AM fungi. It discussed the methodology used in mycorrhizal research and how a combination of morphology and molecular technology had contributed to the recent advances. Advantages, limitations, challenges, and the outlook to the future of mycorrhizal research were also addressed in the concluding part of the literature review.

Chapter 3 described the first research topic, dealing with a field survey, and was titled "Identification of AM Fungi in Field Soils." AM fungal species were identified from the soil samples collected from four soybean fields located in the Story County, central Iowa. The species identification process was mainly based on spore morphology as seen under a microscope, but molecular methods, mainly rDNA fingerprinting with polymerase chain reaction (PCR) protocols, were used to verify and support the morphology work. Spores were identified both directly from the field samples and from trap cultures that yielded healthier spores and allowed additional AM fungal species to be identified by inducing their sporulation. This paper is presented in accordance with the style manual of the Agronomy Journal, where the material is to be submitted for publication.

Chapter 4 was titled "AM fungal Selection by Soybean Cultivars." It presented a greenhouse study conducted in 2003 to investigate the AM fungal population composition of four genetically different cultivars of the same species (*Glycine max* L.) receiving the same field-derived soil inocula. The paper validated the hypothesis that AM fungal communities present in the roots of these cultivars could differ among the cultivars in composition and/or in relative abundance of the AM fungal species that make up these communities. This paper was also written following the style manual of the American Society of Agronomy so it could be submitted to the Agronomy Journal for publication.

Chapter 5 dealt with a paper called "Efficacy of AM Fungi Native to Iowa Field Soils on Soybean Growth." This 2004 greenhouse study investigated the efficacy of isolates of three AM fungal species on three soybean cultivars. The isolates within a species were collected from either the soils of different fields or from different soil types within the fields. The study confirmed our hypothesis that the efficacy of any two AM fungal strains could differ even if the strains belong to the same AM species.

The two latter papers described above are unique in that they dealt with field isolates. There was very little previously published work that investigated actual field isolates in terms of their preference for or selection by host plants or their efficacy for increasing plant growth.

The dissertation concluded with a conclusions chapter after the three studies. The subsequent appendices A, B, and C, contained data collected for the first, second, and third studies, respectively.

CHAPTER 2. LITERATURE REVIEW

Arbuscular mycorrhizal (AM) fungi represent a small but important group of fungi; they form symbiotic associations with most land-plants due to their broad host range, whereby both plants and fungi benefit (Lanfranco et al., 1999). That these associations are beneficial to economically important plants, serving as sources of food, feed, fiber, or shelter, is of great significance. In the last few decades, their studies have been intensified because of the accumulated evidence of their potential beneficial contributions to agriculture and the environment. They are significant plant-growth-promoting organisms, as they not only improve the nutritional status of their hosts but also protect these hosts from pathogens and allow the hosts to survive under adverse conditions. Certain plants, such as orchids, require mycorrhizal associations for their survival, as they depend on the fungus for the carbon (C) source needed for their growth during at least part of their life cycle (Johnson et al., 1997). AM fungi also improve soil structure that is important in soil-water relations.

As obligate symbionts, AM fungi always benefit from the roots of their hosts that represent their sole and ultimate source of C and energy necessary for growth (Jakobsen et al., 2002). In fact, they cannot complete their life cycle beyond spore germination and early hyphal growth in the absence of living roots of host plants (Brundrett and Juniper, 1995). It has been suggested that AM fungi may have lost some functional genes to the host plants and gained access to life-supporting energy and a protected habitat, in analogy to mitochondria and chloroplast organelles in plant cells (Barker et al., 1998). The question is, how much benefit, if any, do the host plants derive from the association? The relationship between the two partners can be mutualistic, where the plant response is positive and plant growth is stimulated in the presence of the association. Commensualism occurs when the host plant does not respond. The fungus can be parasitic to the plants if the cost of harboring the fungus exceeds the benefits drawn from the association (Johnson et al., 1997). It all depends on the balance sheet between the amount of photosynthate the plant has to provide to the fungus and the net gain in growth the plant derives from the presence of the fungus. Current research is endeavoring to discover means of improving mycorrhizal associations through understanding the diversity, ecology, and functions of AM fungi.

Ecological Significance of AM Fungi on Host Plants

This section will briefly discuss a few selected research examples, dealing with investigating functions of AM fungi on host P uptake, disease control, alleviation of heavy metal toxicity, improved soil structure, and how the types of both fungus and plant entering partnership may affect the outcome of the association. The conclusions from these studies will be highlighted.

Improved P Nutrition

Plants require P for their growth and development, as it is a component of nucleic acids, phospholipids, phosphoproteins, certain enzymes, and adenosine triphosphate, the energy currency that drives most metabolic activities in biological systems. Plants have much lower concentration of P, varying from 0.1 to 0.5%, than of N and K (Havlin et al., 1999); therefore, their demand for P is also lower. However, the concentration of P in the soil solution is often too low for optimum plant growth because soil P is relatively immobile and has very low solubility (Paul and Clark, 1989), as it forms complexes with Al, Fe, or Ca, depending on the soil pH. Thus, increased P availability for plants, especially those grown in soil low in labile P, has been considered the most important contribution of AM fungi to enhanced plant growth and health (Bever et al., 2001).

AM fungal hyphae extend from the roots of the host plants farther into the soil than the root hairs, thereby drawing P from a larger volume of soil (Koide and Kabir, 2000). Also, AM fungi, other soil rhizosphere organisms, and plant roots function to release more P and make it available for plant uptake. Predominant plant responses to mycorrhizal colonization are increased yield and improved crop quality due predominantly to improved P nutrition of the host plants. Even in arable lands, where heavy fertilization is applied and availability of P is high, crops with large root systems still depend on AM for absorption of sufficient P and protection against soil-borne diseases (Daniell et al., 2001). Increased availability of P has the potential to increase plant growth, to alleviate heavy metal toxicities, and to minimize diseases effects on the host plants. An experiment by Smith et al. (1994) used leek plants (*Allium porrum* L. cv. Mussel Burgh) inoculated (or not) with *Glomus* (*G.*) *mosseae* and

Glomus sp. and grown in pots in a controlled glasshouse environment. The findings of this study indicated the expression of normal symbiosis, in which both higher dry weight and greater P concentration were observed in mycorrhizal plants compared to their contemporary control plants. Inflow of P to the root cortical cells was generally higher in mycorrhizal plants than in control plants.

Diminished Disease Impact on AM Plants

Experiments under controlled environments indicated that AM fungi exert a protective effect for their hosts against potential pathogens, perhaps by competition for nutrients with potential plant pathogens or by stimulation of plant defenses, possibly through systemic acquired resistance or improved plant nutrition. It has been reported that the roots of sweet orange seedlings grown in the presence of *G. fasciculatum* and *Phytophtora parasitica* (a root-rot pathogen) were healthier and had higher dry weights than the roots of seedlings grown in the presence of the root-rot pathogen alone (Sylvia and Williams, 1992). A significant reduction in incidence of insects was recorded in both susceptible and resistant soybean cultivars in the presence AM fungi (Pacovsky et al., 1985).

Results from a field experiment, however, showed no effects of native AM fungi on the pathogenicity of *Aphanomyces euteiches*, the root-rot pathogen, and on the growth of pea (*Pisum sativum* L.). These negative results may have been caused in part by a low population of indigenous AM fungi in these soils (Bødker et al., 2002). In this study, the pathogen activity was evaluated by measuring the activity of glucose 6-P phosphatase enzyme in its mycelia.

Tolerance to Metal Toxicity in AM Plants

Heavy metal contamination of soils occurs either naturally or anthropogenically, and represents a serious threat to food and health safety. Mycorrhizal fungi alleviate heavy metal toxicity in host plants. Plants grown in moderately contaminated soils acquire greater tolerance to metal toxicity when their roots form mycorrhizal associations, and concentrations of Zn, Cu, and Cd were reduced in their shoots (Christie et al., 2004). For

better management of moderately contaminated soils or reclamation of spoiled soils, it is important to understand the mechanism by which mycorrhizae operate in increasing tolerance of the plants to heavy metal toxicity. Two mechanisms were hypothesized to alleviate heavy metal toxicity of soils: increased plant uptake of P and sequestration of heavy metals in fungal structures. Improved plant nutrition, especially P, can increase plant vigor and allow plants to grow better in contaminated soil. The concentration of metals in shoots can be reduced through the dilution effect of increased shoot biomass. Increased P uptake in mycorrhizal plants increases the concentration of P in plant tissues and that may minimize the toxic effect of heavy metals, especially Zn. The second mechanism involves the retention of heavy metals in fungal hyphae in roots and in the mycorrhizosphere and the restrictive transport of these metals to the shoots (Christie et al., 2004). Root tissue analysis indicated that mycorrhizal roots contained proteins rich in sulfhydryl (SH) ligands that form complexes with metals causing the metals to be deposited in fungal tissue, and consequently not transferred to the shoots (Dehn and Schüepp, 1989).

Mycorrhizal Effect Depends on the Fungus-Plant Combination

The following study demonstrated that different AM fungal species had differential effects on different plant species. Plant growth, shoot P concentration, and root colonization were evaluated in two plant species colonized or not by several AM fungal species. The study showed that the degree to which each of these species was affected by mycorrhizal colonization varied with the host and the colonizing AM species (Burleigh et al., 2002). The P-shoot content of *M. truncatula* was elevated by *G. caledonium* and *G. claroideum*, but that of *L. esculentum* was unaffected. On the other hand, colonization of *Gigaspora (Gi.) rosea* did not increase P shoot content of either host species. In addition, the shoots of *M. truncatula* contained the highest P concentration in the presence of *G. versiforme, G. caledonium*, or *Scutellospora (S.) calospora*, but had lowest P concentration when colonized by *Gi. rosea*. The outcome of this study also supported previous reports that high AM colonization does not necessarily mean that the host plants gained much from the association, as *G. claroidium* and *G. intraradices* had a high degree of colonization but did not translocate higher amounts of P than did other species with lower levels of colonization. This

work also showed that effective translocation of P to the plants should be balanced with a sufficient supply of other nutrients for optimum benefit to the plants. An accumulation of P in the shoots accompanied by a reduction in potential growth suggests that other factors must limit the use of P in plant tissue. Plants may lose too much C to the fungus and be unable to incorporate all of the P in biosynthetic processes because C (not P) is limiting. Plant diversity and plant productivity were positively correlated in a linear relationship in the absence of AM fungi, but the effects of *G. etunicatum* and *G. intraradices* modified significantly the positive correlation between these two variables from linearity to an asymptotic mode. In the presence of *G. intraradices*, the plant biomass reached a very low maximum only after increasing the plant diversity to six types of plants, while *G. etunicatum* allowed the plants to produce a higher maximum biomass when the numbers of different plant species reached ten or more (Klironomos et al., 2000).

Improved Soil Structure

Soil structure is an important physical component of the soil for it affects soil aeration, water infiltration rate in the soil, and resistance to erosion. AM fungi contribute to improvement of soil structure by at least two means. Mycorrhizal hyphae entrap soil particles, creating aggregates that are more resistant to erosion. Extraradical hyphae of AM fungi produce a significant amount of a glycoprotein "glomalin" that increases the stability of the aggregates in soil (Wright et al., 1999). In the presence of AM fungi, more C flow is directed to the roots, increasing root exudates and consequently the bacterial population in the rhizosphere. Also, these bacteria can produce mucigel that can improve soil structure.

Historical Review of Mycorrhizal Research

Fossil records show that AM fungi have co-existed with plants for at least 400 million years, coinciding with the early stages of plant appearance on Earth (Harrison, 1999). Remy et al. (1994) confirmed the existence of AM fungi on *Aglaophyton*, an ancient plant that existed during the early Devonian era and possessed characteristics of both vascular plants and bryophytes. The discovery that the Rhynie chert contains arbuscules, vesicles, and

aseptate hyphal structures similar to those characterizing today's AM fungi provided evidence that they were associated with specialized structures (not true roots) of *Aglaophyton* (Remy et al., 1994). Species producing spores in sporocarps were identified in those fossils, indicating the likelihood that *Glomus* and *Sclerosystis* are closely related to an ancestor of Glomales (Morton and Benny, 1990).

A German plant pathologist, A. B. Frank, was assigned by his government to study the culture of truffles (Paul and Clark, 1989). Instead, he discovered the mutualistic symbiotic relationship between trees and certain fungi he called "mycorrhiza" in 1885 (Harley, 1985). Two years later, he found out that symbioses existed also between nonwoody plants and fungi and he called them "endomycorrhizae." His ideas, however, drew a wave of unfounded and biased criticism from his contemporary mycologists. It was almost a century later that interest in mycorrhizal fungi was rejuvenated in the 1970's, especially when Linnean classification began to be used to classify Glomales in 1974 (Bago et al., 1998).

Mycorrhizal Research in the 19th Century

Mycorrhizal research goes back at least to the early nineteenth century when several outstanding scientific pioneers made valuable contributions in this field, especially A. B. Frank and F. Kamineski (Trappe and Berch, 1985). Rayner (1927) in his monograph cited three important historical periods in mycorrhizal research (1840-1880, 1880-1900, and 1900-1925). A summary of important revelations in mycorrhizal research during each of these periods is provided in the following pages. More details of these periods are available elsewhere (Rayner, 1927; Troeh, 1999).

The highlights of the period dating from 1840 to 1880 were the discovery of massive fungal hyphae covering the feeder roots of trees that turned out to be important in translocation of nutrients and water to trees through ectomycorrhizal associations. A great deal of research was undertaken to resolve the controversy surrounding their nature, and attempts to determine their function and ecology in relation to the plant roots they invade (Trappe and Berch, 1985). Some observers believed hyphae to be arising from non-living things (the concept of spontaneous generation), while others described them simply as "hairy structures" or "thread-like structures." After decades of examination, Theodor Hartig, a

German pathologist, was first to publish, in 1852, a more detailed description of massive hyphae (mantle) masking the feeder roots and the mycelia in the intercellular spaces of the cortical cells (Hartig net) of conifer roots (Harley, 1985). He was unaware of their nature, although Elias Fries (1829), a Swedish mycologist, had already recognized that they were actually fungal structures (Trappe and Berch, 1985). A step forward in mycorrhizal research occurred in 1841 when Edwin Lees suggested that the hairy structures absorbed nutrients from soil and translocated them to the host, but like T. Hartig, he did not discover the fungal nature of the structures of the endophyte. Then, Carlo Vittadini (1842) believed that plants derive nutrients from the endophyte he identified as a fungus. He, however, did not realize that the fungus mutually obtained nourishment from the host (Trappe and Berch, 1985).

Drawings of structures in iris roots made by Karl Nägeli in 1842 fit the description of the arbuscules of AM fungi. Some of his drawings showed states of digestion of arbuscules in plants, so he may have been the first mycologist to actually provide these descriptions. Later, in a follow up of Nägeli's work, Herman Schacht in 1854 rejected spontaneous generation as the means of mycorrhizal endophyte growth inside the host cells, and he also ruled out the existence of endophytes in the vascular system of the host (Trappe and Berch, 1985). In 1874, Hellmuth Bruchmann discovered a dichotomous branching pattern, an important characteristic of mycorrhizal roots. A year later, H. G. Holle discovered that AM fungi are more likely to be present in young roots than in older roots (Trappe and Berch, 1985). Historical records indicated that mutualism between the fungi and roots was probably suggested by Pfeffer in 1877, prior to A. B. Frank, but his idea was not taken seriously because of weak evidence, as cited by Trappe and Berch (1985).

The second period (1880 to 1900) was marked by the work and contributions of F. Kamienski, R. Hartig, and A. B. Frank. Kamienski, a Polish mycologist, published several reviews in 1881 and 1882 (Trappe and Berch, 1985). He, through logical reasoning, explained that nutrients and soluble organic compounds absorbed by plants must pass through fungal hyphae because hyphae completely covered the roots and restricted any direct physical access to the soil. In a paper published in 1894, Frank explained that the fungus acquired more complex N compounds from humus and supplied them to the host, supporting Kamienski's earlier suggestion (Harley, 1985). He also drew an analogy between

mycorrhizae and lichens where symbiosis involves photosynthetic and non-photosynthetic organisms to emphasize the idea of mutualism in mycorrhizae.

Proof of widespread occurrence of mycorrhizae came when A. B. Frank discovered mycorrhizae in non-woody plants that had been presumed to be AM fungal free because their mantles were difficult to detect. They had few hyphae growing into the soil and into the root cortical cells. Frank's view that mycorrhizal fungi were required for the survival of beech trees was strongly opposed by Robert Hartig. R. Hartig was a respected plant pathologist credited for bringing to life in 1886 the work of his father, T. Hartig. Many of his contemporary scientists joined him in dissmissing Frank's idea of mycorrhizal mutualism between trees and the fungal structures T. Hartig had drawn (Harley, 1985).

The work of both Schlicht, a student of Frank, in 1889, and of Janse in 1896 indicated the common occurrence of mycorrhizae in many angiosperms in the tropics. Janse was the first to use "vesicles" to describe the oil-filled thin-walled spheres inside the roots, and Schlicht described the dynamics of infection as the fungus departs from the soil to colonize the roots and noted the scarcity of septae in mycorrhizal hyphae (Mosse, 1985). He also located the site of the mycorrhizal infection in the cortical area of small roots, especially its interior layer, with the main roots not being infected. His observations also indicated lack of colonization in the roots of water plants and in the roots of some plants exposed to temporary flooding. Reluctance in accepting the beneficial contribution of mycorrhizae may have had a somewhat negative impact on mycorrhizal research at the time.

Stahl (1900 as cited by Harley, 1985) made an important contribution by tackling the issue of competition between mycorrhizal fungi and soil fungi and stating that mycorrhizal fungi functioned not only in taking up nitrogenous substances but other ions from the soil as well and translocating them to the host. That was a significant assertion, considering that Justus von Liebig had already established that N, P, and K are essential to plant growth (McNeill and Winiwarter, 2004). Moreover, Stahl attempted to come with a different perspective of how mycorrhizae might support plants. Based on a then well-known theory, stating that nutrient uptake by plants was dependent on the transpiration abilities of the plants (Harley, 1985), Stahl in 1900 reported that plants with more restricted transpiration, due to thick cuticles and to lack of hydathrodes, benefited more from mycorrhizal association than

plants that possessed anatomical features more favorable for high transpiration (Mosse, 1985).

Mycorrhizal Research in the 20th Century

Rayner (1927) considered the time interval of 1900-1925 the modern period of mycorrhizal research. The widespread occurrence of mycorrhizae in nature and major accomplishments in anatomical descriptions of mycorrhizal structures had been established earlier. During this era, mycologists and plant pathologists focused more on descriptive and experimental approaches for expanding knowledge about the composition of mycorrhizae, the ecology of their association with different plant families and species, and especially the effects of mycorrhizae on plant nutrition. In 1900, Dangeard published the first accurate illustration that showed arbuscules and vesicles of AM fungal structures in poplar roots and discovered the multinucleate nature of hyphae. His contemporary, Hasselman, discovered AM associations in plants in the arctic region, which was not expected considering the harsh environmental conditions. Following that, Gallaud, a French pathologist, provided a detailed description of arbuscules as tree-shaped structures in the intercellular spaces at the tips of hyphae and called them "arbuscules" in 1905 (Mosse, 1985).

The difficulty of explaining the reasons for inconsistencies in occurrence in plant responses and the short life span of mycorrhizal roots contributed to the dilemma encountered by scientists advocating mycorrhizae and played in favor of skeptics for decades (Harley, 1985). Also, the challenges of isolating mycorrhizal fungi for more reliable studies led to unconvincing conclusions. Many studies set up to isolate mycorrhizal material ended up supporting the growth of fungi with septate hyphae, leading Peyronel (1923) to suggest the possibility of more than one type of organism existing in the culture (Mosse, 1985).

B. Peyronel started in 1917 to formulate hypotheses about the existence of different mycorrizal species within the roots of a single host and that different hosts may harbor the same mycorrhizal species. He was the first to place AM fungi in the endogonales (Trappe and Schenk, 1982) and to realize that differences in fungal structures, color, and patterns of the sheath and of the Hartig net could be used to determine the type of mycorrhizal fungi involved (Harley, 1985).

E. Melin (1927) and Harley (1937) used this principle and began classifying mycorrhizae (according to Harley, 1985). Many studies were conducted to shed light on what caused conflicting outcomes from mycorrhizal research. A. B. Hatch in 1937 reported a positive correlation between the degree of mycorrhizal infection and N mobilization in a study conducted on trees grown in humic soils. However, he found that in poor soils mycorrhizae resulted in both high root colonization and in increased growth of the host, but in rich soils the increased colonization intensity was not accompanied by an increase in the host growth. The increase was associated with insufficient nutrient availability in woodlands (Harley, 1985). Other inquiries included examination of the effect of light intensity on mycorrhizae, delineation of the developmental stages of plant infection, investigation of the requirements and limitations for infection, and identification of structures involved in infection. McComb (1943) discovered that spores are infective (Harley, 1985). Then came spore germination studies that yielded valuable results such as that some fungi might be obligate symbionts with the possibility of the host being their sole provider for C and growth factors (Harley, 1985). Some even suggested that plants might also be obligate biotrophs, especially in nutrient-deficient soil. Investigators in the field started to develop curiosity about mechanisms by which mycorrhizae increased host growth. Burges (1939) suggested that the fungus might make more nutrients available to the host through releasing them from otherwise insoluble compounds, but Mitchell et al. (1937) and Stone (1950) failed to detect any change in the solubility of N, P, or K in the mycorrhizal roots in their experiments.

The belief that mycorrhizal fungi were pathogenic persisted even after World War I, as W. B. McDougall (1914-1928) and Lange (1934) agreed with R. Hartig. They insisted that humus only provided nourishment to the fungi and made injured tree roots more susceptible to the pathogenic fungi, and they denied the existence of mycorrhizae (Harley, 1985). Some even believed that mycorrhizae might result in causing root rot of the host either directly or by supporting other causative agents. In spite of all that, mycorrhizal enthusiasts continued to defend their point of view and continued to develop more reliable techniques for isolation, culture, and identification of mycorrhizal fungi, and to design better and more productive experiments.

The improvement of cultural techniques seemed to be a necessity, so that was the focus of mycorrhizal research from the 1940's to the 1970's (Mosse, 1985). In the 1940's,

comparative studies of mycorrhizal with nonmycorrhizal plants under manure treatments were dominant, but resulted in drawing doubtful conclusions because the growth medium was always natural soils, which of course harbored a lot of other organisms. Magrou, a Frenchman, obtained in 1946 some early growth of hyphae using root fragments as the starting material (Mosse, 1985). M. Stahl (1949) infected seedling roots with isolated thalli and grew them in sterilized sand and reported to have obtained a 3- to 4-cm growth of hyphae. Barbara Mosse made a valuable contribution by culturing spores from a single fungal species in pots (Schenk, 1985). Subsquent to her achievement, Gerdemann in 1955 established pure cultures of several AM species, and in the process figured out how to use the wet sieving method, previously used for nematode collection, to isolate spores from soil. Many techniques have been provided for spore extraction, but probably the most used is based on density differentials obtained by using solutions with different concentrations of sucrose. Other progress in methodology included the development of clearing and staining procedures for evaluation of root mycorrhizal status that have been made safer. The line intercept method was improved to give a more accurate estimate of percentage of colonization.

The first nutritional benefit of AM fungi for their host to be highly emphasized was enhanced P uptake (Gerdemann, 1964; Mosse, 1973). As time progressed, the research expanded to cover more areas. Studies of the effects of fertilization, manure application, fumigation, cultivation, climate, and topography on the function and distribution of mycorrhizae, just to name a few topics, were investigated mostly based on comparative studies. Research also was directed toward evaluating their effects on plant growth, disease control, alleviation of metal toxicity, and balanced nutrition.

Interest in mycorrhizal research has grown over the last few decades, as indicated by the increased number of publications of books, reviews, and articles and the organization of world conferences. That allowed scientists around the world to engage in vigorous and fruitful discussions, and to share their findings and views about mycorrhizal symbiosis that has a great potential in improving plant production in a safer environment. Taxonomic studies have gained their share in the research spotlight. These have been evolving with improved methods of identification that provide increasingly greater magnifying powers, and

more resolution. The taxonomy along with the importance of the adaptation and use of molecular techniques will be discussed in separate sections of this review.

Classification of AM Fungi and Recent Revisions

Development of Classification of AM Fungi

The first *Glomus* species to be characterized were those producing asexual spores in sporocarps. In the 19th century, based on superficial morphological similarities between sporocarps in *Glomus* species and the sexual spores produced in zygosprangia in *Endogone* species, AM fungi were combined with *Endogone* spp. in Endogonaceae, a single family of Zygomycota (Schüßler et al., 2001). In 1922, Thaxter removed species of the genus *Sclerocystis* from *Endogone* while species of *Glomus* remained included in Endogone (Walker and Schüßler, 2002). The order Endogonales with the family Endogonaceae was suggested to contain three genera, *Endogone, Glaziella,* and *Sclerocystis* (Moreau, 1953 as cited by Almeida and Schenck, 1990). Several species were removed from *Endogone* and assembled in *Glomus* in 1974. Two additional genera, *Acaulospora* and *Gigaspora* were defined, and *Entrophospora* was described five year later (Walker and Schüßler, 2002). Morphological reassessment of the *Gigaspora* group led to assembling selected members of this genus to form a new genus, *Scutellospora*, in 1986.

Extensive morphological, developmental, and germinal studies of AM fungal spores showed fundamental differences between AM fungi and other groups of Zygomycetes, so all AM fungi formerly of Endogonales were placed in Glomales, a new order of Zygomycetes (Morton and Benny, 1990). All members of Glomales are obligate symbionts that form arbuscules and reproduce via intracellular and intercellular hyphae and spores. Morton and Benny further subdivided the Glomales in two suborders Glominae and Gigasporinae. Glominae, the vesicle-forming AM fungi, encompasses two families, Glomaceae and Acaulosporaceae, and are called vesicular-arbuscula mycorrhizal (VAM) fungi (Bever et al., 2001; Daniell et al., 2001; Douds and Millner, 1999). Two genera, *Glomus* and *Sclerocystis*, were grouped in Glomaceae because they produce spores singly, in aggregates, or enclosed in sporocarps while two closely related genera, *Acaulospora* and *Entrophospora*,

characterized by forming a sporiferous saccule, belong to Acaulosporaceae (Morton and Benny, 1990). Gigasporinae has one single family Gigasporaceae whose members produce auxiliary cells instead of vesicles, and it represents the two remaining genera, *Gigaspora* and *Scutellospora* (Douds and Millner, 1999). Similarities between species of these two genera are so strong that their separation was questioned (Morton and Benny, 1990).

Only about 150 species are identified in the six genera of AM fungi despite their ancient association with plants (Bago et al., 1998). There are probably many other AM fungal species that are still unidentified. In fact, the International Culture Collection of Arbuscular and Vesicular Arbuscular Mycorrhizal Fungi (INVAM) possesses about 40 isolates that probably represent several new AM species that do not fit the profiles of any of the identified species (Bever et al., 2001).

Recent Classification of AM Fungi

After examining phylogenetically the sequences of the small subunit (SSU) rRNA gene of AM fungi and of their close-relative, *Geosifon pyriformis*, Schüßler and his colleagues (2001) grouped AM fungi into a new monophyletic phylum "Glomeromycota" and removed them from the polyphyletic phylum "Zygomycota."

Controversy has surrounded the maintenance of the genus *Sclerocystis* that comprised only a single species, *S. coremioides*, characterized by spores organized in a sporocarpic structure with a central plexus that bears resemblance to the species of *Glomus* (Douds and Millner, 1999). Other species of *Sclerocystis* had already been transferred to *Glomus* as a result of observations made on spore and sporocarp development (Almeida and Schenk, 1990). Recently, *S. coremioides* has been placed in the polyphyletic group, *Glomus*, based on data generated from the rRNA gene sequencing analysis (Redecker et al., 2000b). It is interesting to mention that *S. coremioides* was the first species to be described in *Sclerocystis* (Almeida and Schenk, 1990), and the last one to terminate this genus.

Sequence analysis of the 18S RNA gene suggested that two groups of AM fungal species, formerly placed in two different genera *Glomus* and *Acaulospora*, had diverged from AM ancestors much earlier than had either genus to which they were previously assigned. *A. gerdemannii*, *G. gerdemannii*, and *A. trappei* are closely related so they form one group, and

G. occultum and *G. brasilianum* showed an intimate phylogenetic relationship, and they made a separate group (Redecker et al., 2000a). The two groups were placed in two additional proposed families, Archaeosporaceae and Paraglomaceae, each with a single genus *Archaeospora* and *Paraglomus*, respectively (Morton and Redecker, 2001). One group (*A. gerdemannii*, *G. gerdemannii*, and *A. trappei*) was transferred to *Archaeospora*, and the other group (*G. occultum* and *G. brasilianum*), formerly of *Glomus*, became members of *Paraglomus*. All these changes resulted in today's recent classification (See Fig. 2.1).

Selected Criteria for Identification of AM Fungi

Identification and classification of AM fungi were derived from the similarities and differences in morphological characteristics of AM fungal propagules collected from the soil mycorrhizosphere and mycorrhizal roots. This involves a detailed description of the hyphae, spores, subtending hyphae, spore cell walls, vesicles, and auxiliary cells (Manoharachary et al., 2002). There are two types of hyphae, intraradical hyphae that grow inside the mycorrhizal roots, and extraradical hyphae that extend from the mycorrhizal roots into the mycorrhizosphere. Functionally, extraradical hyphae can be infective, absorptive, or runner hyphae.

At the species level, morphological characteristics of the spores, including color, shape, and size, and their wall structures are used to classify AM fungal stains (Douds and Millner,
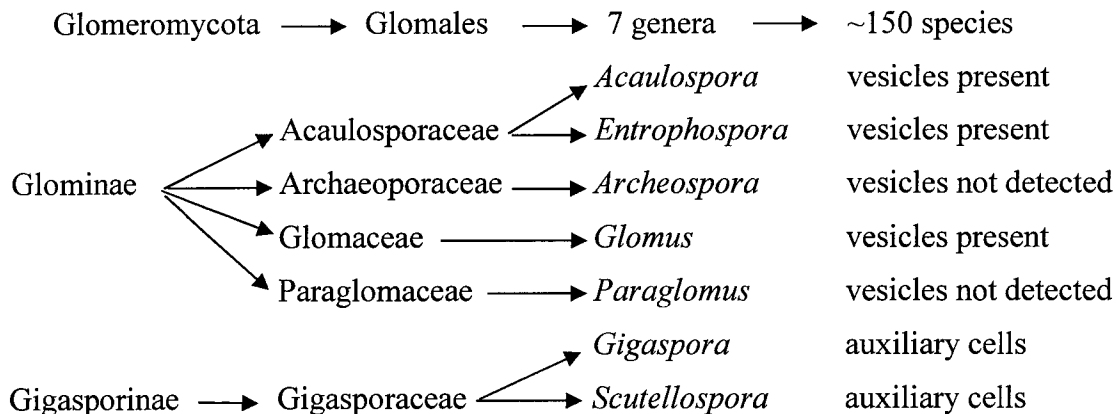
Fig. 2.1. Classification of Glomeromycota (Morton and Redecker, 2001).

1999). The spore cell walls are examined in terms of the number of layers they comprise, flexibility, ornamentation, reactivity to special stains, etc. Keys were previously published, but the World Wide Web site: http://invam.caf.wvu.edu represents a reliable and easily accessible site and was founded by J. Morton and S. Bentivenga of the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) located at the University of West Virginia. Descriptions of individual species, including spore and spore wall characteristics, illustrations, and significant features that are unique to each species are posted and are frequently updated on the site to aid investigators in identifying their own AM fungal isolates.

As mentioned earlier, AM fungi are currently grouped into five families (Manoharachary et al., 2002). Each family and its genera will be briefly described.

Glomaceae

The reaction of arbuscules to stains, trypan blue, acid fuschein or chloral black E, is generally strong but can be very faint. As arbuscules emerge from main hyphae, they thin out as they grow farther from the mother hyphae (Morton and Benny, 1990). Vesicles located at the hyphal tips are likely to be found between rather than inside the cortical cells, and lack of pigmentation requires that they be stained for observation. Hyphae are variable in thickness with more coils near the entries to roots. Connections in the form of "H" between long, straight, parallel, infective hyphae are common (Manoharachary et al., 2002). Subtending hyphae attached to spores are flared to cylindrical. This family has only one genus remaining since the elimination of *Sclerocystis* and placement of its species in the *Glomus* genus. Vesicles inside the roots sometimes develop into spores (Morton and Benny, 1990).

Glomus represents the largest genus of AM fungi, comprising approximately one-half of the AM species identified. *Glomus* spores form at the tips of cylindrical to flared subtending hyphae (Morton and Benny, 1990). Spores can be found in the soil singly or in aggregates, and a few species, including those inherited from *Sclerocystis*, form intraradical spores. Spores enclosed in sporocarps or systematically organized around a central plexus are unique to *Glomus*. The shape of the spores varies from globose to subglobose to oblong with sizes ranging from 40 to 300 μm, rarely exceeding 200 μm (Morton and Benny, 1990). Their thick

walls, hyaline to brown to black, consist of 2 to 4 layers, with different thickness, rare wall flexibility, and variable intensity reactions to stains, such as polyvinyl-lacto-glycerol (PVLG) and PVLG-Melzer mixture. A septum with a pore may form between mature spores and their attached hyphae or an occasional occlusion derived from cell wall thickening may isolate the contents of the spores from those of their stalk. Sporocarps of the former *Sclerocystis*, enclosed in peridiums up to 700 μm in diam., contain spores that are more organized and are attached to a central plexus. High variation in the cell wall structures is considered a major factor in high speciation in Glomaceae (Morton and Benny, 1990).

Acaulosporaceae

Fungal strains belonging to Acaulosporaceae have knobby short infective hyphal strands with vesicles and arbuscules that stain lightly with trypan blue. Subtending hyphae are either absent or "rudimentary" in *Acaulospora*. Swollen subtending hyphae are present in most members of the *Entrophospora* species. *Entrophospora* spores lacking subtending hyphae bear two scars instead of one, whereas spores of the *Acaulospora* species have only one scar (Manoharachary et al., 2002). In both cases, the saccules lose their cytoplasmic content and become more hyaline as the spores mature. Spores attached to saccules mostly occur singly but occasionally form aggregates. Variation in spores is due mostly to types and numbers of flexible layers that develop in their cell walls (Morton and Benny, 1990).

Acaulospora

Spores of the *Acaulospora* species develop laterally on the subtending hyphae of small thin-walled soporiferous saccules that form at hyphal tips and collapse after their contents migrate to their daughter spores (Morton and Benny, 1990). Mature spores become sessile but follow a developmental pattern similar to *Glomus*. Spores, 40-400 μm in diam., are of globose to ellipsoid shapes, hyaline to yellow to reddish brown colors (Manoharachary et al., 2002). The surfaces of the spores may show ornamental structures, such as spines, warts (projections) of different shapes, folds, or reticulations.

Entrophospora

Spores are borne within the subtending hyphae of the soporiferous saccules that provide energy and transfer their contents to the future spores that form from the saccule's neck, and hyphae mostly remain attached to the spores (Manoharachary et al., 2002). The main distinction between *Entrophospora* and *Acaulospora* is the site of the spore as it grows from the neck of the saccule (Ames and Schneider, 1979).

Gigasporaceae

Large spores 300 µm or greater in diam. are borne singly in the soil and are characterized by the bulbous structure that forms on the spore at the base of subtending hyphae. Flexible inner walls form endospores, and the type and number of spore inner walls determine diversity in this group (Morton and Benny, 1990). Germinating spores produce one or more germ tubes. Hyphae are mostly coiled with irregular swelling and lateral knobs that stain strongly in trypan blue. Auxiliary cells, knobby, thin cell-walled spheres, can be present in clusters on coils attached to very fine hyphae in the soil (Manoharachary et al., 2002).

Gigaspora

Large spores varying in shape (globose, subglobose, to irregular) develop on hyphal tips that develop into sporogenous cells (Gerdemann and Trappe, 1974, as cited in Morton and Benny 1990). Only a few species are placed in this genus, and the distinction among their spores is difficult (Morton and Benny, 1990). Auxiliary cells are finely papillate or spiny.

Scutellospora

Scutellospora species form spores that are most often found singly in soil with a wide range in size (55-600 µm in diam.). Spores vary in shape (globose, ovoid, to irregular), and color (hyaline to white to yellow to pink to brown). Some layers of the cell walls may be flexible. Auxiliary cells are broadly papillate or smooth.

Archaeosporaceae and Paraglomaceae

Members of these two families are morphologically indistinguishable from species of their former genera, *Glomus* or *Acaulospora*, but sequencing analyses of 18S rRNA genes indicated not only that they diverged from other AM fungi much earlier than either Glomaceae or Acaulosporaceae (Morton and Redecker, 2001), but also that the two families were distantly related. However, taxa in these two families may be distinguished from other AM fungi at the molecular level by detection of their signature molecules, C16: ω7 cis fatty acid and by an 18S rRNA gene sequence, TGCTAAATAGCCAGGCTGY. Like other AM fungi, *Archaeospora* and *Paraglomus* produce arbuscules and coiled intraradical hyphae, but vesicles have not been detected in either culture or field isolates (Morton and Redecker, 2001). Fungal hyphae inside the roots are difficult to detect using common stains, and although they produce large number of spores, their percentage root colonization rarely exceeds 10%.

Archaeospora

Species that were formerly placed in *Acaulospora* and *Glomus* make up the genus *Archaeospora*. One of the distinguishing features is that species of *Archaeospora* are either monomorphic, with only Acaulosporoid spores, or dimorphic, producing both acaulosproid and glumoid spores, as spores have sufficient morphological characteristics to be classified either in *Acaulospora* or in *Glomus*, respectively. At the moment, *Archaeospora* comprises three species, *Archaeospora (Ar.) trappei*, *Ar. liptoticha*, and *Ar. gerdemannii*. Spores of *Ar. trappei*, are mostly hyaline when young and creamy white at maturity. The 40- to 80-μm diam. spores float in water. Slightly crushed mounted spores show wrinkles in layers of their flexible spore walls. It may be difficult or next to impossible to distinguish these taxa from species of *Paraglomus*, especially in field isolates when mixed communities are involved and spores are not intact. First of all, spores of *Ar. trappei* bear strong morphological resemblance to those of *Paraglomus*, as they have similar color and fall within a close size range (Morton and Redecker, 2001). The soporiferous saccule is hyaline and smaller than the

spores and it positions itself under the floating spores, so it is hard to see. Furthermore, the hyphae connecting the saccule to the spore can be mistaken for a subtending hypha. These are among the reasons why *Ar. trappei* can easily be misidentified as *Paraglomus* (*P.*) *occultum*, or *P. brasilianum*. Identification becomes less challenging when *Ar. trappei* spores are mature and become sessile when the saccules fall off. The taxa *Ar. gerdemannii* is dimorphic producing both glomoid spores and acaulosporoid spores. For acaulosporoid spores occasionally attached to large flaky sacccules (220-300 μm in diam.), the color depends on the number of layers present in their cell walls. Four-layer spores tend to be a darker color ranging from cream to dark orange brown and 160 to 260 μm in diam., while spores losing the two outer layers are bright, shiny white, and generally smaller. Glomoid spores form at the tips of hyphae singly or in aggregates and are much smaller (40-120 μm in diam.) than spores of acaulosporoid species, and have subtending hyphae instead of the pedicel plus saccules as in acaulosporoid species. Their phenotypic features are almost identical to those of *Ar. liptoticha*, except that spores of the latter are larger and can reach up to 260 μm in diam. The other dimorphic species in *Archaeospora* is *Ar. liptoticha* as it produces two distinguishable types of spores, glumoid and acaulosporoid spores, and bears many similarities to its sister dimorphic species *Ar. gerdemannii*. The two dimorphic species differ mainly in spore size.

At the molecular level, species of *Archaeospora* can be distinguished from other fungi by this 18S rRNA gene nucleotide sequence, TCTCKKYTTCGGYSGAGTCC (Morton and Redecker, 2001).

Paraglomus

Species in *Paraglomus*, *P. occultum* and *P. brasilianum*, are strictly monomorphic, producing only *Glomus*-like spores. The grouping of these two species is supported by analysis of ITS and 5.8S gene sequences that revealed that the two species are closely related (Morton and Redecker, 2001). Vesicle-like structures were detected in roots of *P. brasilianum*, but their exact identity is yet uncertain. Thickness of their walls suggested that they might be intraradical spores rather then vesicles. The presence of *Paraglomus* species could be verified by their unique DNA sequence GGCATGTCTGTTTGAGGGCACCA

found at the 3'end of the 5.8S rRNA gene or by amplification of the rDNA region using the ARCH1311 primer that has the following nucleotide sequence: TGCTAAATAGCCAGGCTGY.

P. occultum produces hyaline spores 60-100 μm in diam. with variable shapes. Spore walls have three hyaline layers with the two inside layers occasionally becoming yellow in Melzer's reagent. The outer cell wall layers usually slough off and expose the inner layers to which organic debris may adhere. Specimens of this species from all over the world, including Iowa, are part of the INVAM collection. Spores (60-140 μm in diam.) of *P. brasilianum and P. occultum* species have many overlapping features in size, shape, and cell wall structure.

Strategy for Classifying Field-isolated AM Fungi

Identification of AM genera is based mainly on characteristics of spores and hyphae, including the thickness and staining intensity of hyphae and arbuscules, using special stains such as trypan blue. The identification process includes looking for the presence or absence of vesicles, of auxiliary cells, of special structures such as sporocarps, sporofirous saccules, and bulbous entities, and examining the hyphal status of the spores, as to whether spores are stalked or sessile.

Once an AM strain is placed in a particular genus, more detailed descriptions of spores and ultrastructural examination of spore cell walls are required to assign it to a particular species. Spore color, size, and shape are prerequisites. Spore color can be determined under a binocular microscope with the aid of a color chart provided by the INVAM people. The size can be determined tediously using a micrometer on several spores. More profound examination of spores consists of ultrastructural examination of spore walls. AM species differ in the number of layers a spore wall has and the type of each layer in terms of thickness, flexibility, and reaction to special stains, such as PVLG and Melzer's reagent. These criteria are evaluated by microscopically viewing stained spores mounted on microscope slides.

The criteria listed above for classification of an AM isolate are by no means complete. The extent to which an isolate is examined is context-dependent. Many factors affect the time

it takes to classify the sample and the accuracy at which the sample is classified. The amount of work to be invested in the identification procedure depends, primarily, on the quality and conditions of the spores to be classified, and the qualifications of the investigator. For instance, the healthier the spores are in terms of having essential structures intact, the easier and faster they are to categorize. In addition, the investigator with more expertise and familiarity with procedures and isolates is likely to produce more reliable results. In many instances, there are a few key characteristics that are essential for classification, and the investigator who recognizes them may have his/her work cut short. Another common challenge in identifying AM fungi is distinguishing the stable characteristics from transient ones. In other words, it is difficult to separate genetic characteristics that are likely to be transmitted to the offspring from characteristics that are associated with certain environmental conditions.

Mechanisms of Improved Phosphorus Uptake

Mechanisms for increased P acquisition by plants whose roots are colonized by AM fungi have not been fully understood. This is because it is difficult to sort out the actual contribution of the mycorrhizae due to the complexity of the soil environment and its diverse inhabiting organisms combined with failure to culture AM fungi independently from the host because of their symbiont nature. Possible biological mechanisms for replenishing the soil solution P loss are increased phosphatase activity, release of organic acids, increased CO_2 evolution, and mineralization of organic P compounds, such as inositol phosphates, phospholipids, and phytates (nucleic acids and their derivatives), that account for 10-50, 1-5, and 0.2-2.5% of organic P in soil, respectively (Havlin et al., 1999; Paul and Clark, 1989). This section will emphasize the role of AM fungal hyphae in increased P uptake by the host.

AM Hyphae Expand the Zone of P Absorption

Extension of the hyphal network and absorption of the less mobile P in the soil beyond the depletion zone of plant root hairs may be the most obvious and important mechanism by which AM fungi enhance the growth of their host plants. To test this hypothesis, Rhodes and

Gerdemann (1975) conducted an experiment to determine the maximum distance from which onion plants (*Allium cepa* L. var. Early Yellow Globe) can extract ^{32}P. They found that G. *fasciculatus* increased the zone of phosphorus uptake to at least 7 cm from the roots, many times greater than the 2- to 3-mm zone of the root hairs. However, this distance was the maximum distance available in the chambers used in their experiment and may not represent the farthest point from which mycorrhizal hyphae can absorb P and translocate it to the host plant root.

Mycorrhizal Hyphae Are Major Organs in P Absorption

Supporting evidence for the leading role of AM fungi in P uptake was provided by demonstrating that hyphae and not root hairs of mycorrhizal plants are responsible for increased P content in tissues of mycorrhizal plants. In greenhouse studies, Ayling et al. (2001) designed a set of experiments to investigate the relationship between the root membrane potential and P uptake by leek plants (*Allium porrum* L. cv Vertina). Leek plants grown in soil inoculated with *S. calospora* were compared to those grown in AM fungal-free soil. They found that nonmycorrhizal roots had a more negative membrane potential than mycorrhizal roots inoculated with *S. calospora*. This membrane potential is normally expected to cause the nonmycorrhizal roots to absorb the most P, but shoots of mycorrhizal plants tested higher in P concentration and weighed more than shoots of the control plants. Moreover, high P concentration was maintained longer in shoots of mycorrhizal plants than in those of nonmycorrhizal plants after the last addition of P to the soil in their experiments.

Because a weak correlation between potential difference and P absorption was found in the roots of mycorrhizal plants, the investigators suggested that fungal hyphae, not plant roots, are responsible for P uptake in mycorrhizal plants. However, previous research had indicated that the potential difference of mycorrhizal roots was more negative than that of nonmycorrhizal roots, and this was attributed to the fact that mycorrhizal plant roots took up more nutrients (Ayling et al., 2001). High efficiency of absorption of P through AM hyphae and its translocation from hyphae to plants roots is regulated partially by the functionality of the structures involved in P transport all the way from the soil to the destined root cortical cells of the host plants (Smith et al., 1994). First, optimization of the soil volume from which

hyphae absorb P relies on the fungus producing a hyphal network that is effective in taking up P. Second, P movement through adjacent or connecting hyphae should occur with minimum P loss along the way. Third, P should be successfully transferred to the cortical cells, sites from which P is translocated to the rest of the plant.

Significance of Efficient Transport and Delivery of Absorbed P to Plant Cells

The absorptive power of extraradical hyphae in soil is important, but the role of other AM structures in carrying P and transmitting P is no less significant. For instance, the extent of invaginations formed between arbuscules and the cell membrane of the cortical cells is a contributing factor to the rate of exchange of carbohydrates, nutrients, and water between AM fungi and their hosts. Previous studies examined the P flow from the fungal hyphae to the plants only at the interface site, where arbuscules and the cortical cells interact through the cell membrane. Smith et al. (1994) conducted the first study that investigated the P fluxes beyond the interface site. They expanded the study to include the structures of all participating hyphae, from the tips of extraradical hyphae to the plant cortical cells. Leek (*Allium porrum* L. cv. Mussel Burgh) plants inoculated (or not) with *G. mosseae* (GM) and *Glomus* sp. (CB) were evaluated for mycorrhizal effects. They were grown in potted soils incubated in a controlled glasshouse environment. The rate of P absorption by plants regardless of their mycorrhizal status was highest during early growth stages and decreased with plant age. The values measured in fmol./cm root per second decreased from 270 to 30, 330 to 8, and 180 to 10 during a two-month period of growth for GM, CB, and control plants, respectively. The decline was attributed partially to diminished activity and vigor of roots in soil with time. However, the P inflow to the root cortical cells was generally higher in mycorrhizal plants than in control plants. The rate of flow of P from the fungus to the plants varied from 0.8 to 12.8 nmol./m^2 per second with the assumption that arbuscules are the only structures involved in P transfer between fungal hyphae and root cortical cells of the host plants (Smith et al., 1994).

Evidence from previous inquiries indicated the ability of AM fungal hyphae to hydrolyze organic P compounds, producing P forms suitable for plant uptake. The most convincing findings were the conclusions drawn from the study conducted by Koide and Kabir (2000),

demonstrating that hyphae of *G. intraradices* hydrolyzed two organic P compounds, 5 bromo-4-chloro-indolyl phosphate and phenolphthalein diphosphate, and as a consequence increased the flux of P to the roots of Ri T-DNA-transformed carrots (*Daucus carota* L.).

There is no doubt that more information about the importance of AM fungi in enhancing P acquisition by plants and the investigations aimed at determining and understanding its mechanisms is needed, considering the complexity surrounding the dynamic biological, biochemical, and physical processes predominant in the rhizosphere.

Expression of P Genes in AM Mycorrhizae

At the molecular level, Burleigh et al. (2002) attempted to correlate plant responses in symbiosis with seven AM fungal species to the level of expression of the Pi transporter constitutive genes and P starvation inducible genes in the roots of two plant species, *Medicago truncatula* and *Lycopersicon esculentum*. The two pairs of genes are Pi transporter MtPT2 and P starvation gene Mt4, and Pi transporter LePT1 and P starvation TPSI1 of the former and the latter plant species, respectively. A strong positive correlation was found between the increased shoot P contents and shoot dry weight in response to mycorrhizae in both plant species, with *M. truncatula* being most responsive.

In nonmycorrhizal plants, both the MtPT2 and the Mt4 genes were highly expressed in *M. truncatula*, as was the TPSI1 gene in *L. esculentum* when no P was added to the pots, but these genes were not expressed when adequate P was added to the pots. The LePT1 gene, however, was highly expressed in the roots of *L. esculentum* grown in pots supplied with adequate P, so it was not turned off by high P concentration in the shoots. As for mycorrhizal plants, the expression levels of genes of MtPT2 and Mt4 varied according to the fungal isolate, and the level of expression was negatively correlated with the concentration of P in the shoots of *M. truncatula*, just as one would predict. The expression of the corresponding genes in *L. esculentum* was relatively low, did not vary with the AM isolates, and was not influenced by the level of colonization.

This study (Burleigh et al., 2002) hypothesized that there may be a "number of gene family members" involved in P transport based on the observed patterns of the northern blot analysis. That could be in accordance with the different P transporter genes found in barley

(Smith, 2001), tomatoes, and other plants, grouped in the Pht1 family, that were supposedly responsible for the transport of different forms of phosphates across different membranes in the cortical cells (Smith, 2001). In fact, complementary DNA (cDNA) derived from RNA transcripts of genes encoding for P transport isolated from different plant species have similarities to those previously identified in other fungi, including filamentous fungi, and those in AM fungi (Smith, 2001; Liu et al., 1998b). Smith (2001) reported that AM hyphal networks absorb P from the soil solution by means of proton-coupled phosphate transporters comparable to those identified in the Pht1 family. Similar P transporter genes were cloned from hyphae of *G. veriforme* and have high affinities, allowing them to draw P from low P concentration media such as the soil solution (Smith et al., 2001).

Carbon Flow to the AM Fungi

AM fungi are obligate symbionts because they rely on the C compounds they receive from the host plant. This reliance complicates their study because they are unable to complete their life cycle without connecting to a host in a functional symbiosis. Many attempts to grow them axenically have yielded little progress (Pfeffer et al., 1999).

Mycorrhizal plants translocate 4 to 20% more photosynthate to their root systems than their paired nonmcorrhizal plants (Douds et al., 2000). Much of this photosynthate is used to support and maintain the growth and function of AM fungal structures associated with the plant roots (Grimoldi et al., 2005). In fact, it was reported that plants ranging from angiosperms to mosses may invest as much as 500 billion tons of C each year to nourish the AM fungi colonizing their roots (Bago et al., 2000). However, the AM fungi usually transfer sufficient plant nutrients (P, Zn, Cu, NH_4^+, etc.) and water to the roots to increase plant growth (Johnson et al., 1997). Cost-benefit analyses show that agriculturally important plants generally have a net gain in biomass and improved crop quality from mycorrhizal symbiosis. There has been ongoing research aimed at understanding the nature, pathways, and metabolic activities of the C compounds flowing from the host to the mycelial network of AM fungi that extends from the roots into the soil rhizosphere (Douds et al., 2000). Research updates related to C dynamics during symbiosis, sites of C metabolism, mechanisms of C transport, and distribution of C storage in AM fungal structures will be addressed in this section.

Pre-symbiotic Spore Germination

Neutral lipids constitute 45 to 95% of the AM spore's C reserve, suggesting that most of the C that is transferred to fungal hyphae from the host plants is converted to lipids, and only a small amount to carbohydrates (Bago et al., 2000; Pfeffer et al., 1999). During the pre-symbiotic period, germinating AM fungal spores oxidize mainly neutral lipids, triacylglycerides (TAGs), to derive energy and to produce precursors for development of their germ tubes (Bago et al., 2000). Independent from the host (Harrison, 1999), AM fungal spores are able to initiate the metabolic processes necessary for growth, including DNA, RNA, and protein synthesis for growth of germ tubes and their constituents, including nuclei and chitinous cell walls (Bago et al., 2000). The germ tube elongates through the rhizosphere, increasing the probability of intercepting a compatible root, but if root interception fails, the germ tube ceases growth before the TAG supply is used up (Giovannetti, 2000). A septum is formed to protect the spores, which reenter the dormant state and retain the ability to germinate again when conditions are suitable (Giovannetti, 2000). This strategy gives spores a greater chance of survival and potential success in colonizing roots in subsequent attempts (Bago et al., 2000).

Symbiotic Phase of Mycorrhizae

The establishment of a functional AM symbiosis occurs in several sequential steps. During the asymbiotic phase, the spores or other propagules germinate and develop germ tubes using the energy and C substrates derived from catabolism of their stored TAGs and/or glycogen (Bago et al., 2000). Even without physical contact with the plant roots, AM fungal spore germination may be stimulated by root-emitted substances, including root exudates and volatile organic compounds (Carpenter-Boggs et al., 1995; Harrison, 1999). As the germ tubes approach the prospective host roots, the hyphae undergo extensive branching. These morphological adjustments are believed to be in response to root-emitted signal molecules, such as flavonoids and phenolic compounds (Harrison, 1999).

When the actively branching tips of a growing propagule intercepts a plant root, the formation of an appressorium signals the start of colonization (Giovannetti, 2000; Harrison, 1999). Appressorium formation on epidermal cells occurs only in the presence of a suitable host that produces recognition molecules and not inhibitors in the proximity of a colonizing AM fungus, so this is a critical step for the formation of a successful symbiosis (Harrison, 1999; Giovannetti, 2000). Following the appressorium, a penetration hypha is formed. The infection hypha enters the roots by using either pressure or cell-wall-degrading enzymes to cross the epidermal cells and to reach the cortical cells (Harrison, 1999). Subsequently, fungal hyphae grow into the intercellular spaces of cortical cells developing intraradical hyphae, which then cross the cell walls. Depending on the host-fungus combination, these hyphae either develop hyphal coils and arbuscules in the apoplast spaces (Paris type) or differentiate into only arbuscules (Arum type) inside the cell walls (Cavagnaro et al., 2001). Neither arbuscules nor coiled hyphae penetrate the cell membrane, but they invaginate it, producing a site where the exchange of nutrients and carbohydrates is believed to take place (Bago et al., 2000).

The next logical step after the establishment of hyphal stuctures inside the root cortex is the formation of extraradical hyphae and their proliferation in the soil. Their functions qualify the type of symbiosis and may include the acquisition of nutrients and water and translocation of these nutrients to the host plants. Some of the hyphae may be important in reproduction as they infect more roots (Harrison, 1999). Extraradical (and intraradical in some species) hyphae produce spores, completing the life cycle.

Form and Fate of Carbon Acquired by AM Fungi

Initiation of studies of many organisms is based on extrapolation of observations collected from closely related organisms. Carbon transport in pathogenic fungi gained attention from plant pathologists because of their apparent devastating effects inflicted on economically important crops. In this context, assumptions were made that similarities in C flow exist between saprophytic, pathogenic, and symbiotic fungi, so it has been suggested that sugars are likely to be the compounds that are transferred from the host to the AM fungi (Bago et al., 2000). Studies using isotopic labeling with nuclear magnetic resonance (NMR)

spectroscopy showed that the types of C absorbed by AM fungi are actually 6-C sugars. Intraradical fungal hyphae absorbed glucose and fructose in preference to sucrose from the host roots (Bago et al., 2000; Pfeffer et al., 1999). Increased invertase activity reported in the extracellular spaces supported the evidence that hexose sugars are the main compounds transferred from the host to the fungal mycelia (Snellgrove et al., 1987, as reported by Bago et al., 2000).

Inside intraradical mycorrhizal hyphae, hexose moieties are metabolically converted to lipids and carbohydrates. Extraradical hyphae are not involved in lipid biosynthesis but rather receive lipids from intraradical hyphae (Bago et al., 2002), and they absorb very little or no glucose, fructose, mannitol, or succinate from the soil solution (Pfeffer et al., 1999). Detachment of extraradical hyphae from the roots of the host plant resulted in a fall of TAG concentration in the extraradical hyphae, suggesting that TAG was more likely being catabolized than synthesized (Olsson, 1999). Activities of glycolytic enzymes were almost undetected in extraradical mycelia, which emphasizes that carbohydrate oxidation is not the primary source of acetyl-CoA. However, assays and labeling experiments indicate the presence and activities of enzymes involved in the pentose phosphate pathway, the major source of the reducing power of NADPH, essential to energizing biosynthetic reactions (Pfeffer et al., 1999).

A large portion of the host-supplied hexose was directed toward synthesis of storage lipids in AM hyphae for long term storage (Pfeffer et al., 1999). In this study, the host plant was supplied with ^{13}C labeled precursors for photosynthesis. Subsequent examination of hyphae indicated the presence of ^{13}C TAG. This may be explained by a sequence of events. The labeled host-derived hexose was metabolized to triose sugar (pyruvate) and acetyl coenzyme A (CoA), precursors for lipid synthesis. Fatty acid (FA) synthesis is mediated by the enzymes acetyl CoA carboxylase and other FA synthase complexes. Degradation of fatty acids occurs in mitochondria-yielding acetyl CoA products that are converted to citrates before exiting from the mitochondrion to the cytosol because acetyl-CoA molecules cannot traverse the mitochondrial membrane (Garret and Grisham, 1999). In analogy with other fungi, citrate in the cytosol is converted by the ATP-dependent citrate lyase back to acetyl (CoA) forms that become the substrates for FA synthesis. Biotin-dependent acetyl CoA carboxylase mediates the conversion of acetyl-CoA to malonyl-CoA. Subsequent reactions

for FA synthesis are carried out by the multienzyme complex, fatty acid synthase or simply FAS, condensing additional acetyl CoA to malonyl CoA precursor. The end product, palmitate, may undergo further processes, such as chain elongation or unsaturation (creation of double bonds) reactions. The fatty acids and glycerol molecules assemble to form triacylglycerol units in reactions mediated by acyl transferases, as summarized by Murphy (1991). Hexoses are also immediately converted to disaccharides (trehalose) and to glycogen that represent the major carbohydrate pool in the intraradical hyphae (Bago et al., 2000). More research is needed to provide conclusive evidence to this effect. Identification of sugar transporters will be an important step in understanding the mechanism and the nature of the compounds being transported (Pfeffer et al., 1999).

Cortical cells whose membranes are invaginated by AM arbucules have significantly higher lipid/starch ratios than those of nonmycorrhizal roots (Bago et al., 2000), and the distribution of these lipids varies with both mycorrhizal structure type and the colonizing AM fungus. As spores germinate, TAGs continue to stream to the growing germ tube. Lipid distribution in germ tubes of *G. intraradices* and *Gi. rosea* was examined with *in vivo* microscopy (Bago et al., 2002). The lipid content was highest inside the spores and gradually decreased along the germ tube, with nearly no lipid globules detected at the tip of the tubes produced by *G. intraradices* and *Gi. rosea*. Accordingly, it was estimated that the percentage of the volume of the hyphae occupied by lipid droplets varied between about 16 and 4.5% in areas proximal to the spores down to around 0.3% and none at the tips of the tubes of *G. intraradices* and *Gi. rosea*, respectively (Bago et al., 2002).

A transfer of C can occur between ectomycorrhizal plants connected by mycorrhizal hyphae. This led investigators to examine the possibility of reciprocal movement of C between AM fungi and their host plants. The findings suggest that AM fungi have the ability to draw C from both of the linked plants, but the host-derived C remains in the fungal hyphae and is not transferred to either host. Assumptions are that the fungus obtains hexoses from the host roots through arbuscules and converts them to trehalose, glycogen, or TAG within the root cortical cells and invests it in the formation of new AM structures, including vesicles, spores, and hyphae.

Carbon Transport Systems in AM Fungi

Much needs to be done to unravel the mysteries surrounding the mechanisms of C transport from plants to mycorrhizal fungi (Bago et al., 2000). It has been established that glucose and fructose are preferred to sucrose as the forms of carbohydrates that are transferred to the fungus (Pfeffer et al., 1999), but the mode of transport remains unclear. The transport of these sugars may be active, passive, or both (Bago et al., 2000).

Passive transport based on a concentration gradient is most likely the dominant transport mechanism (Pfeffer et al., 1999). A concentration gradient of hexoses from the apoplast of the cortical cells to the nearby fungal tissue must be maintained for the hexoses to move from a higher concentration medium to a lower concentration medium (Garret and Grisham, 1999). A concentration gradient is usually maintained in biological systems by modifying the substance after it arrives on the receiving side of the system. Using this strategy, AM fungi promptly convert hexoses to glycogen, trehalose, and lipids as these hexoses are deposited in their hyphal or arbuscular tissues and thus keep C flowing from their partner plants (Bago et al., 2000). Hexose transport across membranes by passive diffusion may not be sufficient to sustain the fungal growth, however, so facilitated diffusion is likely to be involved. In this case, specific and high-affinity hexose transporter proteins facilitate the movement of hexoses across the membranes from the cortical cells to the hyphal cells. A high level of expression of a gene encoding for a hexose transporter was detected in cortical cells in areas presumably proximal to intraradical hyhae (Bago et al., 2000).

Active transport of sugars to the fungus may also occur where hexoses actually move against a concentration gradient, so there is a need for energy. Sanders (1988 as cited in Bago et al., 2000) proposed the cotransport of hexoses and H^+, that is common in other fungi and also operates in ectomycorrhizal fungi, as an alternative mode of C transport in AM systems (Bago et al., 2000). Jakobsen et al. (2002) supported the likelihood that C transport to AM fungal structures may depend on a proton gradient across the membranes generated by H^+-ATPase located on the cell membranes of arbuscules; that would allow a proton co-transport of sugars. Fungal H^+-ATPase has been detected only in intercellular hyphae, however, and

this may rule out the arbuscules as a site of C exchange. The peri-arbuscular interface was considered to be a favorable site for C flow from the host due to its being near the phloem (Douds et al., 2000). Whether cell membrane permeability is compromised to allow the passage of sugar molecules is not yet known. Furthermore, the characterization of sugar transporter proteins (if they exist) that move sugars from the cortical cell cytoplasm to the hyphal cytoplasm has not been achieved (Bago et al., 2000).

Distribution and Diversity of AM Fungi in Soils

Geographically, AM fungi are widespread and live in the roots of most land plants due to their low host specificity. Little, however, is known about the extent of their diversity in natural habitats because of the difficulty encountered in identifying field isolates (Dodd et al., 1996). Many factors may affect both temporal and spatial distribution of AM fungi (Anderson et al., 1983; Walker et al., 1982). Among these factors are soil management, topography, drainage class, chemical and physical properties of the soil, and the host plants.

Cultivation has been found to reduce the AM population in the soil in greenhouse studies as well as in field studies. Helgason et al. (1998) demonstrated that the AM fungal diversity in soils subjected to cultivation, fertilization, and/or application of pesticides was significantly reduced compared to that of woodland that had been exposed to minimum soil disturbance. The observed decline in AM fungal population was independent of the host plant and the location. Active hyphae length and spore density were 40 to 50% lower in the top 5 cm of the soil profile in corn fields that are under conventional tillage than in no-till corn fields. The top 15-cm soil layer contained 87 and 84% of hyphae and 76 and 74% of spores collected, respectively, in NT and CT fields (Kabir et al., 1998).

Mycorrhizal colonization was reduced by 80% in soybean roots grown in field plots that received high P fertilization of 112 Kg P ha^{-1}, compared to those grown in plots with no P added (Hicks and Loynachan, 1987). Similarly, high P application lowered AM colonization of roots of clover and lespedeza (Wilson, 1988). Khalil et al. (1992) reported that soybean roots were highly colonized even in soils that tested high in P level. Their hypothesis was that native Iowa AM fungi have evolved and acquired the ability to thrive under high P conditions. Khalil and Loynachan (1994) also reported a significant variation in the

distribution of AM population in Iowa soils both among and within the surveyed fields. Higher spore counts were reported in poorly drained soils than in well drained soils. The authors speculated that increased sporulation might be due to increased organic matter, gravitational movement of spores, or simply stress caused by low oxygen availability or CO_2 toxicity.

The type and certainly the absence (or the presence) of the host plant influence the abundance and survival of AM fungi in the soil. For example, long-term flooding and fallow resulting in few or no living roots significantly reduced the AM fungal population in the soil (Ellis, 1998; Troeh and Loynachan, 2003). Soils of corn plants had higher AM fungal population, expressed in spore counts and most probable number evaluations, than soils of soybeans in a potted field experiment (Troeh and Loynachan, 2003).

Seasonality also affects the distribution and community structure of AM fungi. Spore populations are generally high during the fall season and low during the spring season (Douds and Milner, 1999; Troeh and Loynachan, 2003). Thus, it is preferable to collect spores in the fall, immediately after they are produced because they are less likely to be damaged by parasites. Sampling during a single season, however, may result in selecting against species that sporulate during other seasons because different AM species have their sporulation peaks not only at different seasons but at different times within a season (Bever et al., 2001). For instance, *Scutellospora* species were found to be the first to occur in the roots of bluebell plants and the most abundant while the C is coming from the hypogeous bulbs, but when the plants developed leaves, *Acaulospora* energized by photosynthate C began to replace the declining species of *Scutellospora* (Merryweather and Fitter, 1998b).

AM fungal spores can occur in patches in soil independently of root distribution (Douds and Millner, 1999; Walker et al., 1982), and the number of samples required to adequately represent a soil's AM population depends on the degree of heterogeneity of the location to be sampled. Spores have been detected at a depth of 2.2 m in the soil, but the top 45 cm layer may harbor 70-85% of the spores (Douds and Millner, 1999). Extraradical hyphae can extend at least 8 cm beyond the roots (Rhodes and Gerdemann, 1975).

Walker et al. (1982) investigated the mycorrhizal status of four poplar hybrids planted in two locations in central Iowa. One of their sites at the 4-H Camping Center site in Boone County, Iowa, located on a terrace along the Des Moines river has a young soil profile with

little development of A and B horizons. The other site is in an old meadow on the floodplain, near Rhodes, Marshall County, Iowa. Ten species belonging to three genera (*Acaulospora*, *Gigaspora*, and *Glomus*) were identified in the samples collected from the soil rhizosphere at the 4-H site. The dominant species at this site were *A. spinosa*, *A. scrobiculata*, *G. fasciculatum*, *Gi. rosea*, and *Gigaspora* spp. Twelve AM fungal species were found at the Rhodes site, and the most common species were *A. trappei*, *A. scrobiculata*, *Scutellospora calospora*, *G. albidum*, and *G. occultum*.

Glomus species were the most common AM species encountered in the soybean rhizosphere in twelve soils of central Iowa according to a survey conducted by Khalil et al. (1992). The list of AM species that were common comprised *G. etunicatum*, *G. intraradices*, *G. constrictum*, and *G. mosseae*. Other species that were frequently found in these soils were *Gi. margarita* and *A. spinosa*. Spores of *Scutelospora* were rare and were not identified at the species level.

G. mosseae, a species that sporulated heavily, was dominant in cultivated fields but not found in woodland soils in a study by Helgason et al. (1998). This is logical because cultivation gives species that reproduce mainly through spores an advantage, and hyphae are subject to destruction by tillage in cultivated fields. Low AM diversity may be an indication of reduced function (not activity) of AM fungi in cultivated soils compared to that in woodland soils. Likewise, members of Glomaceae represented 19 of the 24 phylotypes identified in seminatural grassland where two plant species, *Agrostis capillaris* and *Trifolium repens*, were dominant (Vandenkoornhuyse et al., 2002). The remaining taxon units belonged to Acaulosporaceae and Gigasporaceae, but none of the identified isolates were members of Archaeosporaceae or Paraglomaceae because the AM1 primer did not detect these newly defined families.

Selection of AM Fungi by Host Plants

AM fungi have been found to be broadly host specific, but recent investigation showed that hosts may express some degree of preference toward certain colonizing AM fungi (Daniell et al., 2001) and that some AM fungal species have some degree of preference toward certain host plants. Daniell et al. (2001) found that one group of *Glomus* species

dominated in pea roots and a different *Glomus* group in wheat plants. The AM fungal community composition based on sequence variations in the region of SSU rRNA gene amplified using a fungal specific NS31-AM1 primer pair was different for the two plant species, indicating preferences of the host plants for certain types of AM fungi (Vandenkoornhuyse et al., 2002).

Previous research has shown that the AM effect may differ depending on the host plant and the AM fungal species involved. In a field study, potato microplants inoculated with *G. intraradices* yielded less and displayed a poorer crop quality compared with the control while potato microplants inoculated with *Vanimoc*, a mixture of isolates from the United Kingdom, had the highest yield, and the plants inoculated with Endorize, composed of isolates from France, generated the best quality potato tubers (Duffy and Cassells, 2000). This indicates that mycorrhizae may perform best with the right host-fungus combination in a suitable environment. Manipulating the AM fungal species in field soil remains currently unattainable because of the complexity of the ecosystems involved.

Morphological Techniques in AM Fungal Studies

AM fungi reproduce by means of infective hyphae, extraradical hyphae, and asexual spores (Bever et al., 2001). Identification, taxonomic classification, and diversity studies of AM fungi have been conventionally based on the morphological description of the resting, multinucleate spores and the characteristics of their cell walls (Cornejo et al., 2004; Dodd et al., 1996). Determination of the population composition of AM fungi is a prerequisite for understanding their functional diversity in an agricultural ecosystem, and a basis for sound management to optimize the benefits of plants of interest from the symbiosis.

Limitations of Spore Morphology for Identification of AM Field Isolates

The spore morphology method has many limitations in identification and ecological studies (Redecker et al., 2000a). Determination of the diversity of AM fungi has been hindered by the difficulty of identifying AM spores isolated from field soils, especially at the species level, for a numbers of reasons (Sanders et al., 1995). Identifying distinguishing

inheritable genetic characteristics from transient ones is a significant challenge. In many cases transient characters have been erroneously considered among features determining the classification of a species. When the feature disappears, perhaps due to a change in the environment, the same species may be given another identity. The revision of identity of many AM species led to switching species among genera and establishing synonymy among many previously identified species (Morton and Redecker, 2001; Walker and Vestberg, 1998). Morphological differences among certain field isolates are not readily detected because heterogeneity is unavoidable and morphological characters of spores are distorted or lost due to parasitism and physical damage (Bago et al., 1998).

Morphologically similar AM fungal isolates may still be physiologically different and perform different functions in symbiosis (Dodd et al., 1996; Merryweather and Fitter, 1998a) and may have different effects on their hosts (Clapp et al., 1999), so it is important to identify the AM fungi in situ. Stained AM-colonized roots, however, generally have distinguishing characteristics to identify AM fungi only at the family level (Redecker et al., 2000a).

Nonspore-forming AM species of field-collected materials are often overlooked (Douds and Millner, 1999). Detection of some AM fungal species, such as *G. occultum*, in colonized roots is difficult-to-impossible because they do not stain well even though their spore population is high (Morton and Redecker, 2001).

Propagules Unsuitable for AM Identification

Arbuscular fungi produce several types of propagules that inhabit both soil and roots, but most of them are useful in identification only at the genus level under the best circumstances. The assessment of the contribution of an individual AM isolate on plant growth using morphological characteristics of spores collected from field soil is cumbersome because spores collected from soils are generally detached from the hyphae associated with plant roots (Dodd et al., 1996). Morphological means to match spores with intra-radical hyphae from which they were derived are yet to be found, especially when dealing with more than one type of spores. For that reason, a correlation cannot be determined between these spores and the specific AM fungal colonization of plant roots (Daniell et al., 2001). Obligate symbiosis makes the identity of the AM fungus colonizing the root essential to any study of

AM because each isolate may have its own unique function (Merryweather and Fitter, 1998a). Yet, tools for practically collecting AM fungal mycelia from roots or soils and isolating them from mycelia of other fungi under natural systems are still lacking.

Another basic element in identification is to distinguish hyphae of AM fungi from those of other fungi. Young AM fungal hyphae sometimes can be distinguished by the absence of clamp connections and absence of septa, or by production of a special Glomalin protein, and they are usually thicker than hyphae of other fungi. Small AM hyphae, however, are not so easily differentiated from those of non-AM fungi. Hyphae do not provide sufficient distinguishing characteristics to classify AM fungi at the genus level, and discrimination among AM hyphal structures is of limited use to identify AM strains at the family level. For example, accurate identification requires familiarity of the identifier with the procedure and requires repetitive observations of single cultures of individual specimens being studied (Douds and Millner, 1999).

The morphology of AM fungi varies with stage of development and/or with environmental conditions; variations that are genetically inherited should be identified and distinguished from phenotypes related to environmental conditions (Dodd et al., 1996) as a basis for development of more reliable identification techniques. AM fungi have not been successfully cultured independent of the host plant, so the use of morphological techniques necessitates development of homogeneous populations of AM fungi. The best-known approach to achieve this is to propagate AM fungi from single-spore cultures in the presence of a suitable host (Bago et al., 1998), but simple and dependable techniques have not been developed. Currently available techniques for colonization of host roots with a single spore still experiences low success rates (Dodd et al., 1996) and requires much time and labor, especially when field spores are involved.

Quantification of AM fungal tissue in roots is determined usually by estimating percentage of root colonization by AM fungi using the gridline-intersect method (McGonigle et al., 1990). Spores are collected from soil and counted. Correlations between spore abundance and extent of AM colonization have not been established, and spore counts of a certain AM species may not reflect the functional value of this species (Douds and Millner, 1999).

The costs involved in revision and publication of guides for identification of AM fungi are another challenge for improving morphological techniques. Illustrated identification guides have not been updated often enough to include the recently identified AM species or suggested revisions for the existing species, but websites with instructive guides are available (Douds and Millner, 1999).

Molecular Techniques in AM Fungal Research

Recently, researchers have been trying to determine and understand the effects of AM fungi on plant production, plant biodiversity, and stability of the ecosystem in an environment harboring mixed communities. Assessing the effects of individual AM fungal strains on plants has become one of the common topics of mycorrhizal research today. The advent of molecular techniques has been credited for developments that allow more concise, objective, and reliable studies in this field. Among molecular biological techniques that have been applied in AM research are isozymes analysis, fatty acid profiling, and sequence analysis of ribosomal small subunit (SSU) genes (Bago et al., 1998). A special glycoprotein called "glomalin" produced by AM fungi has been discovered, and antibodies to this protein provide a means to distinguish AM fungi from other fungi (Wright et al., 1999). Monoclonal antibodies to glomalin can be used to distinguish strains of the same species, but this procedure has had limited success because of cross reactivity. Another problem is the low productivity of antibodies to antigens of glomalin.

Spores and vesicles store lipids, and some of the fatty acids are unique to specific AM isolates and can be used to identify certain AM species. Thus, fatty acid methyl ester (FAME) techniques may be used for AM identification. Chitin concentration in AM fungal propagules can be used to determine the AM quantification in plant roots, but this technique requires pure material (Douds and Millner, 1999) because chitin is also produced by other fungi. Thus, this method may not be practical for field isolates that include a mixture of unknown species. Bonfante et al. (1996) monitored changes in the expression of α-tubulin genes during mycorrhizal formation in transgenic *Nicotiana tabaccum* plants. They observed the expression of Tubα 3 (but not that of Tubα 1) gene in root cells containing arbuscules and concluded that mycorrhizae induced activity of specific genes in colonized roots. Most of

these techniques will not readily work for identification purposes in field situations with more than one type of AM fungus coexisting in the soils (Kjøller and Rosendahl, 2000).

Recently, polymerase chain reaction (PCR)-based molecular techniques have been adapted and applied to AM fungal identification, classification, and biodiversity studies (Douds and Milner, 1999). These techniques, based on DNA sequencing analysis, overcome many limitations of other alternative methods previously predominant in AM fungal research (Douds and Millner, 1999). PCR is a powerful tool that requires only a small amount of DNA from an organism to conduct a variety of investigations, ranging from identification to phylogenetic studies. This is very important in AM research since AM fungi have not been successfully cultured independent from their hosts (Lanfranco et al., 1999). Also, its use in a natural environment with highly diluted DNA material is convenient. Genes encoding for ribosomal RNA and internal transcribed (ITS) regions of DNA have been most frequently used for identification purposes of AM fungi (Douds and Millner, 1999). Ribosomal RNA (rRNA) genes exist in multiple copies and conserved regions allow access to amplification sites (Simon et al., 1992). Existence of variable sites in the rRNA and ITS DNA regions in fungi enabled researchers to conduct identification and diversity studies based on the sequence analysis of AM fungal rDNA collected from soil or root materials (Anderson et al., 2003). The variations in sequences of rRNA genes have the potential to describe the phylogenic relationships between groups of organisms (Clapp et al., 1999). These DNA-analysis techniques still have limited use for the identification and diversity studies of field-derived AM fungal isolates and are still under improvement.

Types of PCR-based Molecular Techniques

Several molecular techniques, coupled with a PCR protocol, have been developed to objectively and reliably estimate AM fungal population and taxonomic diversities directly in functional colonized plant roots, or in the mycorrhizosphere (Merryweather and Fitter, 1998a). Among these methods are restriction fragment length polymorphism (RFLP), single stranded conformational polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE). Identification can be achieved at or below species levels, which often was difficult-to-impossible with morphology-based characterization. This development is crucial for

understanding the functional diversity of the root colonizers on the hosts (Helgason et al., 1999).

Integrated PCR and Sequencing

Sequencing analysis of cloned (or not) PCR products of the DNA region flanked by a pair of existing suitable primers is often used for a lower taxonomic level identification of AM fungi in roots colonized with an unknown mixture of mycorrhizal strains. The first known nucleotide sequence or primer reported to detect AM fungi with the exclusion of all other eukaryotic organisms was designed by Simon et al. (1992). They used universal eukaryotic-specific primers to amplify 18S rRNA genes from spores of several AM species. The PCR products were sequenced and aligned with published fungal SSU sequences. A region unique to AM fungal SSU genes found on the 5' end was used to create the VANS1 primer. The VANS1 specificity was confirmed by testing it on several AM fungal species in the three families (Glomaceae, Acaulosporaceae, and Gigasporaceae) of Glomales and on non-AM fungal materials (Simon et al., 1992).

Protocols integrating PCR and sequencing have been used for testing the degree of resolution a certain rDNA region can provide for distinguishing taxa in different phylogenetic groups of AM fungi. The regions of SSU DNA of 24 commercial isolates (most of which were acquired from the INVAM collection) of Gigasporaceae were amplified by the use of NS71-SSU1492' primers and directly sequenced (Bago et al., 1998). Sequence analysis resulted in detecting short 6-nucleotide sequence signatures that can be used to differentiate three different groups within *Gigaspora*, *Gi. gigantea* group, *Gi. rosea* group (*Gi. rosea* & *Gi. albida*), and *Gi. margarita* group (*Gi. margarita* & *Gi. decipiens*) from each other (Bago et al., 1998). This study concluded that even though these SSU signature sequences did not distinguish the isolates at the species level as morphologically determined, they would still be useful in population dynamic studies. Previous analyses of *Gigaspora* species using the ITS-RFLP test generated similar results, clustering *Gigaspora* species into groups rather than identifying individual species (Redecker et al., 1997). These observation suggest that *Gigaspora* diverged from Glomales much later than other genera, so its ITS and SSU DNA regions have less variability than those of other genera (Bago et al., 1998).

RFLP Techniques

RFLP methods have been extensively utilized in AM mycorrhizal research, but the quality of information obtained is often not very satisfying because of limited sequence variation in rRNA genes of AM fungi. Mycorrizal fungi in the roots of five plant species at four woodland locations and in the roots of pea, maize, and wheat crops at three farms were identified based on the RFLP patterns of SSU rRNA genes (Helgason et al., 1998). Partial 18S DNA sequences were amplified using the NS31-AM1 primer pair, and cloned PCR products were digested with Hinfl and Alu1. Sequenced RFLP segments indicated that *G. mosseae* and its related taxa were dominant and the mycorrhizal community was much less diverse in tilled soils. The sequence clusters supported by morphological assessment corroborated the presence of three genera, *Acaulospora*, *Glomus*, and *Scutellospora*, in both woodland and field soils. Broad host range of AM fungi was demonstrated by the presence of identical sequences of some isolates in a large number of plant species in the study (Helgason et al., 1998). Low diversity of AM fungi in cultivated fields may be due to the impact of soil management, such as fertilization, fungicide application, and cultivation, rather than crop monoculture.

A parallel study, based on the variation in DNA sequences of genes coding rRNA SSU, was conducted to determine the population composition of AM fungi in roots of wheat, barley, maize, and pea crops in cultivated fields (Daniell et al., 2001). The other objective was to see whether the pattern of AM fungal distribution was affected by plant species (Daniell et al., 2001). Amplified DNA segments using NS1 and AM1 primers were cloned using a PCR script kit. The cloned PCR products were then digested with Hinfl and/or Rsatl to distinguish among different isolates on the basis of RFLP clones (Daniell et al., 2001).

The patterns and sequencing analysis of RFLP clones showed the presence of eight types of sequences that represented the three families of Glomales (Acaulosporaceae, Gigasporaceae, and Glomaceae) based on the previous RFLP grouping established by Helgason et al. (1998) who had designed the AM1 primer. Sequences of the same RFLP pattern were assumed to represent the members of a monophyletic clade, with the exception of the predominant *Glomus* species; they were represented by four different types of sequences, two of which, Glo1A (62%) and Glo1B (20%), were dominant. According to

sequence analysis, *G. mossae* and *G. geosporum*, were present in Glo1A and Glo1 B, respectively. As for spatial distribution, data sequences in some locations suggested that the Glo1A group was the chief colonizer of wheat roots, while the Glo1B group was found to predominate in pea plants. In other locations, both groups appeared to predominate in both maize and wheat. The investigators realized that the study may not have revealed all AM species that existed in their field soils primarily because the AM1 primer was designed for Glomales only and was found to fail to detect members of the two new families of Glomales that were added to the AM fungal group after the AM1 primer was already designed (Daniell et al., 2001).

SSCP Techniques

Among other methods used in AM identification studies is single-stranded conformation polymorphism (SSCP) coupled with PCR. SSCP is a sensitive and rapid method for detecting DNA fragments that are not only identical in length but also have the same nucleotide sequence. A single base substitution in one DNA fragment of a few hundred base-pair sequences can be distinguished from its wild type homologous DNA fragment using SSCP (Simon et al., 1993). This method is based on differential electrophoretic migration of single stranded DNA molecules. DNA molecules of the same length but of different sequences acquire different shapes, and thus, move in the gel at different paces and become separated. In fact, this method allows differentiation between two homologous strands. This method can reduce the need for unnecessary sequencing, since it can identify the potential DNA fragments of interest (Sunnucks et al., 2000).

SSCP is used mostly in clinical studies, but it has been also adapted to other disciplines including mycorrhizal studies. Simon et al. (1993) demonstrated that the SSCP technique could be used for characterization of AM fungi in leek roots. A region of 18S rRNA gene was amplified with four primers that were designed to pair with an AM fungal-specific forward primer, VANS1, using DNA templates from AM spores of known species. The SSU region for these spores had already been sequenced. The four primers, VAACAU, VAGIGA, VAGLO, and VALETC, were designed to detect members of Acaulosporaceae, Gigasporaceae, Glomaceae, and *G. etunicatum*, respectively. The VANS1 primer was labeled

at the 5'end with fluorescein-amidite. The labeled PCR products were denatured with 95% formamide-10 mM NaOH. The denatured DNA samples were loaded on acrylamide gel and electrophoresed. The electrophoregram showed differences among *A. spinosa*, *A. rugosa*, and *Entrophospora* spp. but did not distinguish between *E. columbiana* and other *Entrophospora* spp., just as expected. The SSCP results successfully detected the similarities and differences in sequences of the spore isolates tested. Base substitutions existed in *Acaulospora* species while *Entrophospora* species had identical sequences. Migration differences were not observed in *Gigaspora* species either, and the SSCP technique results agree with the sequencing data. Leek roots were colonized by a different AM species than the ones used in the study, and SSCP and sequencing confirmed that the AM fungi in Leek roots were different than any known AM species used in this study.

In 2000, Kjøller and Rosendahl identified *Glomus* species in roots of four plant species based on differences in patterns and sequences of SSCP fragments of PCR amplification of the large subunit ribosomal DNA region using primers specific for *Glomus* (Kjøller and Rosendahl, 2000). They used the LSU region of rRNA genes because they questioned if the SSCP method would work if SSU genes were used due to insufficient variability in the SSU region to distinguish between closely related AM strains. The SSCP method successfully distinguished between *G. mosseae*, *G. caledonium*, and *G. coronatum* in AM colonized roots. Two years later, the SCCP method was used to investigate the genetic diversity of the ITS region within and among single spores of *G. intraradices* (Jansa et al., 2002). The amplified ITS region of rDNA was determined using primer pair ITS1 and ITS4. Sequencing analysis and SSCP fragment patterns showed higher variability in ITS sequences among single spores than within single spores, but the sequences were all within the range for *G. intraradices*.

DGGE Method

PCR-DGGE has been applied to population diversity studies, especially for uncultured organisms, such as AM fungi (de Souza et al., 2004). The migratory pace of DNA fragments in the polyacrylamide gel depends on their melting points. The melting points are determined by the proportions of purines and pyrimidines that are present in the sequences of DNA segments of interest. The coding region for 18S of rDNA was examined and evaluated for its

usefulness for discriminating among species of Gigasporaceae based on DGGE profiling (de Souza et al., 2004). It turned out that the V3-V4 site of the 18S rRNA gene did not have sufficient sequence variations to discriminate among three *Gigaspora* species, *Gi. margarita*, *Gi. decipiens*, and *Gi.* sp., meaning that all the DGGE fragments from these species were inseparable on the gel. Thus, the DGGE method was not applicable at the V3-V4 region of the 18S rDNA for identification at either interspecies or intraspecies levels. On the other hand, the V9 region located at the 3'end of the 18S rRNA gene expressed sufficient sequence differences that DGGE bands occupied different positions for different species in the study. Moreover, multiple bands were observed for single isolates, indicating multiple sequences within single isolates. The results were reproducible in that DGGE banding patterns remained the same for all isolates within each species and within spores of each isolate. The DGGE patterns divided *Gigaspora* species phylogenetically into two groups. One group comprised *Gi. albida*, *Gi. candida*, *Gi. ramisporophora*, *Gi. rosea* and *Gi. gigantea*, the other group contained *Gi. decipiens*, *Gi. margarita*, and *Gigaspora* sp. The DGGE method allowed differentiation among *Gigaspora* species in soil or plant roots. Detection, however, depends on the relative abundance of the isolate of interest. It may be hard or next to impossible to detect isolates when they represent only 10% or less of the population (de Souza et al., 2004).

Mycorrhizal Research Outlook

Much progress has been made in mycorrhizal research by improving sensitivity, accuracy, reliability, and reproducibility in identification, taxonomic, and phylogenetic studies of AM fungi through adapting molecular techniques that are based on rDNA sequencing analyses (Helgason et al., 1998; Redecker et al., 1997). Multiple AM isolates have been found to coexist in roots of the same or different plant species under field conditions (Clapp et al., 1999; Helgason et al., 1998; Merryweather and Fitter, 1998a, 1998b). Molecular-based methodologies have been used in attempts to identify and characterize individual AM fungal species forming mixed communities in host plant roots and to illustrate the mechanisms involved in AM symbiosis (Harrier, 2001; Harrison, 1999; Smith et al., 2001)

Molecular techniques circumvent many limitations of morphology-based studies. Molecular techniques have the potential to enable researchers to identify and characterize an individual AM fungus in a plant root and classify it at or below the species level. They may even make it possible to evaluate the contribution of each species to plant growth in a terrestrial ecosystem (Rodriguez et al., 2004).

At the moment, there are still difficulties in using molecular techniques when dealing with AM fungal characterization studies under field conditions. Species-specific primers have been difficult to design because rDNA regions vary within a single species or even within single spores (Kuhn et al., 2001; Lanfranco et al., 1999). One of the major concerns in applying molecular techniques for AM diversity studies is the difficulty of designing suitable primers that successfully amplify rDNA only from the target AM fungal species while discriminating against everything else (Anderson et al., 2003). Few primers have been developed for detection of fungal taxa (Redecker, 2000; Simon et al., 1992, 1993; White et al., 1990). Some of these primers either fail to amplify DNA material from the spectrum of organisms they were designed for, or they amplify DNA materials from organisms they were expected to discriminate against (Clapp et al., 1995; Lanfranco et al., 1999). Thus, some investigators had to design their own specific primers either by cloning and sequencing target DNA from their own isolates and or using previously published sequences or sequences deposited in the database (Millner et al., 1998, 2001; Schüßler, 2001). Regions of rRNA genes have been investigated to identify sites that are best suited for determination of AM diversity in natural soils with mixed isolates (de Souza et al., 2004; Jansa et al., 2002).

More work has to be done for understanding the complexity of AM symbiosis and its ecological implications in natural ecosystems, and application of molecular methodologies has the potential to contribute to that work.

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CHAPTER 3. IDENTIFICATION OF AM FUNGI IN SOYBEAN FIELD SOILS

AM fungi in general benefit their host plants when inhabiting roots, but situations where these fungi are neutral or harmful to plants have been encountered, depending on the host-fungus combinations and the surrounding environment (Smith and Read, 1997). Lately, more focus has been directed to determining the significance of individual fungal strains in a functional symbiosis, as a given strain may exhibit different effects on different host plants and different strains may vary in their effects on a given host plant (Kjøller and Rosendahl, 2000). Thus, determination of AM diversity in natural systems is an essential component of understanding the ecology of AM fungi in relation to their host plants and other coexisting living organisms.

Spore morphology has been a valuable tool in taxonomic classifications and diversity studies of AM fungi, but morphology of spores and other propagules has limitations for studies of the functional diversity of AM fungi. Therefore, numerous molecular techniques, often used in combination with morphology, have been exploited to classify AM fungi and to understand their functional diversity. These molecular techniques provide researchers with means for identifying and interpreting variations in the genetic makeup of AM fungi and relating these variations to structure and functions of individual AM fungal species in mixed communities in natural environments.

Morphology-based characterization of AM fungal isolates involves description of fungal structures existing in the mycorrhizosphere. Data about 1) hyphal thickness, coiling pattern, and reactivity to special stains, 2) spore color, shape, size, and their hyphal attachment status, 3) spore cell wall structures in terms of the number, thickness, and flexibility of layers, 4) mode of spore formation (sporogenesis), and 5) observation of spore germination are collected and used to classify AM fungal isolates (Franke and Morton, 1994; Morton and Benny, 1990). The recommended approach for successful spore morphology-based taxonomic classification and diversity studies has been to amplify spores in pot and trap cultures in conjugation with suitable hosts under controlled environments (Clapp et al., 1995). Trap cultures allow for production of healthy spores from AM fungal strains, including those that may not sporulate under field conditions (Bever et al., 2001). In support for this approach, Bago et al. (1998) advised that homogenous populations of freshly

produced spores must be used for constructing taxonomy of certain AM fungal strains isolated from field soils in morphology-based characterization. Freshly collected field spores can be identified by comparing them to those produced in cultures (Franke-Snyder et al., 2001).

Many morphology-based studies have examined AM fungal diversities in wild as well as managed environments with different vegetation, climate, and topography. These studies have indicated that multiple AM fungal species, varying in composition and spatial and temporal distribution, are found in the mycorrhizosphere (Douds et al., 1993; Merryweather and Fitter, 1998a; Troeh, 1999; Walker et al., 1982).

Diversity and spore populations were investigated in two types of soils, a eutric Leptosol (Petrocalcic Calciustoll) and a mollic Vertisol (Typic Pellustert), of central Mexico irrigated with wastewater for 5 and 90 yr (Ortega-Larrocea, 2001). A significantly lower spore population was found in corn fields irrigated with wastewater for 90 yr than in those irrigated for a shorter period, with *G. mosseae* and *G. intraradices* being prevalent and *G. geosporum* and *G. fasciculatum* found in smaller numbers. Vertisols consistently had lower spore numbers than Leptosols. Long-term wastewater treatment increased both P and heavy metal concentrations in the soil and depressed the population of free spores. Sporocarps of *G. mosseae* showed no response to the level of P and heavy metals while sporocarps of *G. sinuosa* showed a positive correlation with the soil content of P and heavy metals.

Microscopy-based identification of AM fungi can be labor intense, time consuming, and prone to error and bias (de Souza et al., 2004). It requires highly renewable doses of energy and patience on behalf of the investigator, and many shortcomings are associated with this microscopy-dependent methodology. In most cases where several AM fungal species coexist, it is almost impossible to make a meaningful ecological assessment, as it is difficult to establish a connection between the soil spores used for identification and intraradical hyphae actually involved in a functional symbiosis in natural settings. Reasons leading to this problem include: spores and hyphae behave independently (Franke-Snyder et al., 2001), spores and hyphae can be broken from the host roots and can be carried away by different dispersal agents, and fungal materials can be lost due to parasitism and physical damage (Bever et al., 2001; Daniell et al., 2001).

Recently, culture-independent molecular techniques, devised for determination of soil microbial diversity, have been adapted for characterization of AM fungi. These include fatty acid methyl ester profiling (Bentivenga and Morton, 1996; Madan et al., 2002), phospholipid fatty acid markers (Sakamoto et al., 2004), isozyme patterns (Bago et al., 1998), monoclonal antibodies toward a mycorrhizal-specific protein (glomalin) (Wright et al., 1998), and sterol profile characterization (Grandmougin-Ferjani et al., 1999). These methods, usually coupled with morphology, provide means for determination of the identity, diversity, and functions of AM fungi.

The use of these methods is restricted, however, especially when the specimens to be characterized are collected from natural ecosystems, because the compounds used in these methods (such as neutral or phospholipid fatty acids, sterols, and chitin) are produced by other organisms besides AM fungi. Thus, their source is not readily determined when the study is carried out in a heterogeneous environment. Also, the compounds have little profile diversity for serving as discriminatory tools among classes of AM fungi, especially at the species level. These methods remain a valuable tool in ecological studies of homogeneous populations of AM fungi. Today, DNA-analysis-based molecular techniques have the lead in providing powerful tools for *in situ* studies of identification and functional biodiversity studies of AM fungi.

Molecular techniques, mainly those involving PCR protocols, have been used extensively because they allow detection, identification, classification, phylogenetic studies, and quantification of AM fungi to be determined not only from spores but also directly from colonized roots (Clapp et al., 1995; Read, 2000). The molecular differentiation among mycorrhizal isolates is based on sequence variations of rDNA and the internal transcribed spacers (ITS) regions. The genes coding for rRNA subunits and ITS have some conserved unique sequences that identify AM fungi in general, as well as sequences that are unique to individual species. Identification of these sequences enabled researchers to develop molecular markers or primers that can be used to detect and identify AM fungi wherever their DNA can be collected.

Several researchers have successfully developed taxon-specific oligonucleotides (TSOs) (Helgason et al., 1998; Millner et al., 1998, 2001a, 2001b; Turnau et al., 2001; van Tuinen et al., 1998; White et al., 1990). The use of PCR approaches based on designed TSOs or

sequencing or both has revealed that numerous AM fungal species coexist in the roots of individual plants in natural ecosystems (Helgason et al., 1998; Kjøller and Rosendahl, 2000; Renker et al., 2003). Reciprocally, individual AM fungal strains have the ability to colonize the roots of plants of different species (Smith and Read, 1997). AM fungi have also been found in the roots of woody plants such as aspen trees along with ectomycorrhizal associations, with AM fungi being prevalent in younger roots and deeper mineral soil layers and ectomycorrhizal fungi prevailing in older roots and at shallower depths where the soil contained more organic matter (Neville et al., 2002). Nine different groups of AM species, with *Glomus* species representing the majority, were detected in the roots of *Littorela uniflora* and *Lobelia dortmanna*, the aquatic plants that live in lakes with oligotrophic soft water, and the diversity was almost as great there as in terrestrial ecosystems (Nielsen et al., 2004).

Design of primers to amplify regions of rRNA genes as well as the ITS regions have enabled researchers to use PCR protocols that are quicker, more reliable, and more bias-free than the older methods (White et al., 1990). Since then, many other methods have been created for expanding the use of PCR to optimally exploit the variations in rDNA sequences between AM fungal isolates for understanding taxonomy, biodiversity, and function of AM fungi in relation to plants and other organisms with which they share the habitat.

Nucleotide sequences of rDNA and ITS have different degrees of variable and conserved regions, depending on the phylogenetic status of the organisms under investigation. The use of PCR coupled with RFLP based on sequencing variations of ITS genes did not provide sufficient resolution among *Gigaspora* species but sufficient genetic variations in this region enabled the researchers to make more concrete distinctions among *Glomus* species (Redecker et al., 1997).

Bago et al. (1998) used sequencing of amplified regions of SSU genes of many identified *Gigaspora* species acquired either from INVAM, Europian Bank of Glomales (BEG), or personal collections. Three six-nucleotide-long signature sequences, corresponding to three groupings of *Gigaspora* species, were identified. Not enough polymorphism was detected on their SSU genes to allow for identification of individual species.

A different approach was taken when PCR in conjugation with DGGE was used to create profiles of the SSU gene of AM fungal species of *Gigaspora* collected from grassland soils

of a cattle farm in Brazil. The sequence variation of V3-V4 located at the 5'end of the SSU gene was useless, but that of V9 allowed identification of individual *Gigaspora* species based on DGGE band patterns (de Souza et al., 2004).

Primer fidelity is another problematic issue in using PCR-based analysis. Designing primers that would amplify exclusively their intended target sequences has been a great challenge. Situations have been encountered where primers fail to amplify rDNA sequences from some of the organisms they were designed for or amplify DNA from other organisms that are out of their scope (Clapp et al., 1995;Vandenkoornhuyse et al., 2002). This could lead to diversity underestimation, confusion, or both. Sometimes even species-specific primers fail to generate the expected PCR responses from known species, so researchers are forced to design their own primers (Millner et al., 2001b). Designing primers that do not always work, assuming the accuracy of everything else, could be attributed partially to misidentification of the AM fungal isolate either by the designer or the user of the primer, or to the multinucleate nature of AM fungi. Hundreds to thousands of nuclei, harboring different genomes, found in single spores or in short lengths of hyphae, can generate high levels of genetic heterogeneity even within a single spore (Clapp et al., 1999; Kuhn et al., 2001). The fact is, there are still AM fungal isolates that have not been identified, and markers or primers have yet to be designed for many others that are classified.

Molecular probes or primers have not been designed for all AM fungal species, but many possibilities to charaterize them exist. Sequences for some AM fungal species deposited in the EMBL/GeneBank (NCBI) sequence database can be used to design primers, and computer software programs, such as Primer Express software designed by Perkin-Elmer Biosystems, Norwalk, CT, are available for this purpose (Alkane et al., 2004). The rRNA and ITS genes have not yet been sequenced for some AM fungal species. Thus, one approach is to use universal primers or any workable primers available to amplify a region of the rRNA genes from the organism to be characterized and sequence that region. The next step is to use blast searches to find homologous DNA sequences in the GenBank and determine the identity and classification of the AM fungal species of interest (Vandenkoornhuyse et al., 2002).

High diversity of AM fungi, their widespread occurrence among plants, and their effects on plant growth, diversity, health, productivity, and the stability of the ecosystem provide

ample justification for studying AM fungi. We need to know how many species exist in an agricultural setting, what each species of the community is contributing to the symbiosis, and how the symbiosis is affected by other biotic and abiotic factors.

The present study assessed the population composition and relative abundance of individual species of AM fungi in soils of Iowa soybean fields. The diversity of AM species was determined not only on morphotypes of spores that were initially extracted from soils, but also in trap cultures that were used to stimulate sporulation of some AM species that might have failed to produce spores in their natural environment. In addition to that, and as time and resources permitted, identification of a few AM species was confirmed with rDNA and ITS fingerprinting analysis, using previously developed TSOs.

MATERIALS AND METHODS

Characteristics of the Soil Sampling Sites

This study was designed to determine the population composition and relative abundance of native AM fungi in soils of soybean fields within Story County, central Iowa. Four field locations (private farms) within about five km south of Ames, Iowa, were used for this study. All the fields had been under corn (*Zea mays* L.) soybean (*Glycine max* L.) rotation crop management and were all within 6 km from each other. The legal descriptions of the field locations were:

Field 1: in the SE ¼ of the NE ¼ of Section 29, T83N, R24W

Field 2: in the NW ¼ of the NW ¼ of Section 29, T83N, R24W

Field 3: in the SW ¼ of the SE ¼ of Section 30, T83N, R24W

Field 4: in the NW ¼ of the SW ¼ of Section 21, T83N, R24W

Sites representing two soil types, Clarion and Webster, that differ in soil drainage classes and are members of the Clarion-Nicollet-Webster association that is dominant in north-central Iowa, were chosen from each field. Clarion is a well drained soil and is described as a fine-loamy, mixed, mesic Typic Hapludoll. Webster is a poorly drained soil and is classified as a fine-loamy, mixed, mesic Typic Endoaquoll.

Soil Sampling

Soil samples were taken after soybean harvest in the fall of 2002 from the topsoils of four fields to a depth of 15 cm with a 10-cm diam. probe. Both Clarion and Webster soils were sampled in each field. Within each soil type, five soil samples were collected from an area approximately 3 m in diam., with 2 m between adjacent samples. The soil probe was positioned so that the probe and the core to be excavated were centered on a plant root system. The soil samples were placed in self-sealing plastic bags and taken to the laboratory for processing. Soil and roots, with the exception of the axial roots, were chopped up and passed through a 6-mm sieve, and the soil-root mixture of each of the forty soil samples was air-dried and then stored in a cool room at 4°C, awaiting analysis.

Chemical Properties of Field Soil Samples

For chemical analysis, a composite sample from each soil type of each field was formed from the mixture of the five samples. Duplicate subsamples of each of the composite samples were analyzed for N, P, K, Ca, Mg, and O.M. at the Soil and Plant Analysis Laboratory, Iowa State University.

Spore Collection and Enumeration

Spore counts were determined from duplicate 50 g subsamples from each soil sample following the methods of Adelman and Morton (1986), Porter et al. (1987), and Sylvia (1994). The soil was suspended in 300 mL of 2% sodium polyphosphate and mixed for 15 s at low speed in a Waring blender as described by Moutoglis et al. (1995). The suspension was washed through stacked 425- and 45-μm sieves with a jet of water. The material left on the 425-μm sieve was examined under a binocular microscopic for large spores and sporocarps, and the material retained on the 45-μm sieve was centrifuged in a 20/60% sucrose gradient at 1300 g for 5 min. The supernatant was pipetted into a 35-μm sieve, washed, and then transferred to a grid-lined Petri dish. Healthy-looking spores were counted in sterile water using a research dissecting microscope at 30 to 50X magnification. The pellets at the bottom of the 60-mL centrifuge tubes were also examined for large spores and spores that were still attached to branched hyphae trapped in the settled soil. The number of spores per g of soil was expressed on the soil dry-weight basis.

Determination of AM Fungal Root Colonization

Fine roots collected from the soil samples were washed with water to remove soil particles and other debris and then air-dried. An amount of 0.25 g of air-dried roots from each sample was soaked in water for 2 h and cleared based on the modified method of Kormanik and McGraw (1982). Root samples embedded in porous filter paper were placed in Tissue-Tek plastic capsules and were cleared with 10% (w/v) KOH for 1 h in a water bath at 90-95°C instead of the 121°C used by Kormanik and McGraw. The roots that did not clear

the first time were subjected to another clearing cycle. Cleared roots were rinsed with several changes of water and subsequently acidified in 1% HCl (v/v) for 30 min and stained with 0.05% (w/v) trypan blue prepared in lactoglycerol composed of equal volumes of lactic acid, gycerol, and water. This was followed by incubation of the stained roots at 90-95°C for 1 h. After the heat treatment, the stained roots were transferred to glass vials that contained 50% glycerol (v/v) to remove the stain from non-fungal structures as described by Phillips and Hayman (1970). Assessment of root colonization was determined on two replicates of about 33 stained root segments 1-cm long in a grid-lined square Petri dish. Microscope slides were placed on the roots and slight pressure was applied for better microscopic observations. The root colonization percentage was determined by a gridline intersect method as described in Giovannetti and Mosse (1980) and Kormanik and McGraw (1982).

Propagation of AM Fungal Spores

In the spring of 2003, two greenhouse experiments were simultaneously conducted for determination of the population composition of AM fungi native to soils of soybean fields. Experiment 1 was designed to evaluate the diversity composition of AM fungi among the soils, while experiment 2 investigated the variability of AM fungal species among five samples of Webster soil in each of two fields.

The spore counts in the initial soil samples were low, but the percentages of AM root colonization were relatively high. Propagation of spores was needed to produce larger numbers of healthier spores for identification purposes and subsequent experiments. Another reason for greenhouse growth was to allow production of spores from fungal strains that may not have sporulated under field conditions (Bever et al., 2001).

Preliminary Single Spore Culture Trials

Attempts were made in the summer of 2002 to mass-produce spores from single spore cultures in preliminary experiments, using spores extracted from prairie soils, field soils, and commercial inocula acquired from INVAM (Morgantown, WV). White clover plants were used as host.

Spore surface disinfection

Spores were surface disinfected, following the procedure described by Shirling and Gottlieb (1966) The spores were immersed in an aqueous solution of 0.05% (v/v) Tween 20 for 2-min periods and then rinsed five times with sterile deionized water in a vacuum filter. The next step involved spore incubation in sterile Petri dishes containing 5% (v/v) chloramine T for 20 min followed with a minimum of 3 rinses with sterile water. The spores were subsequently stored for two months in an antibitotic solution that had been sterilized by passing through a 0.22-μm filter. The antibiotic aqueous solution contained 50 mg chloramphenicol, 100 mg gentamicin sulfate, 200 mg streptomycin sulfate, and 20,000 units of penicillin G potassium per liter.

Germination of spores and plant seeds

Seeds of white clover, *Trifolium repens*, obtained from Dr. Charles Brummer of the Agronomy Department, Iowa State University, were surface disinfected with 10% sodium hypochlorite for 2 min and rinsed with five changes of sterile deionized water. Thirty seeds were placed in each of several Petri dishes containing a mixture of autoclaved finely ground perlite-vermiculite mixture.

Simultaneously, one surface-disinfected spore was germinated in a Petri dish containing Gel-gro medium, following the method described by Fracchia et al. (2001). Ten spores of four morphotypes, including *G. mosseae*-like spores, were selected from surface-disinfected spores extracted from Black Prairie and Ketelson Marsh and their adjacent cultivated fields that are described in Troeh (1999). Ten spores each of *G. mosseae* (DN990), *G. caledonium* (DN986), and *S. calospora* purchased from INVAM were also included in the study.

Only *G. mosseae* spores had a promising germination rate of 50-75%. The germination rates of the other spore types were in the 0-25% range. Some of the spores that failed to germinate were contaminated while others showed no obvious change.

Inoculation and growth of clover seedlings

The 2-wk-old clover seedlings in the perlite-vermiculite mixture were carefully transferred to the top of a Petri dish with a healthy germinating spore. About 30 Petri dishes were set up. About one-half of the Petri dishes were discarded because of seedling die-off by the time they were transferred to pots containing 500 mL of soil-sand, 2:1 mixture, three weeks after the first transfer. The seedlings were incubated in a growth chamber with a photoperiod of 16 h, a day temperature of 25°C, a night temperature of 20°C, and a relative humidity of 60%. Examination of roots of the surviving plants four weeks later indicated that only three Petri dishes contained roots that exhibited 10-20% AM fungal colonization.

Termination of single spore culture experiment

Single spore cultures are time-consuming and labor intensive, with a very low success rate in our experiment. The white clover seedlings failed to maintain their growth, especially after their transfer to Petri dishes containing germinated single spores. The survival of the seedlings was marginal. Two subsequent attempts yielded no better results. Thus, the approach of mass-producing spores from single spore cultures was abandoned due to the low success rate. The outcome did not show promise that a sufficient number of spores to be used in subsequent experiments could be produced in the given time frame.

Trap Pot Cultures

The single spore culture method did not work, so we resorted to using trap cultures as a practical approach. Production of spores was achieved with trap cultures in which pots containing autoclaved soil-sand mixture were inoculated with field soils and were sown to different soybean cultivars as hosts. Soybeans were grown in a greenhouse under conditions expected to stimulate sporulation.

Growth substrate

Low P soil was collected from an uncultivated area next to an abandoned railroad at the Iowa State University Agronomy and Agricultural Engineering Research Farm located in Boone County, Iowa. The soil was excavated from NW 1/4, SE 1/4, Section 9, T83W, R25W of the 5th Principal Meridian. The soil was sieved with a 6-mm sieve and transported to the laboratory where it was placed in autoclavable bags. The soil was steamed three times for 2 h at 100°C. Before each autoclaving, the soil was mixed in the bags and incubated at room temperature for 48 h, allowing for fungal spore germination. Earlier trials of plating autoclaved soil on Potato Agar Dextrose showed that this treatment was sufficient to produce soil medium free of fungal growth. Silica sand was also autoclaved for 2 h at 121°C. The autoclaved soil had 14.5 mg kg^{-1} Bray 1 extractable P, 70.5 mg kg^{-1} NH_4OAc extractable K, 3203 mg kg^{-1} exchangeable Ca, and 421 mg kg^{-1} exchangeable Mg, the organic matter content was 50 g kg^{-1}, and the soil pH in 1:2 soil:water was 6.6.

Preparation of pots

Plastic pots and other non-autoclavable items used in this experiment were sterilized by immersion in 75% ethanol for at least 10 min and rinsed three times with sterile deionized water. Pieces of autoclaved cotton balls were placed in the four drainage holes at the bottom of each pot to prevent soil loss but allow water drainage. The autoclaved soil and silica sand were mixed in a 40:60 ratio on an oven-dry weight basis, and 2 kg of the mixture was placed in each 2-L pot.

The pots were placed on greenhouse benches and leached with autoclaved water every other day for a week to remove toxic substances and salt accumulation that might have resulted from autoclaving. Autoclaving has been shown to increase the concentration of certain toxic ions in soil, such as extractable Mn (Lopes and Wollum, 1976 as cited by Wolf and Skipper, 1994). Also, the autoclaved soil was inoculated with a bacterial suspension free of AM fungal propagules to reduce the possible toxic effect of autoclaving (Rovira and Bowen, 1966) and to reintroduce the native microorganisms to the potted soil. One part of homogenized soil inoculum was diluted with 9 parts of sterile deionized water. The

suspension was filtered through Fisherbrand P5-filter paper at least twice to remove AM fungi and other particles larger than 8 μm. The filtrate was added to the pots at the rate of 0.1 mL per 25 g of soil 1 wk prior to inoculation and planting.

Estimation of field capacity of the potting soil

The field capacity of the potting soil was determined by saturating the soil with water in several pots that contained the same amount of potting soil as those actually destined for soybean growth in this study. The pots were covered with aluminum foil to prevent surface evaporation and then allowed to drain for three days. The pots were then weighed, and their average weight was used to adjust the pot weight in the greenhouse to field capacity when they were irrigated with water twice a week.

Fungal inoculum for experiment 1

A composite sample was made by mixing 800 g of soil from each of the five homogenized soil samples that were taken from each soil type within each field. From each composite sample, 150 g of soil used as inoculum was mixed with the 2-kg soil-sand mixture, previously prepared. The AM-fungal-free soil was emptied into a sterile self-sealing plastic bag and mechanically mixed with the field soil before the bag content was poured back into the pot. An equivalent amount of 150 g from the composite soil sample from the two soil types was autoclaved and added to individual control pots in the same manner as the composite inoculum. All weights in these experiments were expressed on the soil dry weight basis, unless otherwise stated.

Four genetically different soybean (*Glycine max* L. merr) cultivars served as biological hosts for the propagation of indigenous AM fungi from the field soil samples:

> BSR201soybean cultivar was an improved line, and the seeds were obtained from the Iowa Crop Improvement Association, Ames, Iowa.

> Mandarin (strain PI548379), an unimproved cultivar, was provided by Dr. Randall Welson, Curator of the USDA Soybean Germplasm Collection, Urbana, Illinois.

> Iowa2052 and Peking (strain PI548402) soybean cultivars were obtained from the research group of Dr. Walter Fehr, a professor at Iowa State University.

Soybean seeds were surface disinfected by soaking them in 70% ethanol for 1 min and rinsing them with five changes of sterile deionized water. Four seeds were planted in each pot. Two weeks later, the seedlings were thinned to two per pot.

The four soybean cultivars were used as host plants, and all the fields were represented, and each treatment was replicated 5 times. Thus the experimental design for this experiment was as follows:

Inoculated pots: 4 soybean cultivars x 2 soil types x 4 fields x 5 reps = 160 pots.

Control pots: 4 soybean cutivars x 1 composite soil sample x 5 reps = 20 pots

Total = 180 pots.

Fungal inoculum for experiment 2

Each field soil sample was homogenized by chopping the soil and roots into pieces small enough to pass through a 6-mm sieve. After thorough mixing, 150 g of soil was used as inoculum and was mixed with the 2-kg soil-sand mixture, previously prepared. The combination was poured back into the pot. The AM-fungal-free pot soil was emptied into sterile self-sealing plastic bags and mechanically mixed with the field soil before the mixture was poured back into the pot. For controls, 150 g of composite soil samples from either mixture of Clarion or Webster soils was autoclaved and added to pots. All soil weights in these experiments were expressed on an oven-dry weight basis.

Two soybean cultivars, BSR201 and Iowa2052, were used as host plants, and only Webster soil of fields 1 and 4 was represented, and each treatment was replicated 4 times. Thus the experimental design for this experiment was as follows:

Inoculated pots: 2 soybean cultivars x 5 soil samples x 2 fields x 4 reps = 80 pots.

Control pots: 2 soybean cutivars x 4 reps = 8 pots

total = 88 pots

Greenhouse growth conditions

The pots were arranged on the greenhouse benches in a completely randomized block design. Daylight temperature was 30° C, and night temperature was 25°C with a photoperiod

of 16 h. Soybean plants received natural sunlight estimated to represent 85% of the outdoor sunlight supplemented with a photon flux density of approximately 150 μmol m^{-2}s^{-1}.

The pots were irrigated every other day with autoclaved water and received nutrient solution once a week beginning two weeks after planting. An N-free nutrient solution intended for snake beans (Broughton and Dilworth, 1971) was modified for use in this experiment. Half of the recommended amount of P was used, and N was added to make 5% of the nutrient solution (w/v). The volume of liquid to be added to several of the pots to bring them to field capacity was estimated from the field-capacity weights determined earlier. The volumes added to these pots were averaged and the average liquid volume was dispensed to the rest of the pots.

Harvesting plants and processing soil and roots

The plants were grown in the greenhouse until reproductive stage R6-7, when pods start to mature. The cultivars differed in the length of time it took them to reach the maturity stage set for harvest. Mandarin and Iowa2052 were the first cultivars to be harvested, 10 weeks after planting. BSR201 plants were harvested about 4 weeks later when they reached R6-R7, 14 weeks after planting. It took an additional 17 days for Peking, the last cultivar to be harvested, to reach R6-R7.

Harvest involved cutting the plant shoots at the soil surface from each pot. The contents of the pots were loosened, and the large roots were picked from the soil while the small roots were separated from the soil by sieving. Roots were carefully freed from soil, gently washed with water, air-dried on tissue paper, and stored at –4°C for later use.

Spore Morphology-based Identification of AM Fungal Species

Microscopy Observation of Spores

Spores were extracted from 50- to 100-g subsamples of each soil sample by using wet sieving coupled with a 20/60% sucrose-gradient centrifugation, as described above. Spores were washed into glass Petri dishes and examined under a microscope. Healthy spores that

did not show any symptom of parasitism were picked with Pasteur pipets whose openings were adjusted by heat stretching to pick up spores of different sizes. Spores were sorted into groups of similar morphologies (color, shape, size, hyphal attachment status, presence or absence of saccules, etc.) called morphotypes. The spores were counted to determine the relative abundance of each species and the total number of spores g^{-1} on an oven-dry weight basis.

Description of Slide-mounted Spores

Two sets of 20 to 30 spores were placed in separate areas on labeled microscope slides. To one set, two drops of polyvinyl-lacto-glycerol (PVLG) were added; one drop of PVLG and one drop of Melzer's reagent were added to the second set of spores (Koske and Tessier, 1983). A cover slip was placed on each set and localized light pressure was applied to break open cell walls of some of the spores. The slides were incubated at room temperature for at least 3-5 days before being examined under a light microscope. The number of cell wall layers, the reaction of individual layers with the stains, and the flexibility of the layers were observed and recorded. The subtending hyphae, the continuity of the spore cell walls with those of subtending hyphae, the existence of a septum or an occlusion were also considered. These characteristics were compared to the descriptive features of described species posted on the INVAM website. Each morphotype was given the name of the species with which it had the closest match.

The percentage of individual AM fungal species was calculated as a percentage of the total number of spores in the examined soil sample. All the morphotypes present in each examined soil sample added up to 100%. The frequencies of occurrence of AM fungal species in the five soil samples taken from each field were tabulated. The percentage of samples that contained each AM fungal species was calculated as a relative frequency.

Ribosomal DNA-based Identification of AM Fungal Species

The identification of four of the prevalent *Glomus* species was confirmed by analyzing ribosomal DNA from spores and a few roots of soybean plants obtained either directly from

field soil samples or from field-soil-based trap cultures. Variations in sequences of ribosomal DNA regions were used to distinguish among AM fungal species, based on the ribotyping approach.

Spore Genomic DNA Extraction

Many researchers have used commercial kits for extraction of genomic DNA from either spores or colonized roots of AM fungi. The manufacturer's instructions for the use of Ultra Clean^{TM} Soil DNA isolation Kit and UltraClean microbial DNA isolation kit (Mo. Bio. Laboratories, Carlsbad, CA), intended for isolation of DNA from soil or bacteria, respectively, were modified and applied to DNA extraction from mycorrhizal roots (Koide and Dickie, 2002; Ishii and Loynachan, 2004). Several other kits have been used to extract DNA for either roots or spores of AM fungi (Alkan et al., 2004; Kowalchuck et al., 1997). Commercial kits are found to be fast and efficient, but they are expensive. In this study, simpler DNA extraction protocols were used as in Clapp et al. (1999), Jansa et al. (2002), Turnau et al. (2001), and van Tuinen et al. (1998a, 1998b).

Healthy-looking spores of each morphotype were examined, freed from any attached hyphae, and cleaned with 4-5 rinses of sterile nanopure water in microcentrifuge tubes by vortexing and centrifugation (Clapp et al., 1999). Except for *G. intraradices* where 5-6 spores per tube were used, one spore was deposited at the bottom of a 1.5-mL microcentrifuge tube with the aid of a tipped 0.1-2.5 µL Eppendorf micropipet with minimum water volume. Sometimes, the spores were moved to the bottom of tubes by centrifugation. Spores were crushed (cell lysis) in 10 µL of sterile nanopure water with micropestles, or glass pasteur pipets whose ends were flame-sealed. To each tube, an additional 40 µL of sterile nanopure water followed by 15 µL of 20% (w/v in TE buffer) chelex resin 100 (BioRad Laboratories, Hercules, CA) were added. The tube caps were pierced using a flame-sterilized dental probe to avoid pressure buildup inside the tubes that may cause them to open while being heated. The tubes were subsequently placed in a water bath for 10 min at 95-98°C. The tubes were cooled on ice prior to being centrifuged for 5 min at 1400 rpm (12,000 g). The supernatant (crude DNA) was diluted ten times to serve as a

template. This same procedure was applied to extract DNA from 0.25- to 0.5-cm segments of roots. The DNA-containing tubes were stored at –20°C if not immediately used.

PCR Protocols

Identification of *G. mosseae*, *G. intraradices*, *G. claroideum*, *G. etunicatum*, and *Gigaspora* species was performed with nested PCR to amplify regions of 18S rRNA genes of these species in spores or in infected roots as described by Redecker (2000). Universal fungal primers, NS5 and ITS4, were used in the first PCR amplification and produced 1200 bp nucleotides. Species-specific primers paired with universal primers GLOM1310/ITS4 for *G. mosseae*/*G. intraradices*, LETC1670/ITS4 for *G. claroideum*/*G. etunicatum*, and NS5/GIGA5.8 for *Gigaspora* species were used in the second PCR amplification (Table 3.1).

The final concentrations of the reaction mixture components and the PCR conditions for the first and second amplifications were the same as those used by Redecker et al. (1997) (Table 3.2). One μL of 10-fold diluted genomic DNA extract was used as a template, and 1 μL of sterile nanopure water was used for PCR controls. The PCR amplifications were performed as suggested by Redecker et al. (1997). Initial denaturation of 3 min at 95°C was followed by 35 cycles of DNA denaturation for 30 s at 95°C, primer annealing for 30 s at 51°C, and extension for 60 s at 72°C. The PCR reactions were concluded with an extension step of 5 min at 72°C.

Glomus etunicatum was identified by nested PCR performed as described by Millner et al. (2001b) with minor modifications. The identification was based on amplification of ITS regions flanking the 5.8S rRNA gene. The first PCR yielded about 600 bp DNA fragments with ITS1mod and ITS 4 primers. The second PCR was performed using primers GETU1 and GETU2 that are specific for *G. etunicatum* to produce 400-bp DNA fragments (Millner et al., 2001b). Table 3.2 lists the reagents and their final concentrationsin the 12.5-μL reaction mixture used in PCR amplification reactions.

A 5-μL volume of ten-fold diluted genomic DNA extract was used as a template in the 12.5 μL first PCR reaction mixture. The products of the first PCR amplification were diluted 100 fold, and 5-μL aliquots were used as templates for the second PCR amplification. The controls for the PCR runs were treated the same as the samples, except that 5-μL aliquots of

Table 3.1. Primers[†] used in this study for amplification of fungal rRNA genes.

Primer name	Organism	Target sequence	Primer sequence (5'→3')	Reference
LR1	eukaryotes	LSU	GCA TAT CAA TAA GCG GAG GA	Jacquot et al., 2000
NDL22	eukaryotes	LSU	TGG TCC GTG TTT CAA GAC G	van Tuinen et al., 1998b
FLR2	fungi	LSU	GTC GTT TAA AGC CAT TAC GTC	Jacquot et al., 2000
ITS1 F or ITS1	fungi	ITS	TCC GTA GGT GAA CCT GCG G	White et al., 1990
ITS1mod	fungi	ITS	TAG GTG AAC CTG CGG AAG GAT C	Millner et al., 2001b
ITS3	fungi	ITS	GCA TCG ATG AAG AAC GCA GC	White et al., 1990
ITS4	fungi	ITS	TCC TCC GCT TAT TGA TAT GC	"
NS5	fungi	18S	AAC TTA AAG GAA TTG ACG GAA G	"
GIGA5.8R	Gigasporaceae	18S	ACT GAC CCT CAA GCA TGT G	Redecker, 2000
BRAS-86	G. brasilianum	ITS	TGT ATT GGA TCA AAC GTC	Millner et al., 2001a
GBRAS-388	G. brasilianum	ITS	CGC TAT TCA TTG TGC ACT	"
38.21	G. claroideum	LSU	TGG GCT CGC GGC CGG TAG	Jacquot-Plumey et al., 2001
GETU1	G. etunicatum	5.8S	GTA TTC AAA ACC CAC ACT	Millner et al., 2001b
GETU2	G. etunicatum	5.8S	TCG TAA TTG CTT GAT GAG	"
LETC1670	G. etunicatum	18S	GAT CGG CGA TCG GTG AGT	Redecker, 2000
cad 4.1	G. gerdemannii	LSU	TCG AGT ATT GCT GCG ACG A	Turnau et al., 2001
cad 4.2	G. gerdemannii	LSU	CTC AAG TGT CCA CAA CTG C	"
8.22	G. intraradices	LSU	AAC TCC TCA CGC TCC ACA GA	van Tuinen et al., 1998a
5.21	G. mosseae	LSU	CCT TTT GAG CTC GGT CTC GTG	"
GLOM1310	G. moss/intrarad	18S	AGC TAG GCT TAA CAT TGT TA	"
GOCC-56	G. occultum	ITS	CAA CCC GCT CKT GTA TTT	Millner et al., 2001a
GOCC-427	G. occultum	ITS	CCA CAC CCA KTG CGC	"
cad 5.1	G. occultum	LSU	GAA GTC TGT CGC AGT CTG	Turnau et al., 2001
cad 5.3	G. occultum	LSU	TCG CGA AAG CTT GTG	"
ARCH1311	G. occultum	18S	TGC TAA ATA GCT AGG CTG Y	Redecker, 2000

[†] All primers were manufactured by Integrated DNA Technologies, Coralville, Iowa.

sterile deionized water were used instead of templates. Sometimes, DNA templates from other AM fungal species were included as checks.

The PCR tubes were pulse centrifuged before being loaded into a MiniCycler™ (MJ Research, Waltham, MA). The reaction cycles were as follows: The initial denaturation was for 9 min at 95°C followed by 40 cycles of 30 s at 95°C, 45 s at 55°C for annealing, and 60 s at 72°C for extension. The final extension was 5 min at 72°C.

Glomus claroideum, G. intraradices, G. mosseae, and *G. occultum* were identified in AM spores and colonized roots by the 3-step nested PCR, using primers that can detect these species based on variations in sequences located on the large rRNA subunit gene. The chemicals for the PCR reaction mixture and the program for running PCR complied with the method of Turnau et al. (2001) as shown in Table 3.2.

Table 3.2. Reagent concentrations used in PCR amplification of rRNA regions of AM fungal spores and roots in this study for identification and classification of AM fungal isolates.

| | Final concentrations of the PCR ingredients in PCR tubes | | | |
| | Redecker et al., 1997 2-step nested PCR | Millner et al., 2001b 2-step nested PCR | | Turnau et al., 2001 3-step nested PCR |
Reagent	1st and 2nd Amp[†]	1st Amp	2nd Amp	1st, 2nd, and 3rd Amp
Sterile water	Sufficient water was added to adjust the final volume to 12.5 µL/tube			
PCR buffer	1 X (10 X buffer was obtained from Invitrogen, diluted to 1 X)			
$MgCl_2$	1.5 mM	1.5 mM	1.5 mM	1.5 mM
BSA[‡]	0.2 µg µL^{-1}	0.2 µg µL^{-1}	0.2 µg µL^{-1}	0.2 µg µL^{-1}
dNTP mix	200 µM	200 µM	200 µM	200 µM
Primer (F)	0.5 µM	0.25 µM	0.50 µM	0.5 µM
Primer (R)	0.5 µM	0.25 µM	0.50 µM	0.5 µM
DNA Poly[§].	0.05 U µL^{-1}	0.025 U µL^{-1}	0.025 U µL^{-1}	0.025 U µL^{-1}
DNA Template	1 µL	5 µL	5 µL	5 µL

[†] Amp = amplification reaction

[‡] BSA = bovine serum albumin.

[§] DNA Poly. = Platinum Taq Polymerase with the manufacturer's PCR reaction buffer (Invitrogen, Life Technologies, Carlsbad, CA

For the first PCR amplification, 5 µL of 10-fold diluted crude DNA was used as a template, and the target rDNA region was amplified using primers ITS3 and NDL22. For the second PCR amplification, 5 µL of the first PCR amplicons diluted 200 times was used as a template with LR1 and FLR2 primers. Species-specific primers 38.21-FLR2 for *G. claroideum*, 5.21-FLR2 for *G. mossea*, 5.1-FLR2 or 5.3-FLR2 for *G. occultum*, and LR1-8.22 for *G. intraradices* were used in the third PCR amplification. A volume of 5 µL was drawn from 200-fold dilution of the second PCR products and used as template DNA for the third PCR amplification. The DNA template was replaced by 5 µL of sterile nanopure water for the controls.

The PCR amplification was carried out as follows: the initial denaturation for 3 min at 95°C, annealing 60 s at 60°C, and extension for 60 s at 72°C. This was followed by 30 cycles of denaturation for 60 s at 93°C, annealing for 60 s at 60°C, and extension for 60 s at 72°C. The PCR amplification was concluded with a final 5 min extension at 72°C.

Gel Electrophoresis Analysis

Gels were prepared with 1.2% (w/v) low-melting agarose in Tris-acetate TAE buffer (Sambrook et al., 1989). Ethidium bromide was added to the gels at a final concentration of 0.5 μg mL^{-1}. The gel was poured onto a gel bed and allowed to solidify. Then, the gel was placed in an electrophoresis tank containing TAE solution. Aliquots of 3 μL of PCR-amplified products were mixed with a loading buffer, bromophenol blue, and were loaded into the gel wells. The gel was electrophoresed at 57 volts. The DNA bands were visualized and some were photographed with a Foto/Analyst UV transluminator, using PC Image software (Foto/Analyst® Fotodyne, Inc., Hartland, WI). A 100-bp or occasionally a 1-kb DNA ladder was used as a marker.

Statistical Analysis

The data collected for the determination of the relative abundance of AM fungal species were analyzed using a one-way analysis of variance (ANOVA) and least significance difference (LSD) using SAS®\ (Statistical Analysis System) programs (SAS Institute Inc., Cary, NC). Statistical analysis of the relative abundance of AM fungal species and spore count in field soil samples was based on a complete randomized design. The percentage of AM fungal species in soil samples, root colonization, and spore counts in greenhouse pots were analyzed based on a randomized complete block design.

RESULTS

Spore morphology-based identification of AM fungal species indicated the dominance of *Glomus* species in the soils of the four soybeans fields used in this study (Fig. 3.1). Members of other genera were also detected, including those of *Acaulospora*, *Entrophospora*, *Gigaspora*, and *Paraglomus*. The predominant AM fungal morphotypes recognized in these soils included *G. claroideum*, *G. etunicatum*, *P. occultum*, *G. mosseae*, and *G. viscosum*, and minor species that were detected in these soils included *G. constrictum*, *G. geosporum*, *G. intraradices*, species of *Acaulospora*, species of *Entrophospora*, and species of *Gigaspora*. The data for the species richness and composition of AM fungi were obtained by combining the results from identification of spore morphotypes in soil samples and trap cultures.

The distribution of AM fungal species and the relative abundance of native AM fungal species were highly variable in the field soil samples both within and among fields, although the species composition was not very different within or between the sites, with a few exceptions. Some species such as *G. claroideum* and *G. viscosum* were consistently found in the examined soil samples, regardless of the soil type or the field site. The distribution pattern of the other AM fungal species was more erratic, as they were found in some sites and not in others. The relative abundance and the presence or absence of certain species varied even from sample to sample for the same soil type in the same field.

High variability in the percentage distribution of AM fungal species was related to low spore counts combined with the patchy pattern of the distribution of AM fungi in the soil mycorrhizosphere. Spore counts were generally low in the freshly examined soils, especially in fields that tested high in phosphorus.

The community composition of AM fungal species varied widely from one sample to the next over short (2-m) distances. ANOVA statistical analyses using SAS showed that neither soil type nor location had any consistent significant effect on the distribution of individual AM fungal species in the field sites, although the percentage of some species was several times as high in some locations or soil samples as in others.

It was recognized that direct examination of freshly collected soils for AM fungal species might not identify all AM fungal species present in these soils when spore morphology was
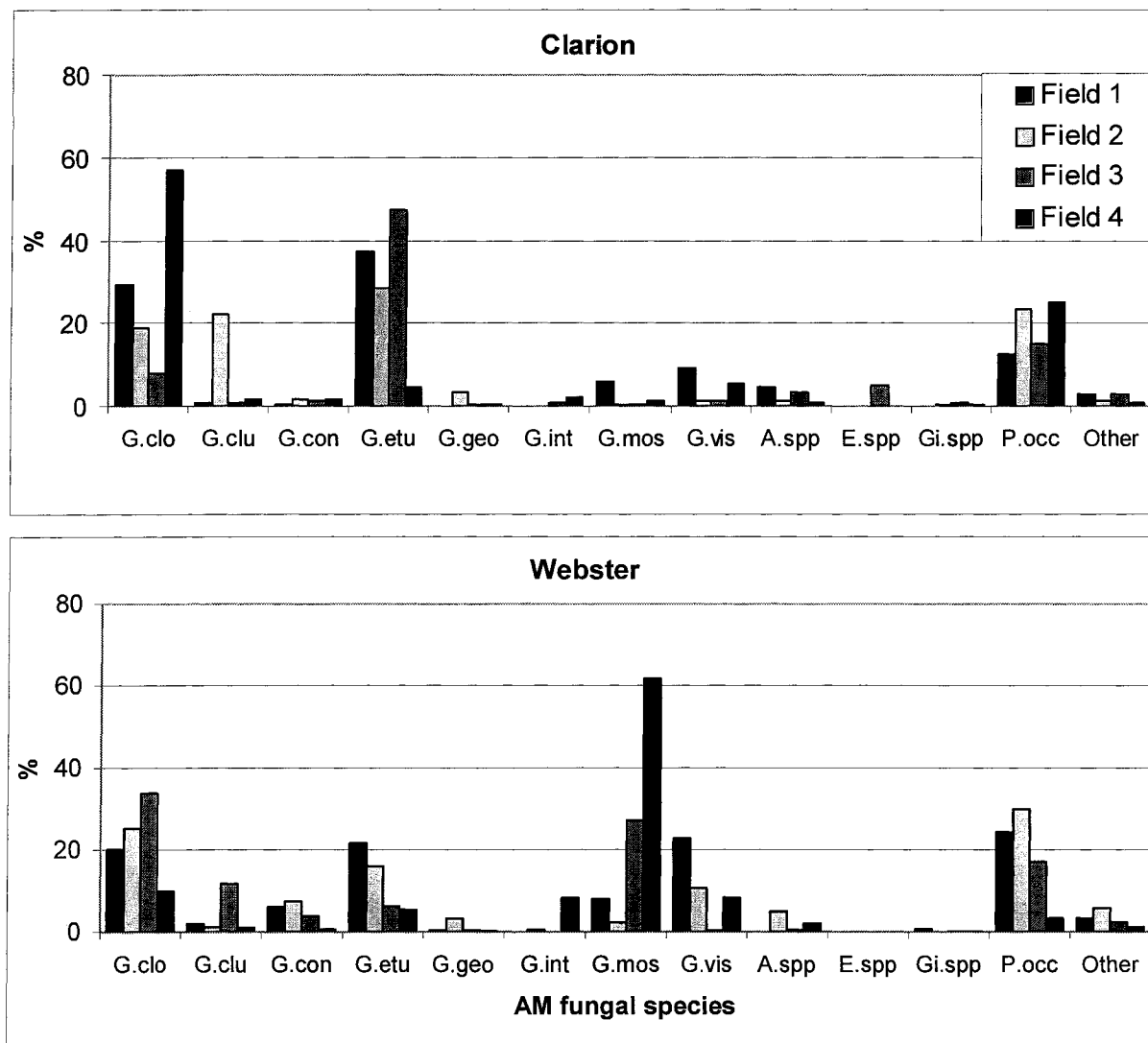
Fig. 3.1. Mean percentages of spores of AM fungal species from field soils as identified in trap cultures using four soybean cultivars. The inoculum came from a composite of the five soil samples for each soil in each field. G.clo = *Glomus claroideum*, G.clu = *G. clarum*, G.con = *G. constrictum*, G.etu = *G. etunicatum*, G.geo = *G. geosporum*, G.int = *G. intraradices*, G.mos = *G. mosseae*, G.vis = *G. viscosum*, A.spp = *Acaulospora* species, E.spp = *Entrophospora* species, Gi.spp = *Gigaspora* species, P.occ = *Paraglomus occultum*, and Other = unclassified AM fungal species.

the basic tool for identification partly because some AM fungal species do not sporulate under certain field conditions. Consequently, the data for AM fungal species initially identified in these soils could lead to an underestimation of the actual population diversity of the AM fungal community in the surveyed sites. On the other hand, some spores collected from the soil might not have been derived from root colonizing fungus at the time of the sampling, as it is difficult to determine the exact age of a spore at the time it is collected. Until simple and quick means of establishing a connection between the detached soil spores and their parental mycorrhizal roots are developed, other approaches such as direct identification of AM fungal species in the roots with rDNA-based molecular techniques have to be used to provide a reliable basis for determination of AM fungal species in field soils. These are expensive and time-consuming techniques. Not all investigators have the resources to use these alternatives, and they were not used in this study.

AM fungal spore numbers were very low in our soil samples, with the maximum number of spores remaining below one spore per gram of soil, and since the root colonization was generally high, the trap culture approach was adopted. Trap cultures allowed for amplification of the preexisting soil spores and induction of sporulation of AM fungal species that were not initially detected in the original soils. Because the soil AM fungal inoculum for the trap culture in this study was basically composed of infected roots, the spores generated in trap culture are considered to have been derived mainly from the active root-colonizing AM fungi in those pots. Therefore, the different types of spores recognized in the pot cultures were likely to represent AM fungal species that existed in the original soils.

The trap cultures led to detection of additional AM fungal morphotypes that included *G. clarum* and *G. consrictum*. Another AM fungal species, *G. coronatum* morphotype, was present less frequently than the others species listed earlier in this section.

Members of the genus *Entrophospora*, mainly *E. infrequens* morphotype, were detected only in trap culture pots that were sown to the Iowa2052 soybean cultivars.

Two other *Glomus* species, *G. fasciculatum* and *G vesiforme*, were occasionally found in the trap-culture potted soils. These were not included in the recorded percentages simply because they were found in low frequency, the spores were too small to count, and they were not recognized as such until later in the study. Other unidentified AM species were mainly

small white spores that resembled *Paraglomus occultum*, *Archaeospora trappei*, and *G. diaphanum*, but were not positively identified.

Original Field Data

Soil Properties

Chemical tests showed that the organic matter content of Webster soils (59-77 g OM kg^{-1}) was about twice as much as that of Clarion soils (31-39 g OM kg^{-1}), as shown in Table 3.3. Mean available Bray P-1 concentrations, expressed in mg P kg^{-1} soil, were higher in soils of fields 2 (143 and 178) and 4 (130 and 203) than in fields 1 (13.5 and 69.3) and 3 (40 and 38) for Clarion and Webster soils, respectively. With the exception of field 3, P levels were generally higher in Webster soils than in Clarion soils of the same field. The Clarion soil of field 1 was low in available P, but the remaining fields were rated very high in P (Iowa State University Extension Service ratings).

At the time of sampling, the gravimetric moisture content of Clarion soils was approximately 10 to 12% in fields 1, 2, and 3, and 19% in field 4. Webster soils had higher

Table 3.3. Chemical analyses[†] of the field soils and their associated AM colonization and spore counts.

	P	K	Ca	Mg	O.M.	pH	Colonization[‡]	Spore count[‡]
			-----mg kg^{-1} -----		g kg^{-1}		%	spores g^{-1} soil
Field 1								
Clarion	13.5	96.0	1909.5	322.0	31	5.81	61.9	0.56
Webster	69.3	221.0	4132.0	654.5	66	5.86	17.9	0.73
Field 2								
Clarion	143.0	440.5	2262.5	246.5	34	6.76	72.2	0.16
Webster	177.5	722.0	3670.5	558.5	67	6.87	60.7	0.10
Field 3								
Clarion	40.0	144.0	1909.5	260.0	32	5.49	71.3	0.20
Webster	38.3	170.0	4808.0	594.5	59	7.52	53.1	0.86
Field 4								
Clarion	130.0	324.8	2325.5	315.0	39	6.32	53.7	0.23
Webster	203.3	523.0	3949.0	637.5	77	6.63	53.2	0.06

[†] Available P determined by the Bray-1 method; available K, Ca, and Mg by exchange with neutral 1M NH$_4$OAc, pH in a 1:2 soil:water ratio by pH meter.

[‡] Percentage of roots colonized by AM fungi and spore counts are based on 2 replicates apiece for each of 5 samples per soil.

moisture contents, ranging from 17 to 18% in field 2, 25 to 27% in fields 1 and 3, and 27 to 28% in field 4.

The pH values were significantly higher (P<0.0001) in Webster soils than in Clarion soils. Average pH values indicated Webster soils rated medium acid (field 1) to slightly acid to neutral (fields 2 and 4) to mildly alkaline (field 3) while Clarion soils were considered medium to slightly acid. Although the ANOVA table showed that the soil samples from different fields had significantly different pH values (P<0.0001), T-tests showed one exception; the mean pH values in fields 2 and 4 were not significantly different. Both pH values (1:2 soil:water ratio) and gravimetric moisture contents (105°C for 24 h) were assessed on duplicate subsamples.

Initial Root Mycorrhizal Colonization

The percentage colonization by mycorrhizal fungi in field-collected soybean roots was significantly different among field locations (P<0.0001), but the mean percentage root colonization was not significantly different between the two soil types (Fig. 3.2). The T-test showed a significant field location effect that was mainly due to the mean percentage root colonization being only about 18% in roots collected from Webster soil in field 1, much lower than that of any other site. In fields 2, 3, and 4, the mean percentage of soybean root colonization by AM fungi was slightly higher in well drained Clarion soils (54 to 72%) than in the wetter conditions of Webster soils (53 to 61%) (Fig. 3.2).

Mycorrhizal Spore Count in Field Soil Samples

Statistical analyses revealed that spore populations in the original field soils, expressed as number of spores per gram of soil, varied significantly (P<0.001) between the two soil types and among the four fields (P<0.0001). T-tests, however, revealed that the means of fields 2 and 4 were not statistically different. Spore counts were generally low in all of the soil samples, not exceeding one AM spore per gram of soil, especially in fields that tested very high in available P (Fig. 3.3).

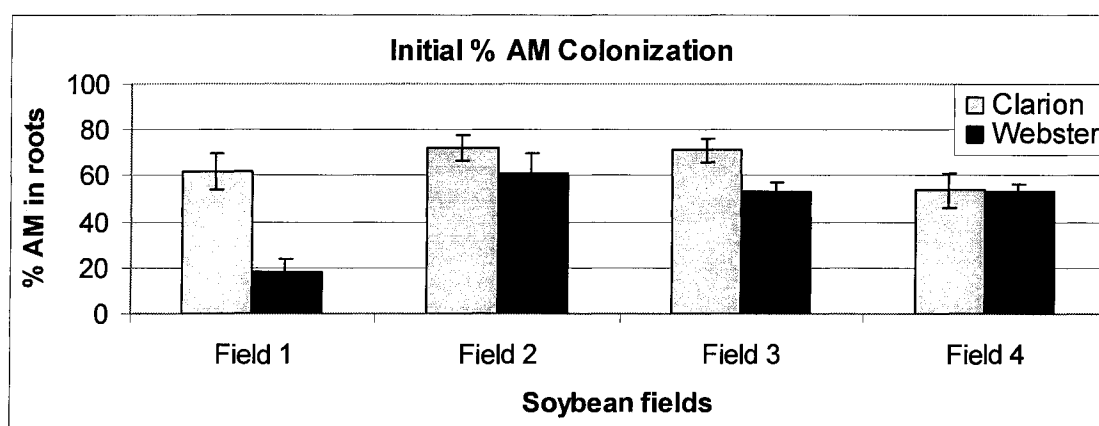Fig. 3.2. AM fungal colonization of soybean roots collected from field soils. Each value represents the mean of five samples. Error bars represent standard deviations.
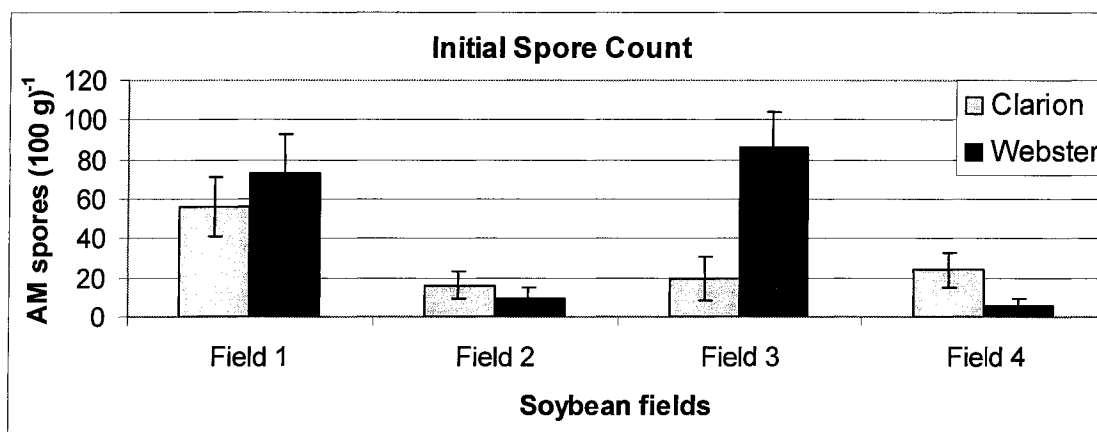

Fig. 3.3. Spore counts in field soils. Each value represents the mean of two counts for each of five samples. Error bars represent standard deviations.
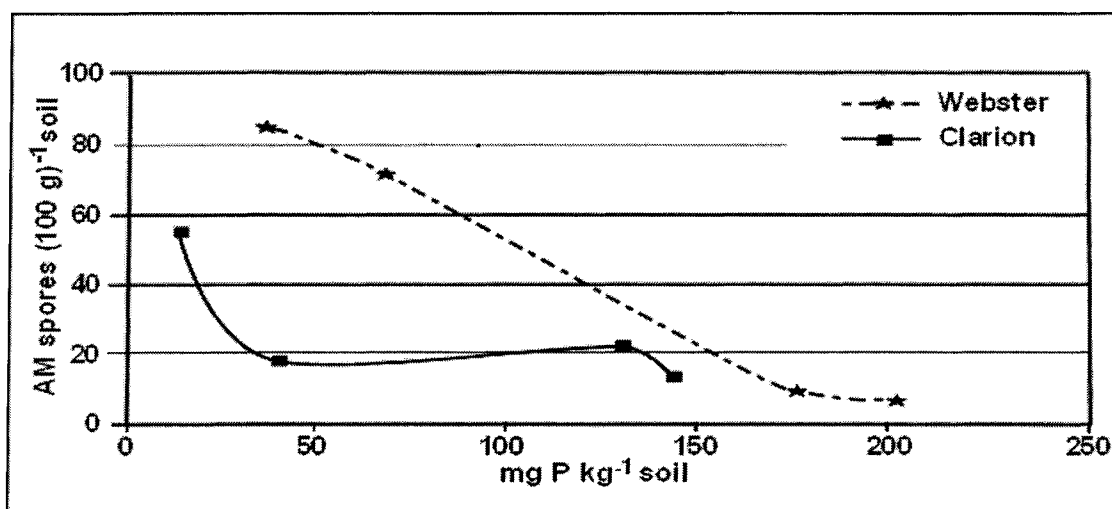
Fig. 3.4. Relationship between soil P level and spore count in field soils.

Mean spore counts were significantly higher in Webster soils than in Clarion soils, but the lowest spore number per gram of soil was recorded in the Webster soil from field 4, which had the highest P level of any soil tested (Fig. 3.4). Average spore counts of the sampled sites varied from 0.16 to 0.56 and from 0.06 to 0.86 spores g^{-1} in Clarion and Webster soils, respectively. Mean available Bray P-1 concentrations, expressed in mg P kg^{-1} soil, for Webster soils were 177.5 in field 2 and 203 in field 4, with the corresponding average spore counts of 0.09 and 0.06 spores g^{-1} soil. But, Webster soils had up to four times as many spores as Clarion soils under lower levels of soil P. Spore counts in these fields were associated with high variability both among samples within each field and among fields.

AM Fungal Diversity Assessment within Fields

Direct examination of the forty field soil samples and of the soils of trap cultures from two soybean cultivars in Webster soils of fields 1 and 4 led to recognition of eight AM fungal species belonging to the genus *Glomus* plus other species belonging to *Acaulospora*, *Gigaspora*, and *Paraglomus* genera (Fig. 3.5).

Statistical examination of the data showed a large variability in the percentage distribution of AM fungal species within and among fields, and the statistical significance of the effects of soil type and location depended on the individual fungal species. For the original fresh soils, the effects of soil and location on the relative abundance of AM fungal species were not statistically significant because the species distribution was highly variable, as shown by high standard deviations around the means and a high coefficient of variance estimated using ANOVA analysis. For example, no significant effect of the soil or field was apparent for two of the prevailing AM fungal species, *G. claroideum* and *G. etunicatum*, despite the apparent large differences in their mean percentages between the soil types among or within fields. For *G. claroideum*, the mean percentage varied from 3 to 13% and 0 to 23% for the fields and soils, respectively, while *G. etunicatum* had corresponding values ranging from 2 to 5% for fields and 0 to 10% for the soil types (Clarion and Webster).

The ANOVA analysis for the effects of soil type and location showed that the mean relative abundance of the dominant species in our soils varied significantly for *G. mosseae* ($P < 0.002$) and *G. viscosum* ($P < 0.001$). The *G. viscosum* morphotype had higher mean

Clarion Soil   **Webster Soil**
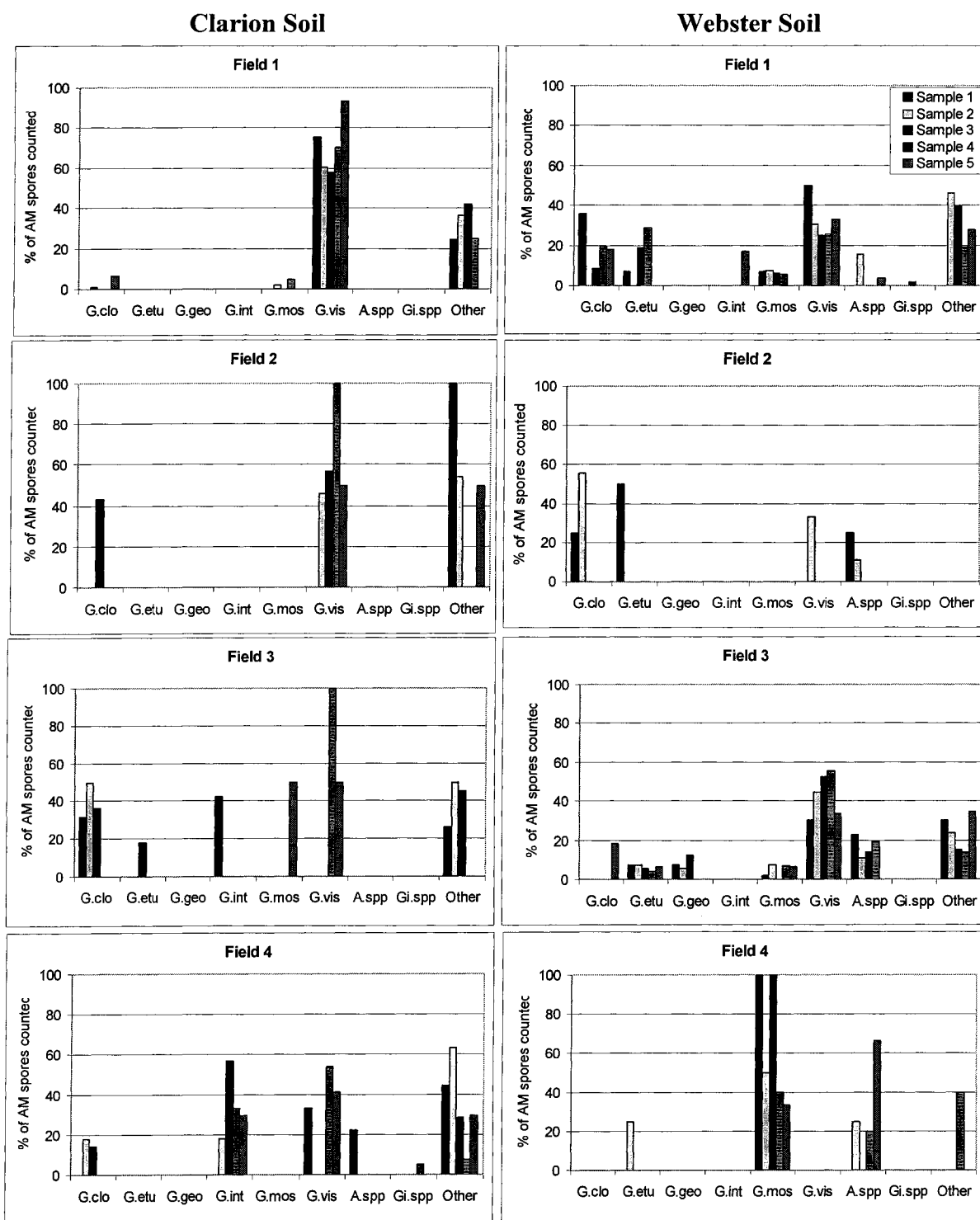
Fig. 3.5. Relative abundance of indigenous AM fungal species identified directly in soil cores from soybean fields; percentages percentages of the total spore counts for each field and soil type. G.clo = *Glomus claroideum*, G.etu = *G. etunicatum*, G.geo = *G. geosporum*, G.int = *G. intraradices*, G.mos = *G. mosseae*, G.vis = *G. viscosum*, A.spp = *Acaulospora* species, and Gi.spp = *Gigaspora* species, Other = unclassified AM fungal species.

percentages in Clarion soils (31 and 44%) than in Webster soils (14 and 20%), whereas spores of *G. mosseae* averaged 18% of the total spores collected from Webster soils but represented only about 2% of the spore population in Clarion soils.

The influence of soil type and location on the distribution of *G. geosporum*'s meager population (found mainly in field 3) was significant ($P<0.01$ and $P<0.04$, respectively) while the distribution of *G. intraradices* ($P<0.03$) differed significantly among fields. Soil type was a major factor in the distribution of *Acaulospora* species, but neither soil type nor location significantly affected the population of species of *Gigaspora*. Similar to *G. mosseae*, spores of *G. intraradices*, *Acaulospora* species, and species of *Gigaspora* were most prevalent in field 4. The cream to yellow spores of *G. claroideum* (Fig. 3.6) with 4-layered cell walls and diameters ranging from 80 to 160 μm were detected in 42.5% of the total samples collected (Table 3.4). Their occurrence and abundance differed from one sample to another within individual fields. For example, this species was detected only in samples 1 and 5 collected from Clarion soils of field 1, and it represented only 1 and 7% of the spore populations, respectively, in these soil samples. For the same soil type, only sample 3 of field 2 had this type of spores, but they represented about 43% of the total spores enumerated from that soil sample. Spores of *G. claroideum* made up 32, 50, and 36% of the total spores counted in samples 1, 2, and 3 of Clarion soils of field 3. In Webster soil of field 1, this AM fungal species was detected in 4 of 5 samples, and its percentages in these samples ranged from 20 to 46% of the spores present in those soils. It was not detected in any of the field samples of Webster soil of field 4, but it was induced to sporulate, and thus was found in samples 1 and 2 in the BSR201 pots of the trap culture. For additional details consult Fig. 3.6.

An average of 37.5% of soil samples contained the small (60-120 μm diam.) deep orange to red spores of *G. etunicatum* (Fig. 3.6), and the distribution and abundance of this species varied from sample to sample and from field to field. It was found only in sample 3 of field 3 of all the Clarion soil samples, but it was more frequently detected in Webster soils. In Webster soil of field 1, spores of *G. etunicatum* constituted 7, 0, 19, 29, and 0% of the total spores recovered from samples 1, 2, 3, 4, and 5, respectively. About one-half of the spores in sample 1 in the Webster soil of field 2 were those of *G. etunicatum*, and this species was not detected in the remaining samples of this soil. Similarly, in Webster soil of field 4, spores of *G. etunicatum* accounted for 25% of the total spores in sample 2, the only sample from field
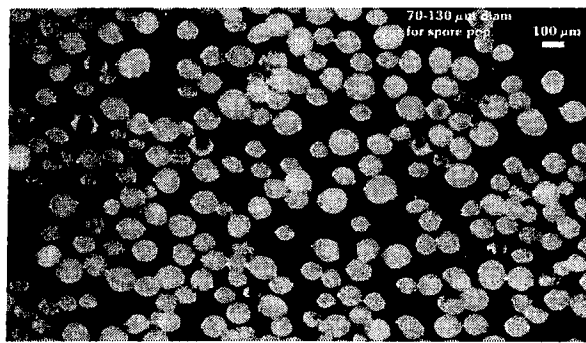
Glomus claroideum
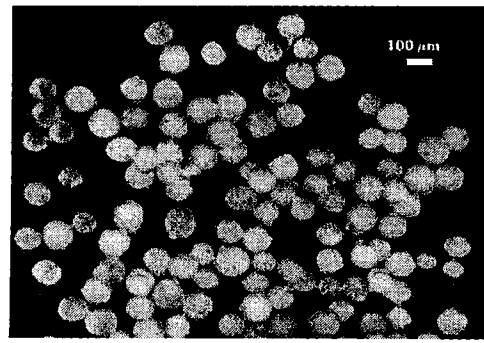
Glomus etunicatum
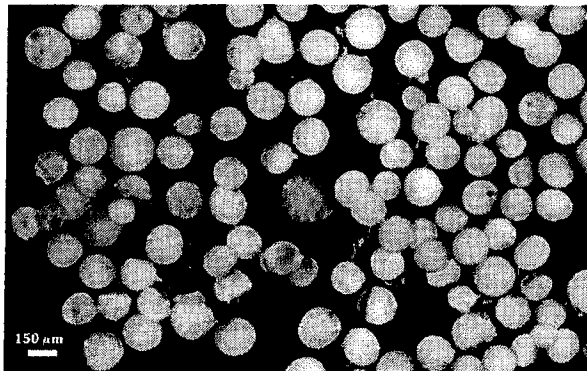
Glomus mosseae
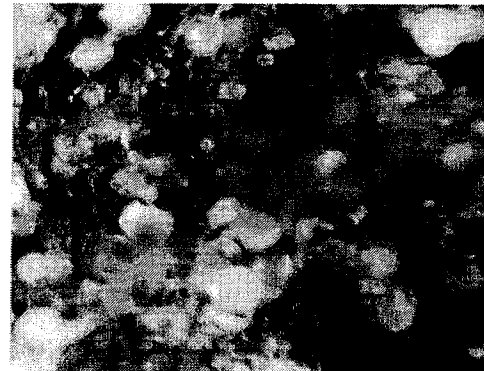
Glomus viscosum (spores 50-120 μm diam.)
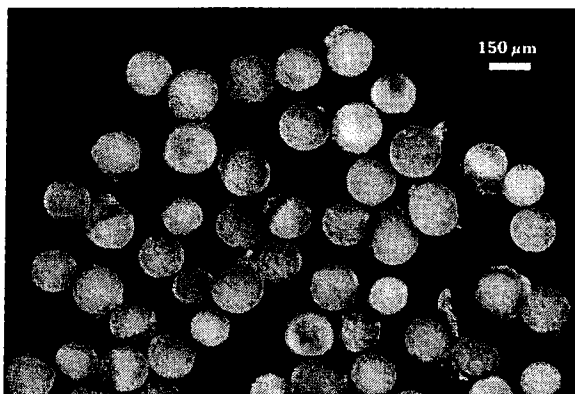
Acaulospora Spinosa
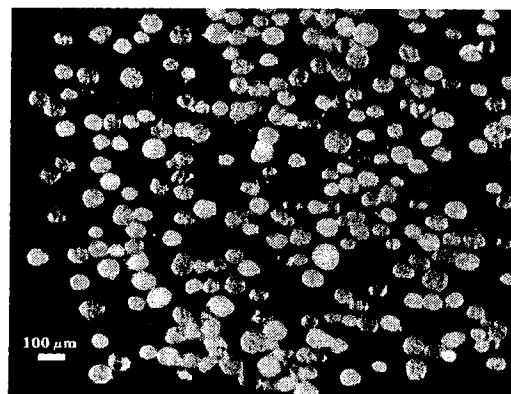
Paraglomus occultum-like
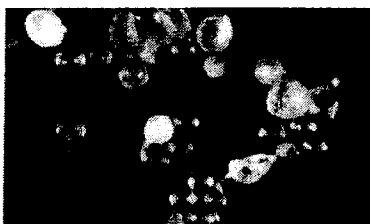
Entrophospora infrequens
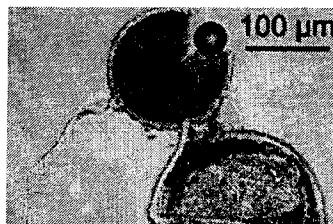
Entrophospora infrequens spore and saccule

Fig. 3.6. Photomicrographs of AM spores from Clarion and Webster soils of soybean fields.

4 that had them. All five samples of Webster soil from field 3 contained spores of *G. etunicatum*, ranging from about 4 to 8% of the spore populations in these samples.

Large (100-260 µm), straw to dark orange-brown spores (and rarely fuzzy yellow-brown to brown sporocarps) of *G. mosseae* (Fig. 3.6) were identified in 50% of all samples (Table 3.4), including 70% of the samples collected from Webster soils. In fact, *G. mosseae* spores were found in all five samples of the Webster soil of field 4 and represented nearly 100% of the spores in samples 1 and 3 and about 50, 40, and 33% in samples 2, 4, and 5, respectively, whereas the spores of this species were not detected in any sample of Webster soil in field 2. Spores of *G. mosseae* in Webster soil of fields 1 and 3 had average representation ranging from about 2 to 8% of the total spore population in 4 of 5 samples in each field, but were not detected in sample 5 of field 1 and sample 3 of field 3 (Table 3.4).

Table 3.4. Frequencies of occurrence of AM fungal species in the five samples taken from Clarion and Webster soils in each field. The last column shows the percentage of the 40 samples where each species was identified.

	Field 1		Field 2		Field 3		Field 4		Average %
	Clarion	Webster[†]	Clarion	Webster	Clarion	Webster	Clarion	Webster[†]	occurrence
G. claroideum	2	4	1	2	3	1	2	2	43
G. clarum	0	1	0	0	0	0	0	3	10
G. constrictum	0	1	0	0	0	0	0	0	3
G. etunicatum	0	4	0	1	1	5	0	4	38
G. geosporum	0	0	0	0	0	3	0	0	8
G. intraradices	0	1	0	0	1	0	4	0	15
G. mosseae	5	5	0	0	1	4	0	5	50
G. viscosum	5	5	4	1	2	5	3	0	63
A. species	0	3	0	2	0	4	1	4	35
Gi. species	0	3	0	0	0	0	1	0	10
Other	4	4	3	0	3	5	5	1	63

[†] Only Webster soils of fields 1 and 4 were subjected to trap cultures using BSR201 and Iowa2052 soybean cultivars.

The small, dirty looking, branched (50-120 μm) spores of *G. viscosum* morphotype (Fig. 3.6) were recognized in 62.5% of the field soil samples (see Table 3.4). They were found mostly in the bottom of the centrifuge tubes, trapped in settled soil and plant debris entangled in their branching hyphae. Spores of this species were found in all 5 samples from Webster soils of fields 1 and 3, and they constituted an estimated 25 to 55% of the total spores recovered from these samples (Table 3.4). About 33% of the spores in Webster soil sample 2 from field 2 were of this morphotype, but other samples in this site as well as the samples collected from Webster soil of field 4 lacked them. As for Clarion soil, the spores of this morphotype were found in all five soil samples of field 1 in approximate proportions of 58 to 93% of the spore populations of those samples. This type of spore was found in 2 to 4 samples of each of the other fields and ranged from about 33 to 100% of the total spores encountered in these samples.

The remaining species had lower abundance and occurred in lower frequencies than the morphotypes listed above. For example, the yellow-brown to dark orange *G. geosporum*-like spores with a size range of 120 to 240 μm were found in 7.5% of the samples. They were detected in Webster soil only in samples 1, 2, and 3 of field 3 in approximately 8, 6, and 13% of the total spores, respectively. Fifteen percent of the samples contained the white to yellow-brown 40-120 μm spores that were identified as *G. intraradices*. About 17% of the spores in sample 1 of Webster soil of field 2 were *G. intraradices*-like spores. Clarion soil of field 4 had the largest representation of this type of spore, as they were present in 4 of 5 samples ranging from 18 to 57% of all spores counted, whereas only sample 1 of field 3 had this type of spore and it represented 42% of the total spores.

Species of other genera, especially sessile *Acaulospora* spores (A.spp) (Fig. 3.6) and the large bulbous spores of *Gigaspora* (Gi.spp), such as *Gi. margarita* and *Gi. rosea*, were also detected in these soils. Spores of *Gigaspora* were found only in 5% of the original field samples, but this value doubles in the data from trap cultures. Species of *Acaulospora* were detected in 30% of the original field samples, and this value increased to 35% after sporulation of these species occurred in pots of the trap cultures. Species of the genus *Acaulospora* included *A. spinosa* that produces cream to pale orange-brown, sessile spores (140-220 μm) with spiny ornamentations. Other *Acaulospora* spores encountered in these

soils were bright red spores (200-350 μm) with light pink soporiferous saccules that were more likely to belong to *A. calossica*.

Unclassified small spores (60-90 μm) that contained mostly colorless (hyaline) to pale cream spores that looked like *P. occultum* were recognized in 62.5% of the samples (Table 3-3). This type of spore was found in all five Webster soil samples of field 3 and ranged from 14 to 35% of the total spores in those samples. They represented 0, 46, 37, 20, and 28% of the spore numbers in Webster soil samples 1, 2, 3, 4, and 5 in field 1. This type of spore was not found in Webster soil samples of field 2, and it represented 40% of the spores in Webster sample 4 of field 4. The occurrence of this spore morphotype was more evenly distributed in Clarion soils than in Webster soils. For example, the spores occurred in all five samples in Clarion soils of field 4 in a wide range of proportions, for they made 44, 64, 29, 8, and 29% of the total spores in samples 1, 2, 3, 4, and 5. At least two samples of Clarion soils in each of the remaining fields had this type of spore.

Identification of the unclassified spores was difficult because of the deteriorated conditions of the field-collected spores. Attempts to identify *P. occultum* revealed the difficulty associated with separating this species from other look-alike species, including *G. eburneum*, *G. brazilianum*, *G. aggregatum*, *G. diaphanum*, and/or *A. trappei*. Mounted spores of some mophotypes showed heterogeneity in cell wall structure even though they were selected on the basis of having similar characteristics in shape, size, and color when observed microscopically. The structures of cell walls and hyphal attachments of the mounted spores were not sufficiently resolved under the light microscope to provide positive identification of any of these species.

AM Fungal Diversity Assessment among Fields

Examination of the original field soils revealed that *G. claroideum*, *G. etunicatum*, *G. mosseae*, and *G. viscosum* were abundant and *G. clarum*, *G. geosporum*, *G. intraradices*, *G. coronatum*, *Acaulospora* species, and *Gigaspora* species were present in lesser frequency. The trap cultures using four soybean cultivars resulted in the production of large numbers of healthy spores of AM fungal species in the greenhouse pots and allowed sporulation of some species that were not detected in the original field soil samples. Additional species identified

from the trap cultures included *G. constrictum*, *G. vesiforme*, and *Etrophospora infrequens* morphotypes, whose spores were not found in the original field soils (Fig. 3.7).

The population composition of AM fungi was slightly different among the fields. Morphotypes of *G. claroideum*, *G. etunicatum*, *G. mosseae*, *G. occultum*, *G. viscosum*, and *Acaulospora* species were detected in both soil types of the four fields surveyed in this study, and their widespread distribution was independent of the soil type or field location. For example, spores of *G. viscosum* morphotype were detected in at least some of the original soil samples from each site based on the direct examination of soil samples and trap culture, and they often represented the highest percentage of the spore population in those soil samples, especially in Clarion soils (Fig. 3.7). The lowest frequency of its spores was observed in Webster soil of field 2 because its spores were collected only from 1 of 5 samples, but this does not prove its absence from other samples because it might have existed in the host roots and/or soil in the form of hyphae. The individual *Acaulospora* species were found in a few samples. As an example, *A. calossica* spores were encountered in both soil types in field 2.

The distribution of other species seemed to be limited to certain fields or one soil type. For example, the haloed, yellow-brown, medium-size (100-260μm) spores of *G. clarum* were found in all fields but were not detected in Webster soil of field 2. Spores *G. intraradices* were found only in Clarion soils of fields 3 and 4 and Webster soils of fields 1 and 4. The small brown spores of *G. fasciculatum*, associated with a hyphal network in grape-like clusters, were observed in soils derived from pots inoculated with composite samples collected from Clarion soil of fields 1 and 2. The *G. vesiforme* spore morphotype with masses of loose sporocarps containing small light-brown spores loosely aggregated by mycelia were detected in Clarion soil of field 2 and Webster soil of field 4. The spore population of the large bulbous *Gigaspora* spores was present in very low numbers, and these spores were identified in Webster soil of fields 1, 3, and 4 and Clarion soil of all four fields. One unique event was that one category of spores that were assigned to *E. infrequens* was found only in Clarion soil of field 3, and was associated only with the roots of Iowa2052 soybean cultivar. Spores identified as *Scutelospora verrucosa* were detected in one sample of Webster soil from field 1 where BSR201 was used as the host plant.
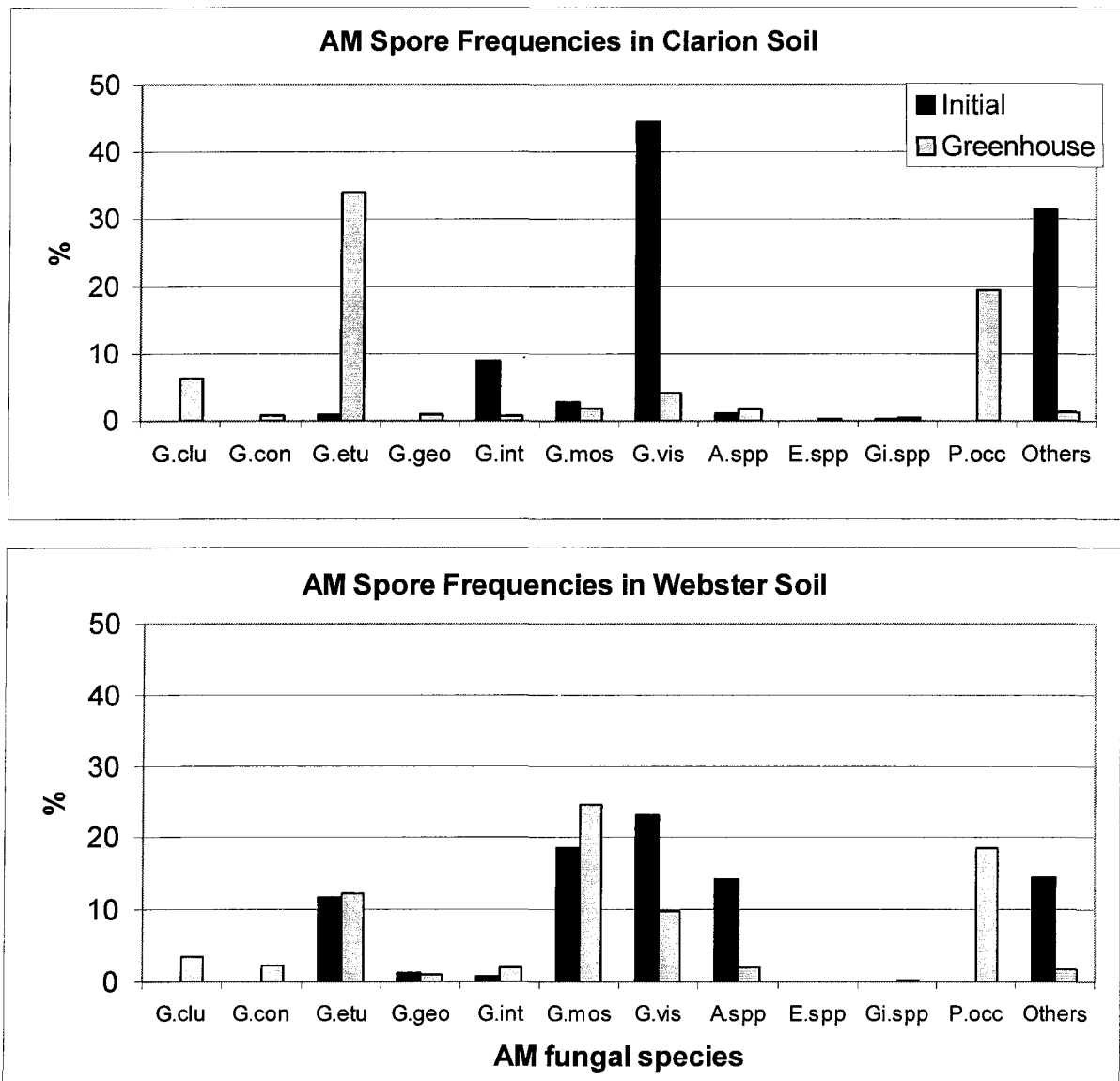
Fig. 3.7. Mean percentage distribution of AM fungal species identified in field soils before and after greenhouse trap cultures. The initial soil data represent averages for twenty soil samples and the trap cultures were averages for four soybean cultivars. G.clo = *Glomus claroideum*, G.clu = *G. clarum*, G.con = *G. constrictum*, G.etu = *G.etunicatum*, G.geo = *G. geosporum*, G.int = *G. intraradices*, G.mos = *G. mosseae*, G.vis = *G. viscosum*, A.spp = *Acaulospora* species, E.spp = *Entrophospora* species, Gi.spp = *Gigaspora* species, P.occ = *Paraglomus occultum*, and Others = unclassified AM fungal species.

Ribosomal DNA-based Identification of AM Fungal Species

Amplification of a target region of rDNA from genomic DNA extracted from single spores of *G. claroideum*, *G. etunicatum*, *G. intraradices*, and *G. mosseae*, using species specific primers, and *Gigaspora* species using a generic-specific primer yielded PCR products of appropriate sizes, supporting the spore morphology-based identification of these fungi.

The success rate of PCR amplification of a target DNA using specific primers for AM fungal species identification depends on the species and the primers used. The following statistics pertain to the PCR results obtained after some adjustments to make the PCR protocol work to assess the presence of a specific AM fungal species in roots or to confirm the identify of a spore morphotype. These statistics are given in Table 3.5.

Appropriate 1000 bp (base pairs) DNA fragments were obtained by amplification of a target region of 18S rRNA gene of *G. intraradices* and *G. mosseae* with published primer pairs, GLOM1310 and ITS4 in nested PCR (Fig.3.8).

Twelve replicates of *G. intraradices* spores were processed for PCR, and no product was obtained when single spore genomic DNA was used as a template. When genomic DNA was extracted from 5 to 6 spores, about 40% of the reactions yielded PCR products. Slightly higher success rates, 50-60% of PCR reactions, were obtained when mycorrhizal colonized root segments were used as a source of DNA. Replicates of 5-6 spores of *G. intraradices* and single spores of *G. mosseae* were PCR-analyzed for their 25S rDNA sequences using species-specific primer pairs LR1/8.22 and 5.21/FLR2, respectively (Fig. 3.9). Appropriate PCR DNA segments of 455 bp were generated for *G. intraradices* and produced the expected DNA fragment lengths of 364 bp for *G. mosseae* from both roots and spores (Table 3.5).

The *Gigaspora*-specific primer pair NS5/GIGA5.8R resulted in 80% of the total 11 PCR replicates generating the expected fragment lengths for genomic DNA from single *Gigaspora* spores. Primer pair LETC1670/ITS4 failed to amplify the desired PCR products from single- or multiple-spore genomic DNA of *G. claroideum* or *G. etunicatum*, despite the many trouble-shooting approaches that were taken.

Amplification of the *G. etunicatum*-diagnostic 400-bp DNA fragments (Fig. 3.9) of the 5.8S rRDNA and its flanking ITS region from *G. etunicatum* single spores and colonized

Table 3.5. Success rates for generation of PCR products in ribotyping of AM fungal spores and roots for identification and classification of AM fungal species in field soils.

AM fungi	Primer pairs			AM. fungal spores		Success rate %	AM. fungal roots		Success rate %
	set 1	set 2	set 3	Initial	Products	%	Initial	Products	%
Gigaspora spp.	NS5/ITS4	NS5/GIGA5.8	NA[†]	11	9	82	NA	NA	NA
Glomus:									
G. claroideum	NS5/ITS4	LETC1670/ITS4	NA	45	none	0	NA	NA	NA
G. etunicatum	"	"	NA	60	none	0	NA	NA	NA
G. intraradices	NS5/ITS4	GLOM1310/ITS4	NA	20 sets	8	40	6	11	54
G. mosseae	"	"	NA	44	33	75	37	21	57
G. etunicatum	ITS1mod/ITS4	GETU1/GETU2	NA	23	17	74	18	12	67
G. claroideum	ITS3/NDL22	LR1/FLR2	38.21-FLR2	35	30	86	33	21	64
G. intraradices	"	"	LR1-8.22	20	11	55	10	6	60
G. mosseae	"	"	5.21-FLR2	16	13	81	57	34	51

[†] NA = not applicable
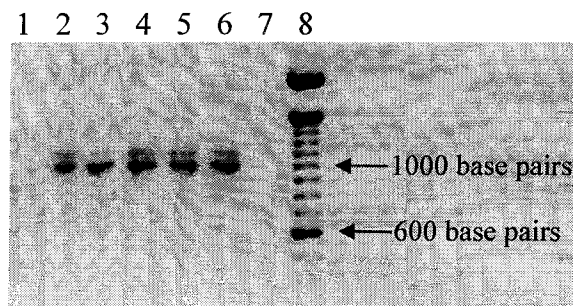
Fig. 3.8. Results from gel electrophoresis of PCR 1000 bp DNA fragments generated in 2-step nested-PCR reactions of 18S rDNA of AM fungal spores isolated from soybean field soils using GLOM1310/ITS4. Lane 1: control, lanes 2 to 4: DNA bands from *G. mosseae*, lanes 5 to 7: *G. intraradices*. Lane 8: 100 bp DNA ladder.
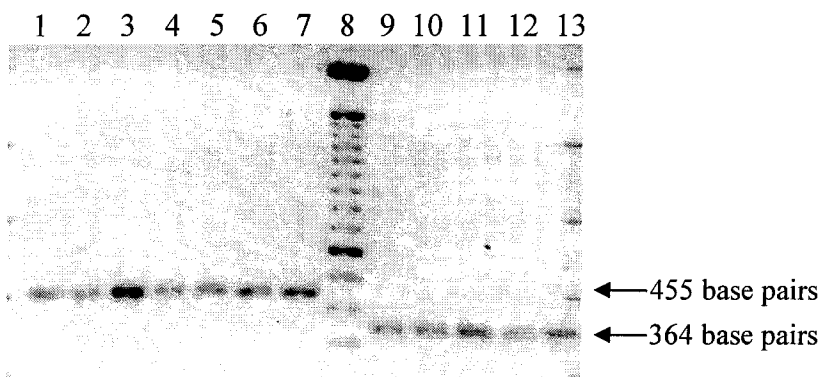
Fig. 3.9. PCR products derived from the amplification of the 25S rDNA region from single spores of AM fungi from field soils in a 3-step nested PCR. Lanes 1-7: 455 bp DNA fragments for *G. intraradices*. Lane 8: molecular marker 100 bp DNA ladder. Lanes 9 to 13: shows 364 bp DNA fragments from *G. mosseae*.
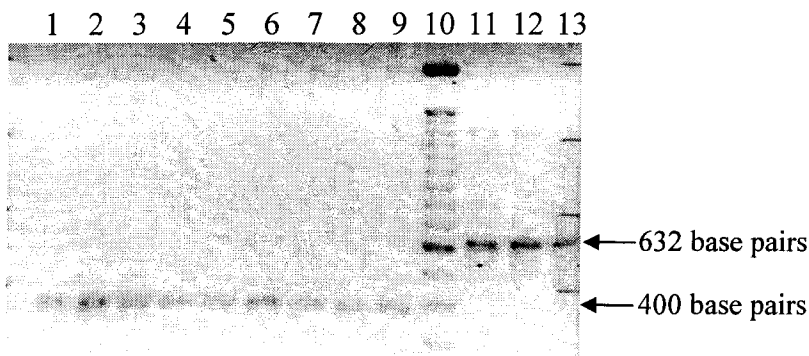
Fig. 3.10. DNA profile produced from PCR amplification of the rDNA region of single spores of *G. claroideum* (3-step nested PCR) and *G. etunicatum* (2-step nested PCR) from field soils. Lanes 1 to 9: 400 bp-DNA fragments obtained from PCR amplification of 5.8S rDNA and ITS region for *G. etunicatum*. Lane 10: 100bp DNA marker. Lanes 11 to 13: 632-bp nucleotides from the 25S rDNA region of *G. claroideum*.

roots was achieved with a GETU1/GETU2 primer pair in a nested PCR protocol, after exhausting trials to get the procedure to work (Table 3.5).

The *G. claroideum*-specific primer pair (38.21/FLR2) designed to amplify the 25S rDNA region produced DNA nucleotides of approximately 632 bp in a 3-step nested PCR protocol from about 85% of the 35 replicates of single spore genomic DNA (Fig. 3.10).

Species-specific primer pairs were used for diagnosis of *G. occultum* based on the diagnostic 597 bp (cad5.1/FLR2) and 296 bp (cad 5.3/FLR2) sequences located on the 18S rRNA gene in a 3-step PCR protocol. No amplification products were generated even after PCR troubleshooting steps were undertaken. It was decided that cloning would be an appropriate approach to determine the identity of the isolate, but time and financial resources did not provide for this activity.

The rDNA fingerprinting was used to test or confirm the presence of *G. claroideum, G. intraradices, G. etunicatum,* and *G. mosseae* in the roots collected from the field soil samples and from the trap culture pots (data not shown).

DISCUSSION

This study highlights the extent of variability in the composition and abundance of the AM fungi in two types of soil (Clarion and Webster) in four cultivated fields located in central Iowa. Eleven species morphotypes of *Glomus* were categorized: *G. claroideum*, *G. clarum*, *G. constrictum*, *G. coronatum*, *G. etunicatum*, *G. fasciculatum*, *G. geosporum*, *G. intraradices*, *G. mosseae*, *G. viscosum*, and *G. vesiforme*. Several types of spores of other genera were recognized in these two soils, including *Acaulospora callosica*, *A. feavea*, *A. spinosa*, *A. verrucosa*, *Entrophospora infrequens*, *Gigaspora margarita*, *Gi. rosea*, and *Gi. gigantea*, and *Paraglomus occultum*. Species of *Acaulospora* and those of *Gigaspora* were grouped into *Acaulospora* species (A.spp) and *Gigaspora* species (Gi.spp) for graphing the data.

The composition and frequency distribution of these AM fungal species exhibited higher variability within the fields than among the fields in this study. Previous research indicated that the distribution of AM fungi depends on several factors, including soil management, soil organic matter, soil fertility levels, soil moisture, soil pH, and more importantly, the host plants. AM fungi are obligate symbionts that cannot complete their life cycle independently of a suitable host (Douds et al., 1993; Ellis et al., 1992; Ortega-larrocea, 2001).

Results from this study indicated that *G. claroideum*, *G. etunicatum*, *G. mosseae*, *G. viscosum*, and *P. occultum* were prevalent in both types of soil in all four fields. These have been among the prevalent *Glomus* species in several previous diversity studies (Cousins et al., 2003; Franke-Snyder et al., 2003; Johnson et al., 1992, Nielsen et al., 2004). Statistical analysis of the data indicated the mean percentages of the spore population of *G. claroideum*, *G. etunicatum*, *G. mosseae*, and *G. viscosum* in the trap culture pots were significantly different among fields and also between the two soil types. Two less dominant AM fungal species, *G. constrictum* and *G. geosporum*, were also found in the two types of soil in each field. Only Webster soil of field 2 seemed to not show the presence of *G. clarum* while *G. coronatum*, *G. fasciculatum*, *G. intraradices*, and *G. vesiforme* were detected in some fields but not others. *E. infrequens* was detected only in Clarion soil of field 3. Different species of *Acaulospora* and *Gigaspora* genera were recognized in different fields.

The composition of the AM fungal population was most variable at the smallest scale studied (samples spaced 2 m apart), according to microscopy spore morphology identification of AM fungal species in freshly collected field soil samples. Trap culture pots inoculated with individual soil samples from Webster soil from fields 1 and 4 produced much larger numbers of spores than the original soil samples and led to the identification of additional species of AM fungi.

The combined data from the original soil samples and the trap cultures identified four AM species, *G. etunicatum*, *G. mosseae*, *G. viscosum*, and *P. occultum*, as being present in all five samples from each of these four fields. These four species represented the majority of the AM spores in these soils. The frequency of the remaining *Glomus* species varied among samples from the same field. Adjacent samples harbored different groups of AM fungal species, as shown in Fig. 3.4. This could be explained by patchy distribution of AM fungi within microsites where many interactions among AM fungal species occur. For instance, an aggressive species such as *G. mosseae* or G. *viscosum* could prevent infection by subsequent AM fungal species through several strategies, such as pre-emptive exclusion, induced systematic resistance, or occupying all of the available entry sites (Hepper et al., 1988).

The dominant *G. viscosum* spores remained attached to their branched hyphae, and some of these hyphae were still bound to the root pieces. Their resistance to breaking away from their hyphae might be an important feature that allowed them to compete well in these soils. Spores (mainly enclosed in single or double-spore sporocarps) of *G. mosseae* were found mainly in Webster soils. They represented the highest percentage of spores in Webster soil of field 4 where spores of some other species were not detected. This soil had the highest P concentration and the highest gravimetric moisture of any of these soils at sampling time, and these conditions favor the growth of other microorganisms, including parasites that might attack and deteriorate the AM fungal spores in the soil. Perhaps the sporocarpic structures protected these spores against potential pathogens.

Looking at the graphs (Fig. 3.5), it seems that there is a negative correlation between the spore populations of *G. mosseae* and *G. viscosum* in that where one species dominates the other one diminishes. Assuming that the spore abundance of these two species reflects their actual populations in these soils, this could be explained by many interactions. One explanation could be that these two species use the same limiting resources and compete for

a site, and the first one to colonize the roots reduces or prevents the other's access to the same site in what is described as pre-emptive exclusion (Hepper et al., 1988). Spores of *G. mosseae* were not identified in any of the five samples initially colleted from Clarion soil of fields 2 and 4 and Webster soil of field 2, but trap-culture results proved that *G. mosseae* existed in the roots of at least one sample in each of those fields.

The pH values place these soils between medium acid (5.6) and mildly alkaline (7.8), and this pH range was suitable for these AM fungal species. Isolates of AM fungi function best where their spores germinate well, and *G. mosseae* spores germinate in soils with a pH range of 6 to 9 (Giovannetti, 2000). This may contribute to the higher spore population of *G. mosseae* in Webster soils than in Clarion soils. Higher soil organic matter in Webster soils could have stimulated sporulation of certain AM fungal species, especially *G. etunicatum* and *G. mosseae*.

On the other hand, high P could have reduced sporulation of several AM species in fields 2 and 4, but the percentage root colonization was less affected by high P, probably because other AM fungal propagules were better protected inside the host roots, but the reasons for low root colonization in Webster soil of field 1 remains unclear. *Glomus* species dominate in cultivated fields, probably because they can propagate in soil via both spores and broken pieces of hyphae while propagation of members of *Gigaspora* and *Scutellospora* require either spores or intact mycelia; hyphae damaged by cultivation are useless (Daniell et al., 2001). Moreover, *Glomus* species have an additional competitive advantage as they exhibit the capability to mend broken mycelia through anastomoses to reconstruct a hyphal network that has been damaged by cultivation, a phenomenon not yet known to occur in *Gigaspora* or *Scutellospora* species (Giovannetti et al., 2003). The occurrence of these species in Webster soils of fields 2 and 4, further indicated the adaptation of these species to high P levels, as well as the occasional flooding of these soils that sometimes restricts the oxygen supply needed by these aerobic organisms for respiration.

Khalil and Loynachan (1994) suggested that the reasons for high spore populations in Webster soil could be due to gravitational movement of spores from upslope, but higher average spore counts were recorded in microplots of Webster soil than in Clarion or Nicollet when a 3-cm freeboard prevented this effect in a field experiment (Troeh and Loynachan, 2003). Others have speculated that sporulation occurs as an energy investment for AM fungi

when growth of hyphae is limited, and this is perhaps an important effect when AM fungi are propagated in trap cultures.

Large populations of AM fungi in high P soils were previously reported (Khalil et al., 1992). This might be explained by the ability of these fungi to adapt to high P soils and to compete with coexisting organisms, or they may have used different resources than the antagonistic organisms (Bever et al., 2001).

In a similar study, Walker et al. (1982) reported *G. mosseae*, *P. occultum*, and *Acaulopora* species to be among the dominant AM fungi associated with poplar trees in several sites in central Iowa, while Cousins et al. (2003) found that *G. etunicatum*, *G. mosseae*, and *P. occultum* were dominant in their soil samples collected from the metropolitan area of Phoenix, Arizona. One of the differences between those two studies and this study was that those two studies found *G. fasciculatum* to be more frequent in their soils than in this current study. Twenty-six species of AM fungi were identified in field soils under sorghum [*S. bicolor* (L.) Moench] and soybean [(*Glycine max* (L.) Merr.] at the University of Nebraska that included almost all species recognized in our study plus species formerly classified as *Sclerocystis* (Ellis et al., 1992).

CONCLUSIONS

The use of trap cultures increased the number of spores produced and the number of AM fungal species identified in this study. When the spores identified from the trap cultures were added to those from the original soil samples, the results revealed that the largest number of species (11) categorized in these soils belonged to *Glomus*. Members of other genera, *Acaulospora*, *Gigaspora*, *Entrophospora*, and *Paraglomus*, were also found in these soils.

The distribution and composition of AM fungal species exhibited higher variability within individual fields than among the fields. The occurrence and the abundance (expressed in percentage of total spores) of individual species varied among soil samples collected 2 m apart.

Five species, *G. claroideum*, *G. etunicatum*, *G. mosseae*, *G. viscosum*, and *P. occultum*, were detected in all the samples collected and consequently existed in all the participating fields, perhaps because they were fast growers and were the first to occupy the most susceptible infection sites of the host roots. The mean spore population percentages varied from approximately 3 to 25, 25 to 70, and 25 to 40% of the total spore populations, respectively, for *G. claroideum*, *G. viscosum*, and *P. occultum* in Clarion soils, and from about 10 to 23% for *G. etunicatum*, and 20 to 63% for *G. mosseae* in Webster soil. The majority of the remaining species were found in very low numbers in some of the samples derived from both soil types within a field. The absence of spores of certain AM species in a fraction of the samples further emphasizes the variability within small areas of the field.

The composition of AM fungal species was less variable among the fields than it was within individual fields, perhaps because surveying a larger area in search of a certain AM fungal species makes it more likely that the targeted species will be found. The richness of AM fungal species was similar among fields, and field 3 harbored the largest number of species in both its Clarion (12) and Webster (11) soils, including *E. infrequens* that was detected only in this soil. Only *G. intraradices* was present in one soil (Clarion) and lacking in the other (Webster) soil in this field. Clarion soil of fields 1 and 2 had similar numbers (10) of species, but in field 2 the frequency in pot cultures and the abundance in both original samples and trap cultures of five of these species were very marginal, especially in field 2. Some AM fungal species, such as *G. clarum* and *G. intraradices*, were missing from these

latter soils. Only eight AM fungal species were identified in Webster soil of field 2, the fewest of any soil examined, and species such as *G. clarum* and *G. intraradices* were missing from this soil. The high-P Webster soil of field 4 showed ten AM fungal species with *G. mosseae* being dominant, as it produced up to 90% of the spore population in this soil.

Some AM fungal species had a tendency to produce relatively more spores in the field than in the greenhouse pots; others sporulated better in pots. *G. viscosum* had the largest spore count in the original field samples as its spores represented at least 50% of the total spores in the soils when present. In pot cultures, however, its spore population rarely exceeded 20%. The opposite was noted in the behavior of *G. etunicatum*. Its spore population percentage at least doubled in pot cultures as compared to the field soils. Species that proliferate in pots are good candidates for mass-producing inocula or material for identification and other studies.

FUTURE WORK

Members of Glomeromycota confer substantial contributions to their host plants as they improve growth, increase disease resistance, increase competitive abilities, maintain their diversity and contribute to ecosystem stability (Bever et al., 2001). Study of the composition of AM fungi is the key to understanding their ecological and symbiotic relationships with the host plants.

The current study examined the soil samples collected from soybean fields in the fall when sporulation was considered to reach its peak, so the spores would be harvested from an optimum number of AM species. However, previous research indicated that sporulation of certain AM fungal species is seasonal and depends on an array of factors, principally the host and the edaphic factors. Thus, repeated soil sampling at pre-determined time intervals, especially during the growing season, would more accurately account for the species that reside in a certain habitat.

Molecular approaches, such as rRNA fingerprinting for direct detection of AM fungal isolates in the host roots using designed species-specific primers, could produce more reliable results as it accounts for the species actually active at the time of the investigation. Primer development lags far behind the number of species being discovered in the soil. Thus, the recommended approach is to profile the AM fungal species through sequencing analysis of appropriate regions of rDNA genes. There are numerous PCR-based methods, such as TTGGE, that are useful in identification of AM fungal isolates in roots.

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CHAPTER 4. AM FUNGAL SELECTION BY SOYBEAN CULTIVARS

Fungi of Glomeromycota colonize the roots of terrestrial plants under a myriad of biotic and abiotic stresses and confer different types and degrees of effects on host plants (Burleigh et al., 2002; Johnson et al., 1997). The multinucleate nature of AM fungi provides a redundancy in genes of similar functions so that an alternate gene may compensate for another gene's loss of activity (Jansa et al., 2002) and helps explain their abilities to override the host defense systems, overcome harmful mutations, and maintain vigor. Since AM fungi express diverse functions toward their hosts, it is likely that different degrees of affinity and compatibility exist between the two partners involved in the symbiosis. These partnerships could be affected by environmental and/or genetic factors.

Numerous studies have indicated that certain plant species host some AM fungal species in preference to others. This propensity of plants to select for certain types of fungi was observed both in surveys of natural ecosystems and in studies conducted with controlled conditions (Burleigh et al., 2002; Helgason et al., 1998). Gollotte and coauthors (2004) reported that a group of AM fungi colonizing the roots of *Lolium perenne* was different in composition than the group of AM fungi found in the roots of *Agrostis capillaris* when these two grass plants were grown in monoculture, replacing the native grasses. The response of these two grass species to AM fungi led the investigators to suggest that some plants may be able to select the types of fungi to be their partners in symbiosis. Plants express different growth responses in the presence of some AM fungal strains, but plants that host a larger number of AM fungal species receive more benefits than those hosting smaller numbers of species, probably due to their complementary functions (van der Heijden et al., 1998). What really drives the selection process remains unknown.

Establishment of an AM fungal symbiosis and its effects on plant growth depend on the genotypes of both the fungus and the plant in the partnership and also on the environmental conditions of their habitat. It would be interesting if we could provide some predictions about the types and the abundance of AM fungi that exist beneath a piece of land just by looking at its vegetation. Both field and laboratory experiments conducted in different parts of the world have provided some understanding of the interaction of AM fungi with their hosts. Yet a great deal of the mysterious and complex world of AM fungal ecology remains to be

unraveled because these interactions are also influenced by other organisms, soil biochemical and physical properties, and the dynamics of this assemblage (Johnson et al., 1992).

Helgason and his colleagues (2002) investigated the interactions of five plant species with four species of AM fungi. All plants and AM fungi except one AM fungal species used in this laboratory experiment were derived from a woody site in North Yorkshire (UK). Having failed to grow the field isolate *S. dipurpurescens* in pot culture, the investigators acquired the isolate from INVAM (Morgantwon, WV). The conclusions of this study suggested that individual selected species of AM fungi native to the same field bestowed different benefits and occupied different niches and proportions of their host plants. The degree of colonization, P uptake, and growth benefits varied with the plant-fungus combinations. For example, sycamore (*Acer pseudoplatanus*) seedlings showed no root colonization in the presence of *A. trappei*, *Glomus* sp., or *S. dipurpurescens*, but *G. hoi* not only infected 60% of its roots but also increased its shoot P content and growth. The roots of only three of five tree species were infected by *S. dipurpurescens*, with a low percentage root colonization averaging about 7%. The *S. dipurpurescens* fungus caused a minimal increase in P content and seedling growth of wood sage (*Teucrium scorodonia*) that received much higher benefits from other *Glomus* species. The remaining species, *A. trappei* and *Glomus* sp., coexisted in the roots of all plants used in the study with the exception of those of *Acer pseudoplatanus*, and caused different growth responses in their hosts (Helgason et al., 2002). This study provided evidence suggesting that plants may express some degree of selectivity toward members of AM fungi. Plants are likely to enter into a symbiosis with beneficial AM fungi and prevent or at least depress the growth of potentially parasitic AM fungi in their roots.

The presence of three species, *G. caledonium*, *Glomus* sp. type E3, and *G. mosseae*, was evaluated and quantified in the roots of leek plants with dual inoculations by band patterns of glucosamine protein separated by gel electrophoresis (Hepper et al., 1998). In paired inoculations where potted leek plants received inocula of two AM fungal strains as a mixture of soil and infected roots, one *Glomus* species simulating an indigenous AM fungal strain was mixed with a volume of a substrate and placed in the bottom part of the pot. The second species was used in analogy with an introduced strain. It was mixed with diluent soil but added in only about 1/10th of the volume of the indigenous-simulating strain and was placed

in a layer above the soil mixture that contained the indigenous-simulating strain (Hepper, 1998). Lastly, leek seeds were placed on top of the inoculated soil and covered with additional soil mixture. Leek plants were grown in the pots for three months.

Hepper et al. (1998) noted that the least competitive AM strain was *Glomus* sp. type E3. Its presence in the roots continued to decline during plant growth in the presence of either *G. caledonium* or *G. mosseae*, even when it was present as the indigenous species. By the final harvest, only traces of it were detected in the roots when it was used as the indigenous strain with *G. mosseae* as the introduced strain. It was not detected in the roots in the other dual inoculations at the last harvest date. When leek plants were inoculated with single strains, all three species increased plant growth, but the growth response to *Glomus* sp. type E3 was minimal. The most competitive strain was *G. caledonium*. It was able to occupy a portion of leek roots when it was introduced along with an indigenous *G. mosseae*, but neither of the two other species was detected in the roots when *G. caledonium* was used as the indigenous strain.

This study emphasizes the existence of competitiveness among AM fungal species and showed that some AM fungal species compete better than others for the host roots. In extreme cases, a strong competitor can invade and proliferate in the cortical cells of the host roots and exclude others. Competitive organisms use fast growth and reproduction, secretion of inhibitory growth substances, and induction of host resistance to invasion by a subsequent organism to survive the hostility of the members of the established communities and thereby occupy available susceptible infection sites (Hepper et al., 1988). There is nothing unusual about plants selecting for AM fungal strains with highly competitive traits and with potential benefit to the host. Thus, the selectivity for a plant-fungus combination depends on both the host and the mycosymbiont.

Formation of a mycorrhizal relationship between AM fungi and plant roots is influenced by both the host and soil conditions, but the extent of the effects of either the host or the soil may be affected by the composition of the AM fungal communities involved. A field experiment was conducted to determine the effects of five 4-yr-old grass monocultures grown in plots of different topsoil-subsoil-sand mixtures ranging from 0 to 100% topsoil in Cedar Creek National History Area in east-central Minnesota (Johnson et al., 1992). This study identified a total of 19 AM fungal species in the site. It also revealed that spore

abundance of *G. aggregatum* and *G. leptotichum* was high in the soil regardless of the host or the soil mixture status while the spore population and distribution of a group of eight species were mainly affected by the host plant species. The soil mixture affected the relative abundance of another set of eight species. Host-soil interaction significantly affected the population of *G. etunicatum* and *G. macrocarpum*. Although soil factors affect the composition of certain AM fungal populations, the abundance of the majority of AM fungi is strongly affected by the plant community, supporting the hypothesis that hosts have a significant effect on the composition of AM fungi residing in their roots (Johnson et al., 1992).

The present greenhouse study attempted to find out whether different cultivars of the same plant species (soybean) show any propensity to select or favor certain AM fungal species derived from field soils.

MATERIALS AND METHODS

Study Sites and Soil Sampling

In the fall of 2002, soil samples were collected from each of two types of soils, Clarion (a fine-loamy mixed mesic Typic Hapludoll) and Webster (a fine-loamy, mixed, mesic Typic Endoaquoll) in four soybean fields in Story County, Iowa, USA. Soil cores were excavated with a 10-cm diam. probe to a 15-cm depth from the soil surface, in such fashion that the root systems of the plants were included in the cores. Five samples within a circle about 3 m in diam. were taken from individual soil types within each field. The samples were about 2 m apart, and the fields were located within 6 km of each other. The samples were homogenized by chopping up and thoroughly mixing the soil and roots of each sample and were air-dried and stored in self-sealing bags in a 4°C walk-in cool room. Chemical properties and other information about the sampling sites and samples are included in chapter 3 of this dissertation.

Greenhouse Experimental Setup

In the spring of 2003, a greenhouse experiment was conducted to test whether four genetically different cultivars of soybeans select or have preference for different AM fungal strains. This was based on a determination of the population composition and relative abundance of AM fungal species associated with individual cultivars inoculated with AM fungal propagules derived from field soils.

Soil Medium and AM Fungal Inoculum

AM-fungal-free diluent soil was prepared by sterilizing low P soil at 100°C for 1 h three times with 48 h incubation intervals between subsequent sterilization cycles. Silica sand was also autoclaved at 121°C for 2 h. Autoclaved soil and sand were mixed in the ratio of 40:60 soil:sand (w/w). More details about the preparation and properties of the diluent used in this experiment can be found in chapter 3 of this dissertation.

Pots and other nonautoclavable materials used in these experiments were washed, disinfected with 70% ethanol for 10 min., and then rinsed with sterile deionized water. Autoclavable materials and glassware were autoclaved at 121°C for 20 min. An amount of 2 kg of autoclaved soil-sand mixture was placed in individual 2-L plastic pots. Prior to planting, the autoclaved soil-sand mixture in pots was subjected to detoxication by repeated washings and was reinoculated with indigenous AM-free microbes as described in the material and methods section of chapter 3 of this dissertation.

Inocula were taken from composite soil samples from each soil type within each field. The composite sample was a representative sample of chopped soil and roots from each soil type within each field, and 150 g of the composite inoculum was mixed with the 2-kg soil-sand mixture and poured back into the pot. Also, 150 g of the soil composite from the two soil types of all four fields was autoclaved and added to individual control pots. All soil weights in these experiments were expressed on an oven-dry weight basis.

Soybean Cultivars and Planting

Four soybean cultivars, BSR201, Iowa2052, Mandarin, and Peking, were used in this experiment. Soybean seeds were surface disinfected by soaking them in 70% ethanol for 1 min. The seeds then received five rinses of sterile deionized water.

The surface-disinfected soybean seeds were planted at a 1.5-cm depth at a rate of four seeds per pot. Two weeks later, the seedlings were thinned to two seedlings per pot. More detailed descriptions of the soybean cultivars used in this experiment can be found in chapter 3 of this dissertation.

Each of the four soybean cultivars was inoculated with field soil representing each of the two soil types from each of the four soybean fields, and each treatment was replicated five times. Thus, the experimental design for this experiment included a total of 180 pots as follows:

Inoculated pots: 4 soybean cultivars x 2 soil types x 4 fields x 5 reps = 160 pots.

Control pots: 4 soybean cultivars x 1 composite soil x 5 reps = 20 pots

Total = 180 pots.

Soybean Growth

The growth conditions for soybean cultivars in the greenhouse were described in chapter 3 of this dissertation. The pots were placed on greenhouse benches in a randomized complete block design to offset the light and temperature gradients. Every other week, the pots within individual blocks were rearranged according to a randomization program. Plants were incubated in the greenhouse until the seedlings reached reproductive stage R6-R7. Different cultivars reached the target stage at different times, so they were harvested accordingly at different times.

Plant Harvest

Harvest involved cutting the plant shoots at the soil surface in each pot. The contents of the pots were loosened, the large roots were picked from the soil by hand, and the small ones were separated from the soil by sieving. The roots were carefully freed from soil and gently washed with water, air-dried on tissue paper, and stored at $-4°C$ for later use.

AM Root Colonization Assessment and Spore Enumeration

Root colonization was determined in 0.25 g of fine air-dried roots that had been cleared using the method of Kormanik and McGraw (1982). The roots were stained following the method described by Phillips and Hayman (1970). More details can be found in the method and materials section of chapter 3 of this dissertation. The root colonization percentage was determined in duplicate 30 to 40 1-cm root segments by a gridline intersect method, as described by Giovannetti and Mosse (1980) and Kormanik and McGraw (1982).

Spores were collected from two individual 50-g soil samples to enumerate AM fungal spores in each pot. Healthy-looking spores were counted in square grid-lined Petri dishes under a light microscope using the methods of Adelman and Morton (1986), Porter et al. (1987), and Sylvia (1994). The results were given in number of spores per gram of soil, on the basis of oven-dry weight.

Identification of AM Fungal Species in Potted Soils

Clean, intact spores (free of visible parasites) extracted from well-mixed soil from individual soybean pots were separated into groups of spores based on microscopic observations. Spores with the same morphological attributes, size, color, hyphal attachment, presence of saccules, etc., were placed in each specific morphotype.

Spores within each morphotype were counted to represent the relative abundance of different AM fungal strains in individual soil samples. Two sets of 20 to 30 spores from each morphotype were placed on two sections of a labeled microscope slide. One set of the spores was mixed with two drops of polyvinyl alcohol lactic acid glycerol (PVLG) (Koske and Tessier, 1983). A mixture of Melzer's reagent and PVLG (1:1 v/v) was added to the other set of spores. The mounted spores were left in these stains for a minimum of five days before being subjected to microscopic observation to optimize reaction time of the spore cell walls with the stains (personal communication from Dr. Joseph Morton, West Virginia State University).

Data from microscopic examination of mounted spores, including the number of cell walls, the relative thickness of individual cell walls, the existence or absence of septa in subtending hyphae, and the reactivity of cell walls to mounting stains, were recorded for each morphotype. For references, the recorded observations were compared to species descriptions available at the website of the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM).

RESULTS AND DISCUSSION

The trap cultures of AM fungi using four soybean cultivars resulted in production of a large number of healthy-looking AM fungal spores in the pots. It also allowed sporulation of some species for which the spores were not initially detected in the original field soil samples.

AM Root Colonization and Spore Enumeration

Percentage mycorrhizal colonization of the four greenhouse-grown soybean cultivars harvested at R7/6 reproductive stage showed no statistically significant difference between Clarion and Webster soils used as inoculum (Fig. 4. 1). Perhaps, this lack of a soil effect was because of the wide distribution of fungi that readily colonized the soybean roots and the inoculum derived from the soil samples represented only about 1/10 (w/w) of the media in the pots and its effect was overwhelmed by the substrate (soil-sand mixture) representing the remaining 90% of the growth media.

Data analysis using ANOVA showed a significant difference (P<0.01) among the four soybean cultivars in the proportion of root tissue colonized by AM fungi. Average percentage AM fungal root infection varied from 91 to 92, 83 to 86, 78 to 89, and 38 to 74%, respectively, for Peking, Mandarin, BSR201, and Iowa2052 in Clarion soils (Table 4.1). The corresponding values in Webster soils were 78 to 93% for Peking, 81 to 90% for BSR201, 65 to 88% for Mandarin, and 56 to 85% for Iowa2052. Iowa2052 consistently had the lowest AM fungal root infection while Peking scored highest in the proportion of its roots that tested positive for mycorrhizal colonization (Fig.4.1).

Spore enumeration showed a large increase of spore density in pots of all four soybean cultivars compared to the initial spore counts in fresh field soils (Fig. 4.2). Mean spore counts in the original field soils were low, ranging from 0.16 to 0.56 and 0.06 to 0.86 spores g^{-1} soil, respectively, for Clarion and Webster soils. The spore counts in the pot mixtures were larger when the host roots were grown in pots inoculated with Clarion-derived soil-root mixture than in those that had Webster-derived inocula. In the Clarion soils, the spore density varied
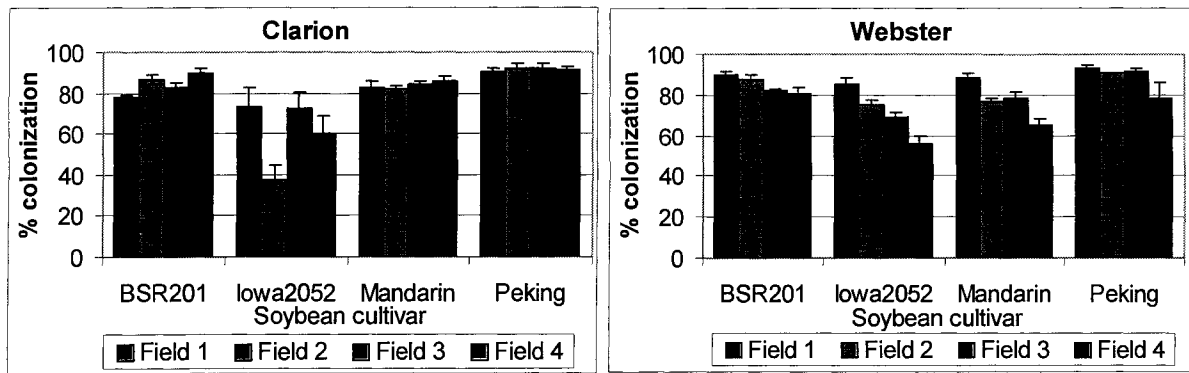
Fig. 4.1. Percentage root colonization of four soybean cultivars used in trap cultures in greenhouse pots. Each value represents the mean of five replicates. The error bars represent standard deviations.
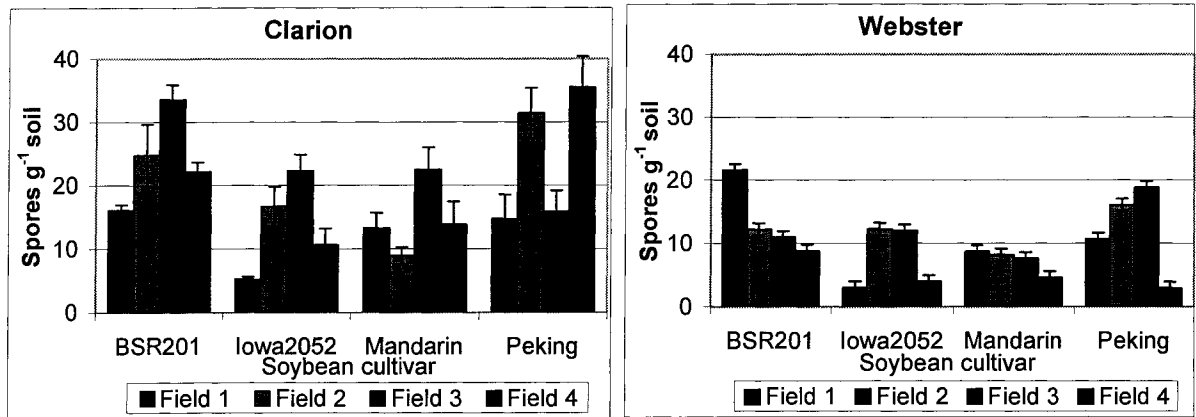

Fig. 4.2. Spore counts of four soybean cultivars used in trap cultures in greenhouse pots. Each value represents the mean of five replicates. The error bars represent standard deviations.

Table 4.1. Mean percentage AM colonization (mean ± SEM, n = 5) in roots of soybean cultivars grown in pots inoculated with Clarion and Webster soils from soybean fields.

	------------------------% AM fungal colonization-------------------------				
Clarion	BSR201	Iowa2052	Mandarin	Peking	Pooled
Field 1	78.2 ± 0.4	73.7 ± 4.2	83.2 ± 1.1	90.7 ± 0.6	81.5 ± 1.1
Field 2	86.7 ± 0.9	37.6 ± 2.9	82.2 ± 0.6	92.3 ± 0.9	74.7 ± 0.8
Field 3	83.0 ± 0.9	73.0 ± 3.5	84.1 ± 0.8	92.4 ± 0.9	83.1 ± 0.9
Field 4	89.5 ± 1.3	60.3 ± 3.6	86.3 ± 1.1	91.7 ± 0.5	82.0 ± 1.0
Pooled	84.4 ± 0.4	61.2 ± 1.6	83.9 ± 0.4	91.8 ± 0.3	80.3 ± 0.4
Webster	BSR201	Iowa2052	Mandarin	Peking	Pooled
Field 1	90.3 ± 0.4	84.9 ± 1.6	88.1 ± 1.1	92.9 ± 0.8	89.1 ± 0.5
Field 2	87.3 ± 1.1	75.2 ± 0.9	77.1 ± 0.6	90.4 ± 0.3	82.5 ± 0.4
Field 3	82.3 ± 0.5	68.9 ± 1.0	78.0 ± 1.4	91.1 ± 0.8	80.1 ± 0.5
Field 4	80.8 ± 1.1	55.9 ± 1.7	65.3 ± 1.3	78.1 ± 3.4	70.0 ± 1.0
Pooled	85.2 ± 0.4	71.2 ± 0.6	77.1 ± 0.5	88.1 ± 0.9	80.4 ± 0.3
Both soils	84.8 ± 0.3	66.2 ± 0.8	80.5 ± 0.3	90.0 ± 0.4	80.4 ± 0.3

Table 4.2. Average spore counts (mean ± SEM, n = 5) in media used to grow soybean cultivars in pots inoculated with Clarion and Webster soils from soybean fields.

	--------------------------------Spores g^{-1} soil--------------------------------				
Clarion	BSR201	Iowa2052	Mandarin	Peking	Pooled
Field 1	16.1 ± 0.4	5.2 ± 0.2	13.3 ± 1.1	14.7 ± 1.7	12.3 ± 0.5
Field 2	24.7 ± 2.2	16.7 ± 1.4	8.9 ± 0.6	31.4 ± 1.8	20.4 ± 0.8
Field 3	33.5 ± 1.1	22.3 ± 1.1	22.5 ± 1.6	15.9 ± 1.5	23.5 ± 0.7
Field 4	22.1 ± 0.7	10.6 ± 1.2	13.8 ± 1.6	35.5 ± 2.2	20.5 ± 0.8
Pooled	24.1 ± 0.6	13.7 ± 0.5	14.6 ± 0.5	24.4 ± 0.8	19.2 ± 0.3
Webster	BSR201	Iowa2052	Mandarin	Peking	Pooled
Field 1	21.6 ± 0.7	3.0 ± 0.4	8.7 ± 0.5	10.7 ± 0.6	11.0 ± 0.3
Field 2	12.2 ± 0.5	12.3 ± 1.1	8.2 ± 0.7	16.1 ± 1.8	12.2 ± 0.6
Field 3	11.0 ± 0.6	12.0 ± 1.1	7.6 ± 0.9	18.9 ± 1.6	12.4 ± 0.6
Field 4	8.8 ± 0.9	4.0 ± 0.4	4.6 ± 0.8	2.9 ± 0.5	5.1 ± 0.3
Pooled	13.4 ± 0.3	7.8 ± 0.3	7.3 ± 0.3	12.1 ± 0.5	10.2 ± 0.2
Both soils	18.7 ± 0.4	10.8 ± 0.3	10.9 ± 0.3	18.2 ± 0.4	14.6 ± 0.2

from about 36 (field 4) to 15 spores g^{-1} soil (field 1) for Peking, 34 (field 3) to 16 spores g^{-1} (field 1) for BSR201, 22 (field 3) to 9 spores g^{-1} (field 2) for Mandarin, and from 22 (field 3) to 5.25 spores g^{-1} (field 1) (Table 4.2). The spore population in potted soils was more

sensitive to soil conditions than root colonization, as the spore population was significantly affected by both the soil type (P<0.01) and the host involved (P<0.05).

Many organisms use sporulation as a means for survival under stressful conditions. AM fungi, on the other hand, tend to sporulate under greenhouse conditions where adequate nutrients are provided to the host, there is little competition with other organisms, and harsh, fluctuating field conditions are usually considerably reduced. Moreover, the fungus is contained in pots where the roots of the host plants are readily accessible. All this would increase the probability of contact between the AM fungal propagule and a root.

A possible explanation for high sporulation in pots is that the fungus expends energy synthesizing hyphal structures. In the field, the expansion of external hyphae is relatively unrestricted and can consume all of the available energy. However, in pots the expansion of external hyphae is limited, as the hyphae tend to hit the pot walls. That may cause the fungus to switch to producing spores after a certain hyphal density is achieved. This phenomenon can be explained either as a response to stress as the fungus has limited space to produce hyphal structures, or sporulation can be interpreted as an alternative mechanism for the fungus to store excess energy.

Distribution of AM Fungal Species as Affected by the Host

Data collected from this greenhouse experiment indicated that the spore distribution and spore population density (spores g^{-1} soil) of AM fungal species differed among the four soybean host cultivars, BSR201, Iowa2052, Peking, and Mandarin (Figs. 4.3 and 4.4). Spores of at least twelve AM fungal species were identified in association with the roots of the four soybean cultivars used in this study, and the majority of these species belonged to *Glomus*, the largest genus in the Glomeromycota phylum. These species included *G. claroideum* (G.clo), *G. clarum* (G.clu), *G. constrictum* (G.con), *G. etunicatum* (G.etu), *G. fasciculatum*, *G. geosporun* (G.geo), *G. mosseae* (G.mos), *G. vesiforme*, *G. viscosum* (G.vis), *Entrophospora infrequens* (E.spp), and *Paraglomus occultum* (P.occ). Members of *Acaulospora* (A.spp) and *Gigaspora* (Gi.spp) genera were also found in the trap cultures, and their spore populations were reported only at the genus level. Most frequently encountered species of these two genera were *A. spinosa*, *A. calossica*, *Gi. gigantea*, *Gi. margarita*, and

Fig. 4.3. Species diversity and spore abundance of AM fungi in trap cultures with soybean cultivars grown in media inoculated with Clarion soil. Each entry represents an average of five replicates, and each replicate is an average of two measurements. The error bars represent standard deviations.
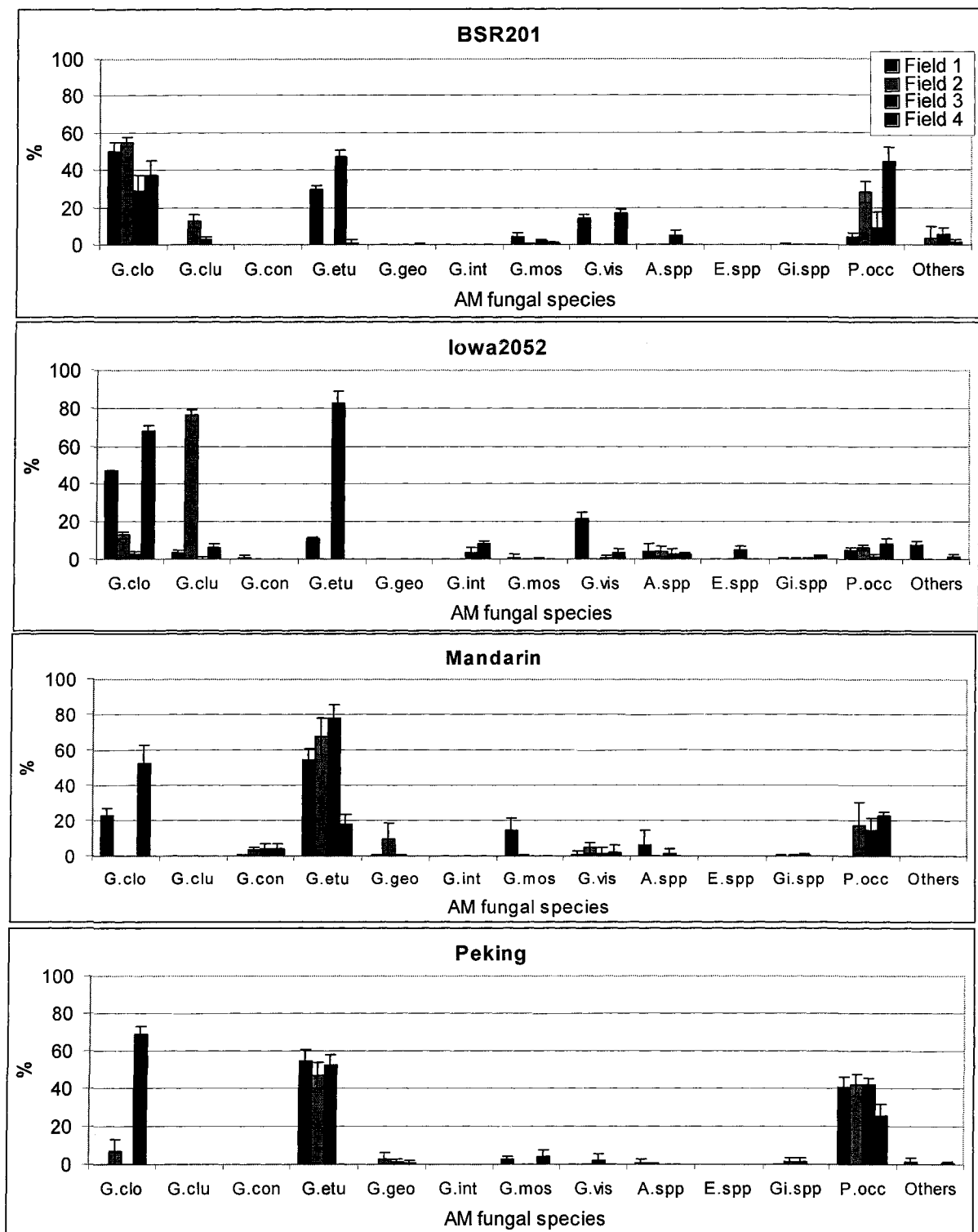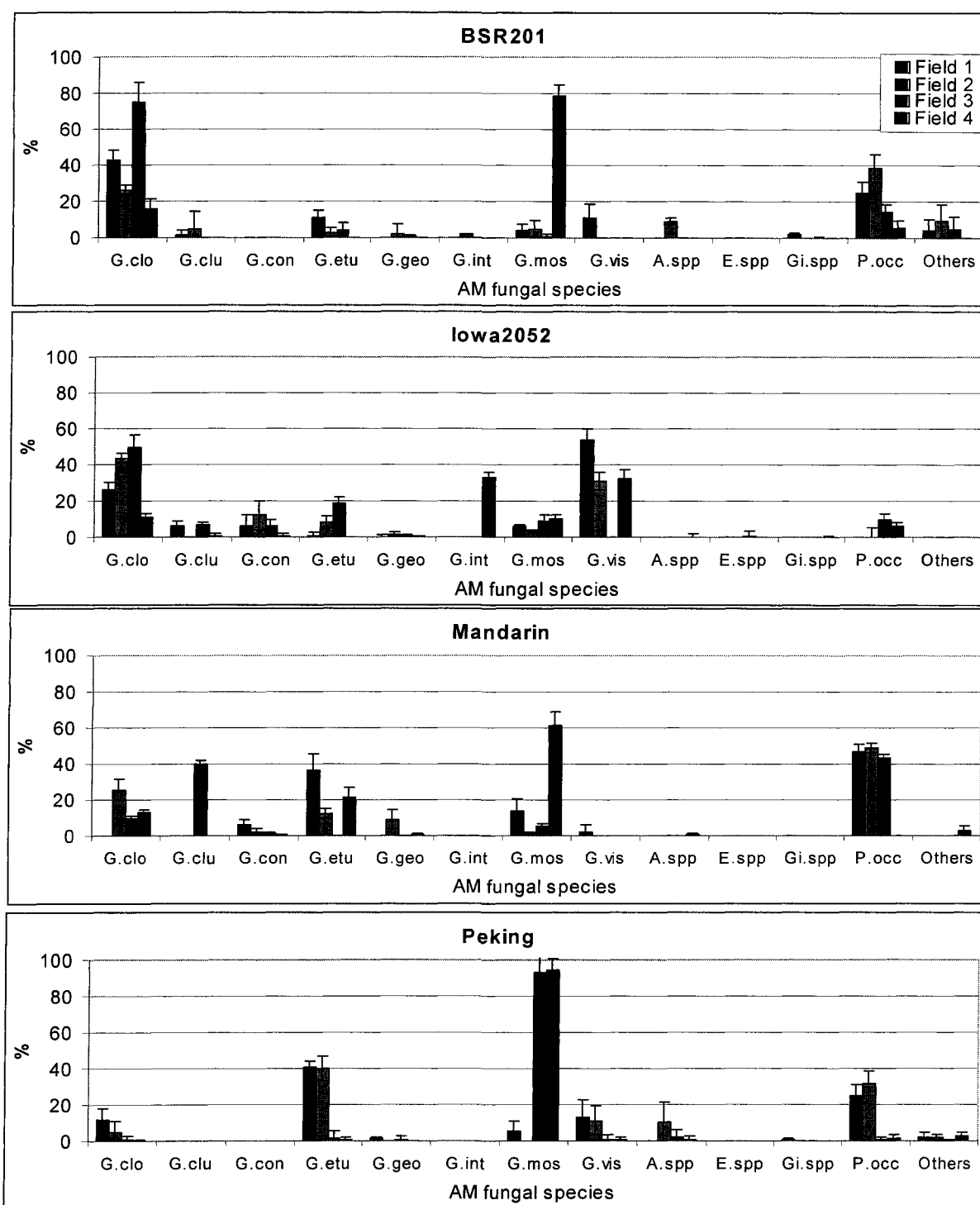
Fig. 4.4. Species diversity and spore abundance of AM fungi in trap cultures with soybean cultivars grown in media inoculated with Webster soil. Each entry represents an average of five replicates, and each replicate is an average of two measurements. The error bars represent standard deviations.

Gi. rosea. Some small spores whose identity was uncertain could possibly have been *A. trappei*, *G. eburneum*, *G. luteum*, *G. aggregatum*, *G. spurcum*, or *P. brazilianum*.

Five AM fungal strains were present in the pots in relatively high population, regardless of the host. Three were consistently detected in the pots of all four cultivars, but their spore populations varied with the host type. Spores of the four remaining AM fungal species were detected in pots of some cultivars but were absent in pots of other cultivars (Table 4.3).

The predominant AM fungal species in all of the trap-culture pots were *G. claroideum*, *G. etunicatum*, *G. mosseae*, *G. viscosum*, and *P. occultum* (Figs. 4.3 and 4.4). Each of these five AM fungal species typically represented more than 10% of the total AM spore population. Spores of *G. clarum* were not initially found in the field soils, but *G. clarum* was induced to sporulate in the pot culture, and represented a fairly large portion of the total spore population in the pots. The predominance of *Glomus* species is in agreement with several studies that have attempted to determine the distribution and the population composition of AM fungi in Iowa fields. A number of *Glomus* species have been identified, including *G. albidum*, *G. constrictum*, *G. geosporum*, *G. fasciculatum*, and *G. mosseae*. Other species commonly found in field soils include *Acaulospora spinosa*, *A. trappei*, *Gigaspora* species, *Paraglomus occultum*, and *Scutellosepora* species that were associated with soybeans, corn, poplar seedlings, or even prairie grasses (Khalil et al., 1992; Troeh, 1999; Walker et al., 1982). All of these studies have relied on diagnostic work based solely on the spores directly extracted from the field soils. This technique is likely to underestimate the population diversity of AM fungi in those soils because many species have been found to not sporulate under field conditions (Gollotte et al., 2004; Franke-Snyder et al., 2001).

Table 4.3. Presence of AM fungal species found in pot cultures of soybean cultivars.

	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.vis	A.spp	E.spp	Gi.spp	P.occ
BSR201	x	x	-	x	x	x	x	x	x	-	x	x
Iowa2052	x	x	x	x	x	x	x	x	x	x	x	x
Mandarin	x	x	x	x	x	-	x	x	x	-	x	x
Peking	x	-	-	x	x	-	x	x	x	-	x	x

Statistical analysis of the data from this study indicated that the mean percentages of *G. claroideum*, *G. clarum*, *G. etunicatum*, *G. mosseae*, and *G. viscosum* in the spore population of the trap culture pots representing different fields were significantly different among fields, between the two soil types, and among the soybean cultivars at the 1% level of confidence. The mean percentage distribution of spores of *P. occultum* varied significantly among the fields (P<0.0001) and among cultivars and their interactive effects, but was not significantly different between the two soil series.

The most abundant AM fungal species in these trap cultures was *G. claroideum*, as it was produced in the roots of all four cultivars and its spores represented approximately 46, 36, 16, and 13% of the total spores in the pots sown to BSR201, Iowa2052, Mandarin, and Peking, respectively. Spores of *G. clarum* had their largest representation of about 13% in Iowa2052 pot soils, and represented only 4% in Mandarin pots and 3% in BSR201 pots. None were recognized in pots sown to Peking. The average percentages of *G. etunicatum* spores were 38, 33, 18, and 13%, respectively, for Mandarin, Peking, Iowa2052, and BSR201. Peking pot soils contained the largest estimated average number of spores of *G. mosseae* that made up 27% of the total spores recovered. On the other hand, spores of *G. mosseae* approximated only 13, 13, and 4% of the spore population in Mandarin, Iowa2052, and BSR201, respectively.

Similar to *G. mosseae*, spores of *P. occultum* constituted their largest estimated portion of the spore population in pot soils of Peking (30%), followed by Mandarin (26%), BSR201 (23%), and finally Iowa2052 (6%) pots. Spores of *G. viscosum* were produced mainly in pots of Iowa2052 plants where they represented an average of 20% of the spore population. This species only made up about 4% or less in the pots sown to the remaining cultivars.

G. claroideum, *G. etunicatum*, and *P. occultum* were among the most aggressive AM fungi in pot cultures as they were the most frequently found and the most abundant species in trap cultures (Figs. 4.3 and 4.4). The relative proportion of *G. viscosum* spores in pots, on the other hand, declined relative to their occurrence in the original field soils (compare Figs. 4.3 and 4.4 to Fig. 3.5). The main difference between this species and the three preceding species was the relative amount of hyphae. Spores of *G. viscosum* were found bound to a greater amount of hyphae. The remaining AM fungal species usually represented less than 10% of the total spore population in the examined soils, so they were considered to be minor species.

They included *G. constrictum, G. geosporum, G. intraradices, Acaulospora* species, *Entrophospora* species, and *Gigaspora* species. The field location, soil type, and cultivar factors all significantly affected the average percentage distribution of the three *Glomus* species and of the *Entrophospora* species listed above. The occurrence of *Acaulospora* species and *Gigaspora* species appeared to be independent of all these factors in this study, and their spores occurred in small percentages in association with some cultivars while they were not detected in other cultivars. For example, *G. geosporum* produced spores that represented less than 1% of the total spore population in Peking, BSR201, and Iowa2052 pots, and no spores were recognized in Mandarin pots, while spores of *G. constrictum* had their largest occurrence in pots sown to either Iowa2052 or Mandarin, and spores of *G. constrictum* were absent from pots sown to BSR201 or Peking. *G. intraradices* spores were found in Iowa2052 pots at the rate of 6% of the total spores recovered, represented only 0.3% of the spore population in BSR201 pots, and were not found in pots of the other two cultivars.

These results are in agreement with previous findings that the composition and the abundance of AM fungal species residing in plant roots depend on the plant-fungus combination (Gollotte et al., 2004; Helgason et al., 2002). That is probably related to the difference in the compatibility between the host and the colonizing species or in competitive ability traits of the coexisting species (Hepper et al., 1988).

CONCLUSIONS

Colonization of soybean roots by AM fungi was not significantly affected by soil type in this study. The mean percentage root colonization varied significantly among cultivars. Spore population on the other hand was significantly affected by both soil type and soybean cultivar. Thus, the argument suggested earlier that the inoculum effect (either from Webster or Clarion) might be masked by the effect of the diluent soil (the same for all pots) might not be valid because the soil type was significant in the distribution of the spore population. Perhaps, the more logical explanation is that the assessment of root colonization dealt with fungal structures inside the roots where the soil would have little or no effect, but the spores outside the roots were exposed to the effect of the soil

This study concluded that the majority (about 70%) of species identified in trap cultures were associated with the roots of each of the four cultivars, but their abundance differed with both plant cultivar and fungal species. Five out of twelve species encountered in our soils were rated aggressive as they not only were detected in all pots but represented a large proportion of the spore populations in those soils. Spores of several other species, mainly those of the genera other than *Glomus*, occurred in much lower counts than those of the predominant AM fungal species.

The absence of certain species in pots growing certain soybean cultivars might be related to the selection of these cultivars against those AM species. For example, Peking had the lowest species richness among the cultivars, with only ¾ of the encountered species. Spores of *G. clarum*, *G. constrictum*, *G. intraradices*, and *E. infrequens* were not detected in trap culture pots of Peking. Neither spores of *G. intararadices* nor those of *E. infrequens* were found in Mandarin culture pots. The occurrence of *G. constrictum* and *E. infrequens* in BSR201 pots was not confirmed. The Iowa2052 cultivar had the highest AM fungal species richness, as its pots had all the species found in the pots of other cultivars, and it also had *E. infrequens* that was not detected in any other pots. Could it be that the other cultivars had mounted a defense against *E. infrequens*?

FUTURE WORK

Identification of AM fungal species in the roots in addition to spore identification should provide more reliable results in AM fungal diversity studies to establish the right connections between free-soil spores and the root-inhabiting fungi (Clapp et al., 1995). In our study, however, the soil inoculum was composed mainly of colonized roots and hyphae fragments, and the spores produced in pot cultures were presumed to have been freshly produced in our pot cultures. The many-fold increase in spore population, compared to the spore population in the original field soil, supported this claim.

Different species have been found to sporulate during different seasons and under different soil conditions, so soil sampling at different seasons and the use of trap culture techniques under different environments could increase the species richness identified in a study, and could provide more accurate information regarding the selective traits of these cultivars for or against certain AM fungal species.

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CHAPTER 5. EFFICACY OF NATIVE AM FUNGI ON SOYBEAN GROWTH

In mycorrhizal symbiosis, both the photosynthetic plants and the obligate symbionts benefit through bidirectional transfer of nutrients between arbuscular structures of AM fungi and the cortical cells of the host roots (Bago et al., 2000; Pfeffer et al., 1999). About 90% of land plants form mycorrhizal associations with AM fungi. They receive a myriad of benefits that include promoting growth, enhancing uptake of P, Zn, Cu, NH_4^+, and water, increased disease resistance, enhanced plant competitive ability, increased plant productivity, and maintaining plant diversity (Ruiz-Lozano et al., 2001; Sylvia et al., 2001; van der Heijden et al., 1998). Plants provide reduced C that is required for the survival, development, maintenance, and functioning of the AM fungi. From the released C, AM fungi can synthesize other organic compounds such as palmitic acid in intraradical hyphae only in the presence of active roots of the host plants that provide them with necessary signals to activate the fatty acid synthase (FAS) complex to make palmitic acid (Trépanier et al., 2005).

The net effects of AM fungi on plants are evaluated on the basis of a cost-benefit balance sheet of the symbiosis (Grimoldi et al., 2005). Sometimes the C cost to the plants for sustaining AM fungi can be too high in comparison to the benefits received by the plants (Olsson et al., 2005). AM fungi are most noticeable for their role in improved P nutrition as their extraradical hyphae ramify into the soil matrix, thus increasing the absorption of P and other low mobility nutrients. P is often the most limiting nutrient to crop growth, and application of P is not always effective because P is highly reactive in soils (Nielsen et al., 1998; Smith and Read, 1997). Plants not receiving an adequate P supply suffer from growth retardation and have high root-to-shoot ratios. Increased P content of plant tissues through AM symbiosis, however, does not always result in increased plant dry weight when root respiration is very high, or when C or some other element becomes limiting.

Communities of AM fungi regulate plant population density, as AM fungi increase the survival rate of plants where the population is low, but this effect decreases as plant density increases. Thus, AM fungi contribute to stabilizing and maintaining certain levels of plant populations (Koide and Dickie, 2002). Furthermore, mycorrhizal plants tend to produce more flowers through branching, and consequently produce more seeds that tend to have a high P

concentration and high vigor, germinate faster, and compete better in the soil than the seeds from nonmycorrhizal plants.

The structure, composition, and establishment of plants are controlled by the interactions of the plant roots with the microbial communities that find an oasis where root exudates are abundant in the endorhizosphere, rhizoplane, and ectorhizosphere (Vandenkoornhuyse et al., 2002). AM fungi are important members of the soil microbial communities and in most circumstances have a mutualistic relationship with plants, but situations where their effects were neutral or detrimental to the host plants have been encountered. It has been demonstrated that some AM fungi have special functions toward their hosts and that AM fungal species differ in their ability to increase P uptake, plant growth, and crop quality due to their functional diversity (Garcia-Garrido, 2000). As an example, *G. intraradices* reduced the flowering rate as well as the yield of potato plants, but two other AM fungal strains, *Vaminoc* and *Endorize*, increased potato crops. Plants inoculated with *Vaminoc* produced the highest yield while *Endorize* produced the highest quality seeds, so it is crucial to find the appropriate match between plants and the AM fungal isolate for the intended purpose (Duffy and Cassells, 2000).

Mycorrhizal dependency varies with both the hosts and the infecting AM fungal strains. Three soybean cultivars responded positively but differently to colonization by *Gi. margarita* (Khalil et al., 1999). Mycorrhizal Soja plants had the greatest response as their average total shoot P and shoot dry weight were significantly larger than those of nonmycorrhizal plants and they had the highest value (0.52) for P use efficiency. Mycorrhizal plants of Mandarin and Swift cultivars had higher total P and shoot dry weights but were not statistically different from those of nonmycorrhizal plants. The effects of AM fungi on plant growth, however, vary not only with the plant-fungus combination but also with soil physical and chemical properties defined by the five soil forming factors, and with soil management practices (Allen et al., 1987). Thus, in agricultural systems where mixed microbial communities predominate, it becomes difficult to determine the contribution of individual AM fungal strains in the community (Smith and Read, 1997).

This study evaluated the growth effects of individual AM fungal strains native to Iowa soils on different soybean cultivars in a greenhouse experiment.

MATERIALS AND METHODS

The growth response of three genetically different soybean cultivars to spores of three AM fungal taxa extracted from soils of soybean fields of central Iowa was evaluated in a greenhouse experiment in the fall of 2004.

Characteristics of Soil Sampling Sites

The sampling sites were two of the four private farms located in Story County, central Iowa. More information about the locations of the sampling sites, chemical and physical soil characteristics, and sampling strategies is given in the materials and methods section of chapter 3 of this dissertation.

Greenhouse Experiment

The effects of three species of *Glomus*, *G. claroideum*, *G. etunicatum*, and *G. mosseae*, were assessed on 10-wk-old seedlings of three soybean cultivars, BSR201, Iowa2052, and Peking, based on shoot dry weight, root dry weight, plant height, number of seeds, and seed pods in each pot.

AM Fungal Spores

AM fungal propagules in the form of a soil-root mixture were propagated in 2-L black poly-tainer pot cultures (Nursery Supply Inc, Orange, CA) using four soybean cultivars in the greenhouse. Plants were harvested at the R7 reproductive stage. AM fungal species that produced high spore populations were chosen for the efficacy study. A more detailed description of the propagation procedure is provided in the material and methods section of chapter 3 of this dissertation. Spores of *G. mosseae* were originally from Webster soil of field 4 and had been propagated using Mandarin (G.mos.W4.Man) soybean. Those of *G. etunicatum* (G.etu.C1.BSR, G.etu.W4.Man) were collected from Clarion soil of field 1 and Webster soil of field 4 and had been propagated on BSR201 and Mandarin soybean,

respectively, from the two soils. Spores of *G. claroideum* (G.clo.C1.BSR, G.clo.C4.BSR, G.clo.C4.Pek, G.clo.W1.Man, and G.clo.W4.Man) came from Clarion soil of fields 1 and 4 propagated on BSR201 soybean, from Clarion 4 using Peking soybean, and from Webster soil of fields 1 and 4 propagated on Mandarin soybean.

Preparation of Soil Medium and Pots

Disinfection of pots and other materials and preparation and sterilization of the soil and sand mixture used as a potting substrate were performed as described in Chapter 3 of this dissertation, except that the substrate used in this study was composed of a soil:sand 60:40 (v/v) mixture.

After the 2-L pots were filled with the soil:sand mixture and autoclaved, they were leached with 500 mL of sterile water every other day for 1 wk to remove sterilization-induced toxic elements (Lopes and Wollum, 1976 as cited by Wolf and Skipper, 1994). Microbial inoculum was applied to individual pots after 1 wk of leaching to reestablish the AM-fungal-free indigenous microbial population lost to autoclaving (Rovira and Bowen, 1996). The weight of the pots at field capacity was based on the moisture content of the potted soil after 3 d of drainage. The pH value of the soil mix was about 6.6 in 2:1 water:potting mix. Further details about this section are provided in chapter 3 of this dissertation.

Pregermination of Soybean Seeds

The soybean seeds were surface disinfected by immersion in 10% sodium hypochlorite for 2 min and rinsed with five changes of sterile deionized water. The surface-disinfected seeds were germinated on germination paper. Germination paper sheets were autoclaved and moistened by spraying them with sterile deionized water. Seeds were placed on two layers of autoclaved germination paper and were covered with a third sheet. The papers with the seeds were rolled into a tube and fastened with rubber bands. The seeds inside the rolled papers were incubated in germination trays in the dark at room temperature (about 25°C) for 1 wk.

Inoculating and Planting Pots

About 500 mL of potting mix was removed from each previously filled 2-L pot to a separate container. The middle soil of the pot was mixed with 100 spores of *G. mosseae* or 120 spores of *G. claroideum* or *G. etunicatum*. A larger number of spores of the latter two *Glomus* species were used because previous experience had indicated that spores of *G. mosseae* germinated better than those of *G. etunicatum*, and the larger size of *G. mosseae* spores gave a comparable volume with a smaller number. Two healthy pregerminated seedlings of BSR201, Iowa2052, or Peking were transplanted directly into the inoculated soil. Subsequently, the 500-mL of potting mix was poured back into the pot. The planted pots were immediately transported to the greenhouse where they were watered as needed to keep them near field capacity.

Greenhouse Conditions and Watering Schedule

Soybean seedlings were incubated in the greenhouse for 10 wk under a light intensity of about 960 μmol m^{-2}s^{-1} as a combination of greenhouse natural sunlight supplemented with approximately 150 μmol photon m^{-2}s^{-1}. The day/night temperatures were 25/27°C with a photoperiod of 16 h. The pots were irrigated as needed to near field capacity with sterile distilled water, and nutrient solution (Broughton and Dillworth, 1971) containing only 1/2 of the recommended P and 70 mg L^{-1} KNO$_3$ was applied with every third watering.

Soybean Harvest and Data Collection

The 10-wk-old soybean plants were cut at the soil surface level. The shoots were cleaned of soil and placed in brown paper bags. The plant height, number of pods, and number of seeds per pod were recorded for each pair of plants in each pot, and the shoots were replaced in the bags. The contents of the pots were carefully separated into roots and soil by sieving or hand picking the roots. The roots were washed with water, blotted in paper towels, and placed in small paper bags. Both roots and shoots were dried in the oven at 70°C for 72 h to

obtain their dry weights. The response of soybean cultivars to AM fungi was expressed in terms of shoot dry weight and was calculated by the formula of Planchette et al. (1983).

The percentage root colonization was evaluated on duplicate sets of 30 to 35 1-cm root segments. The roots were cleared with 10% KOH (w/v) for 1 h at 95-100°C. After water rinsing, the roots were acidified with 1% HCl (v/v) and then stained for 30 min at 95-100°C with 0.05% (w/v) trypan blue dissolved in equal volumes of glycerol, lactic acid, and water. Subsequently, the roots were transferred to glass vials containing 50% glycerol in water for destaining for at least a day (Phillips and Hayman, 1970). The percentage root colonization was determined by the gridline intersect method as explained in chapter 3 of this dissertation. Root colonization was evaluated as the percentage of intersections where colonized roots were observed.

Statistical Analysis

The percentage root colonization by AM fungal species, shoot dry weight, and root dry weight of plants were analyzed in a complete randomized block design (CRBD). A one-way analysis of variance (ANOVA), least significance difference (LSD), and Pearson correlation analysis were performed using SAS®\ (Statistical Analysis System) programs (SAS Institute Inc., Cary, NC).

RESULTS and DISCUSSION

The results of this study indicated that the 10-wk-old soybean cultivars, BSR201, Iowa2052, and Peking, produced significantly (P<0.0001) higher shoot dry weight and higher number of seeds when they were inoculated than when they were grown free of AM fungal inoculum. There were also significant differences among the weights of the three cultivars.

Spores of *G. claroideum* (G.clo), *G. etunicatum* (G.etu), and *G. mosseae* (G.mos) were isolated from trap culture pots inoculated with either Clarion (C) or Webster (W) soils of fields 1 and 4 and were propagated on BSR201, Mandarin, or Peking. Paired comparison of the means using F-Tests indicated that all three soybean cultivars produced significantly higher shoot dry weights (g pot^{-1}) and that BSR201 and Iowa2052 set more seeds (seed pot^{-1}) than their corresponding control (nonmycorrhizal) plants. Plants of certain genotypes, including soybean, have been shown to produce a larger number of flowers when inoculated with AM fungi than when grown without AM fungal inoculum, and production of more flowers is likely to result in a larger number of seeds (Koide, 2000).

Characteristics of Soybean Cultivars

The three soybean cultivars, BSR201, Iowa2052, and Peking, belong to different genetic lines and had significantly (P<0.0001) different responses to individual AM fungal species in terms of shoot dry weight, root dry weight, and seed production as recorded for individual pots. They also differed among each other in height (P<0.0003).

Average plant height (from soil surface to highest node on the stem) at harvest for Peking was significantly higher than that of either BSR201 or Iowa2052. The respective heights for Peking were 31 to 56 cm with an average of 38 cm for inoculated plants and 22 to 39 cm with an average of 30 cm for control plants. BSR201 plant heights were 21 to 45 cm with an average of 33 cm for inoculated plants and 21 to 41 cm with an average of 31 cm for control plants. Iowa2052 plant heights were 17 to 39 cm with an average of 28 cm for inoculated plants and 22 to 35 cm with an average of 28 cm for control plants. The average height of BSR201 plants was higher than that of Iowa2052 plants, but the difference between the two

means was not statistically significant. The difference in height among these cultivars was mostly attributed to their genetic nature, but mycorrhizal inoculation slightly increased the average plant height for BSR201 and Peking but not for Iowa2052 plants.

The recorded stage of development (Ritchie et al., 1997) of the 10-wk-old plants at harvest was easily distinguishable among the three soybean cultivars, with inoculated plants being slightly more mature than their corresponding control plants for each cultivar. Most of the Iowa2052 plants were smaller and more mature than those of the other two cultivars. The indeterminate Peking plants were mostly at early reproductive stages ranging from full bloom (R2) to early pod formation (R3). Though smaller, the plants of Iowa2052 were the most mature of the three cultivars, as most of them had developed pods with seeds starting to mature (R6/R7). Plants of BSR201 were generally less mature than those of Iowa2052 but more mature than those of Peking. About one-half of the BSR201 plants had pods with full seeds (R6), and the developmental stages of the remaining plants ranged from late flowering (R2) or early pod formation (R3) to mostly full pod formation (R4). Plants inoculated with AM fungal species were found to mature faster than nonmycorrhizal plants (Ozbec, 1998).

Verification of Root Colonization by the AM Fungal Isolates

All of the inoculated plants were mycorrhizal, and all of the examined roots of uninoculated plants (controls) had zero percent AM fungal colonization. Also, large numbers of spores were produced by each strain (spore count not performed). The mean percentage root colonization was statistically ($P<0.0001$) different among the cultivars. The average percentage AM fungal root colonization of Peking (33%) was significantly ($P<0.0001$) greater than that of BSR201 (30%) and that of Iowa (24%) (Fig. 5.1).

Fungal strains of the same or different AM fungal species colonized different proportions of the roots of different host cultivars. The *G. etunicatum* strain, G.etu.C1.BSR, consistently colonized high proportions of the root tissue in all three cultivars, with means ranging from about 38% for Iowa2052, to 44% for BSR201 and 45% for Peking. *Glomus claroideum*, G.clo.W1.Man, had higher mean percentage root colonization for Peking (48%) and for BSR201 (45%), but a lower value was recorded for Iowa2052 (20%), compared to G.etu.C1.BSR. The overall mean percentage root colonization was not significantly different
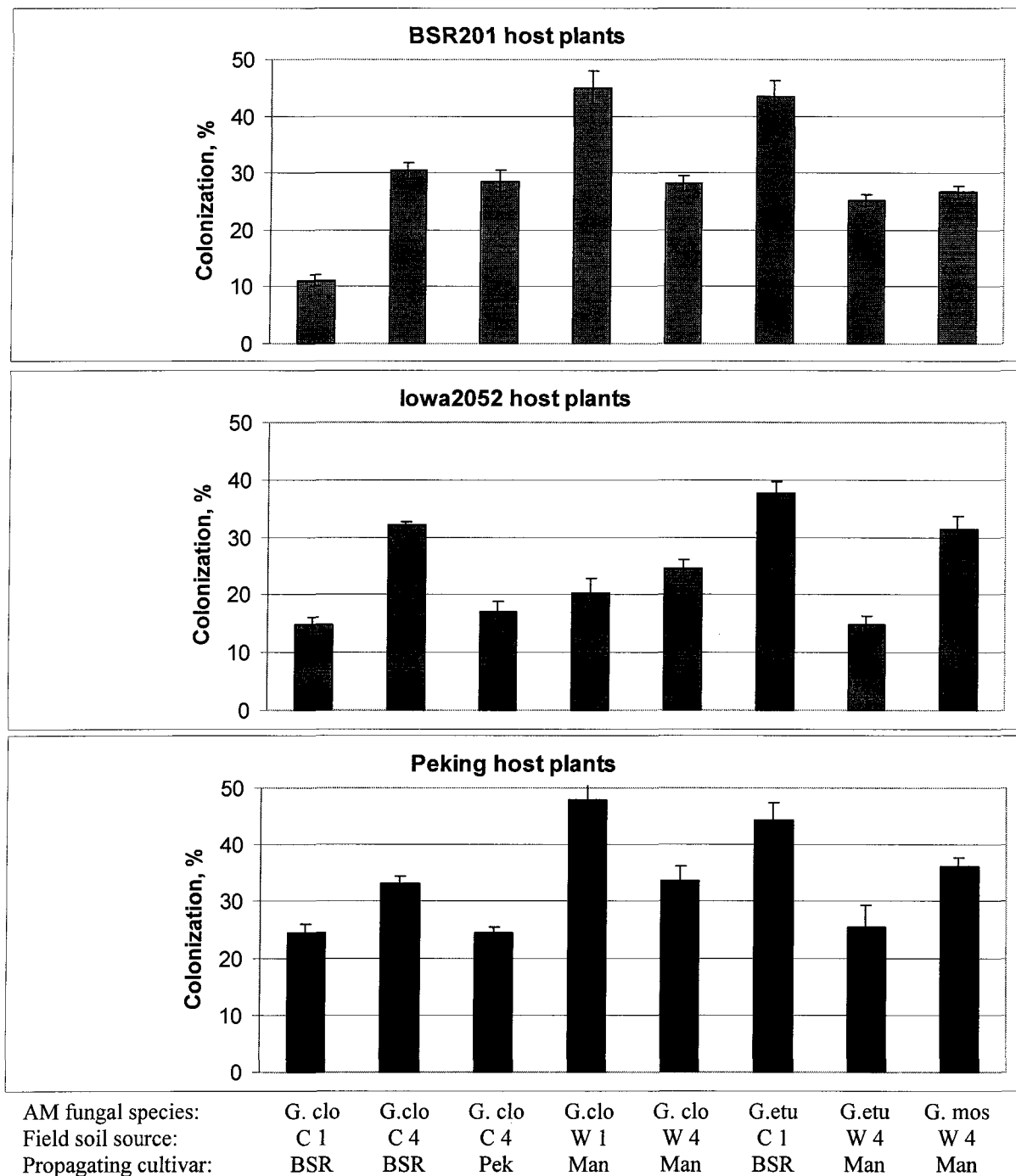
Fig. 5.1. Average percentage root colonization of three soybean cultivars inoculated with
strains of *Glomus claroideum* (G.clo), *G. etunicatum* (G.etu), and *G. mosseae* (G.mos)
derived from Clarion (C) and Webster (W) soils of fields 1 and 4 and propagated on
BSR201, Peking, or Mandarin soybean cultivars. Each entry represents an average of
results from five greenhouse pots with two plants in each pot. The error bars represent
standard deviations.

for G.clo.C4.BSR and G.mos.W4.Man, and their approximate values were 30, 32, and 33%
for G.clo.C4.BSR, and 27, 31, and 36% for G.mos.W4.Man, respectively, for BSR201,
Iowa2052, and Peking cultivars. The isolate G.clo.W4.Man had values similar to those for
BSR201 (28%) and Peking (34%), but relatively lower percentage values for Iowa2052
(25%).

This study showed that the amount of root colonization depended not only on the AM
fungal isolate but also on the host plant and the soil environment where isolated. We used
two isolates of *G. etunicatum*, G.etu.C1.BSR that was isolated from a low P soil of field 1
and G.etu.W4.Man that came from a very high P soil of field 4. The isolate from the low P
soil grew better inside the host roots and occupied a higher portion (42%) of the roots than
the isolate from the high P soil (22%). Similarly, G.clo.W1.Man (37%) (from a high P soil,
but not nearly as high as that of field 4) did better than G.clo.W4.Man (29%). There was an
exception where the isolates from field 4 (G.clo.C4.BSR (32%) and G.clo.C4.Pek (23%)) did
better than the comparable isolate from field 1 (G.clo.C1.BSR (17%). It seemed that the
colonizing abilities of these eight AM fungal isolates were influenced more by the P level of
the soil from which they were derived than they were by the soil series (Clarion vs. Webster).

Assessment of Pot Cultures for Spore Production

Examination of soil pots of each cultivar after plant harvest showed that homogenous
spores of the same introduced species were produced (exceeding 50 spores g^{-1} soil) from
seven isolates, G.clo.C1.BSR, G.etu.C1.BSR, G.clo.C4.BSR, G.clo.C4.PEk, G.clo.W1.Man,
G.clo.W4.Man, and G.mos.W4.Man. The pots from the eighth isolate inoculated with
G.etu.W4.Man, however, were contaminated with other spores of similar size but whiter
color that represented about 20 to 25% of the spores in the soil pots of all three cultivars.
These contaminating spores had morphological characteristics similar to those of *G.
vesiforme* and might have been derived from contaminating hyphal fragments that escaped
detection during cleaning of the inocula for this *G. etunicatum* strain.

Effects of the Propagated Field Isolates on Shoot Dry Weight

Paired analyses using ANOVA indicated that the differences in shoot dry weight were significant (P <0.0001) among cultivars (Table 5.1). The largest values being recorded were in Peking pots (averaging 8.59 g pot^{-1} for inoculated plants), medium in BSR201 pots (averaging 7.22 g pot^{-1}), and lowest in Iowa2052 pots (averaging 6.18 g pot^{-1}). The smallest increase attributable to AM fungal inoculation was recorded in Iowa2052 plants whose roots were inoculated with *G. etunicatum* isolates that originated from Webster soil of field 4 (Fig. 5.2).

All of the individual AM fungal strains resulted in significantly higher shoot dry weights of their host plants than of the control plants (Table 5.1). The largest increases occurred in plants inoculated with *G. claroideum* (G. clo.C4.BSR and G. clo.W4.BSR) and the smallest increase was recorded in plants grown in pots inoculated with G.etu.W4.Man. Plants inoculated with spores of G.mos.W4.Man produced significantly (P<0.05) higher mean dry weights than did plants in the presence of G.etu.W4.Man but slightly lower weights than those produced by plants inoculated with G. clo.W4.Man.

This study indicated that G.clo.C1.BSR, G.clo.C4.BSR, and G.etu.C1.BSR typically increased plant growth more than G.etu.W4.Man and G.mos.W4.Man. Plants in the presence of *G. mosseae* spores from G.mos.W4.Man produced statistically equal or higher shoot dry weights than plants that were inoculated with *G. clastroideum* or *G. etunicatum* spores from G.clo.W4.Man or G.etu.W4.Man.

The effects of AM fungal strains in this study differed with the host. Isolates of *G. claroideum* and *G. etunicatum* from Clarion soils generated higher shoot dry weights on Peking host plants (9.17-9.86 g pot^{-1}) than on plants associated with the remaining fungal strains. The G.clo.W4.Man (8.47 g pot^{-1}) and G.mos.W4.Man (8.57 g pot^{-1}) strains performed equally on Peking host plants.

Contrary to Peking plants, BSR201 plants produced significantly higher dry weights (8.33 and 9.60 g pot^{-1}) when they were inoculated with the *G. claroideum* strains, G.clo.W1.Man and G.clo.W4.Man, than when they were inoculated with the other strains. No significant difference existed among shoot dry weights produced by BSR201 plants when

Table 5.1. Statistical comparison of shoot dry weights averaged over three cultivars of soybean plants grown in the greenhouse with AM fungal inoculum of the indicated species obtained from soils where the indicated soybean plants were grown.

Inocula comparison		Difference	95% confidence	
Source	Source	between means, g pot^{-1}	limits	
G.clo.C4.BSR[t]	Control	3.93***[‡]	3.62	4.24
G.clo.W4.Man	Control	3.68***	3.41	3.94
G.etu.C1.BSR	Control	2.80***	2.54	3.06
G.clo.C4.Pek	Control	2.77***	2.51	3.03
G.mos.W4.Man	Control	2.74***	2.48	3.01
G.clo.C1.BSR	Control	2.64***	2.38	2.90
G.clo.W1.Man	Control	2.35***	2.09	2.62
G.etu.W4.Man	Control	1.63***	1.37	1.89
G.clo.C4.BSR	G.etu.W4.Man	2.30***	1.93	2.67
G.clo.W4.Man	G.etu.W4.Man	2.05***	1.72	2.38
G.clo.C4.BSR	G.clo.W1.Man	1.57***	1.20	1.94
G.clo.W4.Man	G.clo.W1.Man	1.32***	0.99	1.65
G.clo.C4.BSR	G.clo.C1.BSR	1.29***	0.92	1.66
G.clo.C4.BSR	G.mos.W4.Man	1.18***	0.81	1.56
G.etu.C1.BSR	G.etu.W4.Man	1.17***	0.84	1.50
G.clo.C4.BSR	G.clo.C4.BSR	1.16***	0.79	1.53
G.clo.C4.Pek	G.etu.W4.Man	1.14***	0.81	1.47
G.clo.C4.BSR	G.etu.C1.BSR	1.13***	0.76	1.50
G.mos.W4.Man	G.etu.W4.Man	1.11***	0.78	1.45
G.clo.W4.Man	G.clo.C1.BSR	1.04***	0.71	1.37
G.clo.C1.BSR	G.etu.W4.Man	1.01***	0.68	1.34
G.clo.W4.Man	G.mos.W4.Man	0.93***	0.60	1.26
G.clo.W4.Man	G.clo.C4.BSR	0.91***	0.58	1.24
G.clo.W4.Man	G.clo.C1.BSR	0.88***	0.54	1.21
G.clo.W1.Man	G.etu.W4.Man	0.73***	0.39	1.06
G.etu.C1.BSR	G.clo.W1.Man	0.45*	0.11	0.78
G.clo.C4.Pek	G.clo.W1.Man	0.41*	0.08	0.75
G.mos.W4.Man	G.clo.W1.Man	0.39*	0.06	0.72
G.clo.C1.BSR	G.clo.W1.BSR	0.28	-0.05	0.62
G.clo.C4.BSR	G.clo.W4.Man	0.25	-0.12	0.62
G.etu.C1.BSR	G.clo.C1.BSR	0.16	-0.17	0.49
G.clo.C4.BSR	G.clo.C1.BSR	0.13	-0.20	0.46
G.mos.W4.Man	G.clo.C1.BSR	0.10	-0.23	0.44
G.etu.C1.BSR	G.mos.W4.Man	0.06	-0.27	0.39
G.etu.C1.BSR	G.clo.C4.Pek	0.03	-0.30	0.36
G.clo.C4.Pek	G.mos.W4.Man	0.03	-0.31	0.36

† See text for the meaning of the abbreviations.
‡ *Indicates statistical significance at the 0.05 confidence level.
   ***Indicates statistical significance at the 0.001 confidence level.

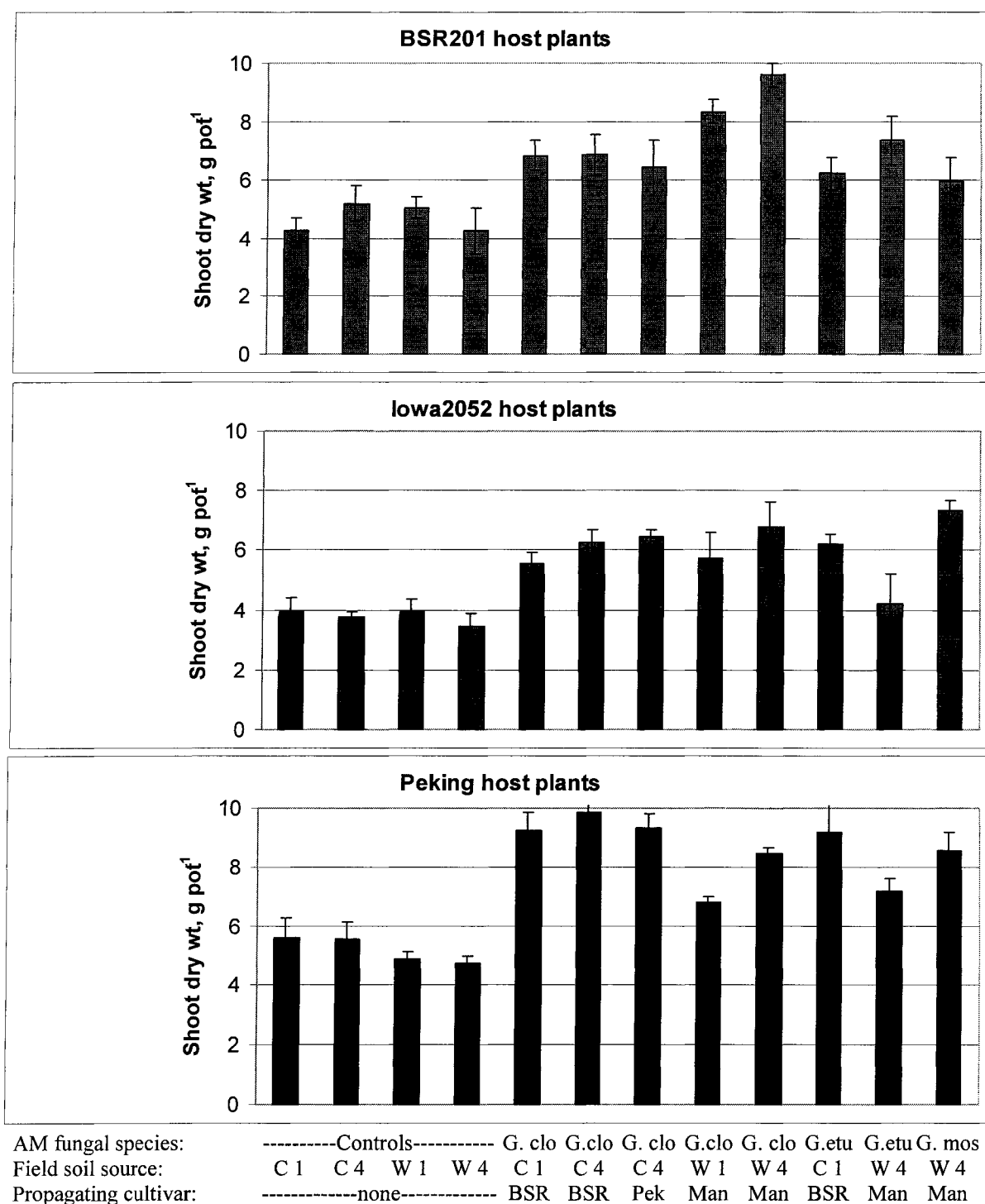Fig. 5.2. Shoot dry weights of three soybean cultivars inoculated with strains of *Glomus claroideum* (G.clo), *G. etunicatum* (G.etu), and *G. moseae* (G.mos) derived from Clarion (C) and Webster (W) soils of fields 1 and 4 and propagated on BSR201, Peking, or Mandarin soybean cultivars. Each entry represents an average of results from five greenhouse pots with two plants in each pot. The error bars represent standard deviations.

they were inoculated with G.clo.C1.BSR, G.clo.C4.BSR, G.clo.C4.P, or G.etu.C1.BSR that were isolated from Clarion soils of our two fields.

Several strains, including G.clo.C1.BSR, G.clo.C4.BSR, G.clo.C4.Pek, G.clo.W1.Man, G.clo.W4, G.etu.W4.Man, and G.mos.W4.Man, affected the dry weight of Iowa2052 plants in nearly the same way that they did the weights of Peking plants (Fig. 5.2). The effect of AM fungal species was more pronounced in the Iowa2052 cultivar when plants were grown in the presence of either G.clo.W4.Man or G.mos.W4.Man. With the exclusion of the weight of plants inoculated with G.etu.W4.Man, no significant difference was apparent among shoot dry weights of Iowa2052 plants whose roots were colonized by the following strains, G.clo.C1.BSR, G.clo.C4.BSR, G.clo.C4.Pek, G.cloW1.Man, or G.etu.C1.BSR and whose weights ranged from 5.54 to 6.45 g pot^{-1}.

This study suggested that the performance of certain AM fungal species in plant growth may depend not only on the present host-strain combination but also on the previous host. A study by Pawlowska and Charvat (2004) was conducted to test the sensitivity and the survival of *G. etunicatum* and *G. intraradices* to different toxicity levels of the heavy metals cadmium (Cd), lead (Pb), and zinc (Zn). These two species of AM fungi had been previously cultured and grew equally under low toxicity levels of these heavy metals. Exposure to higher concentrations of heavy metals showed that *G. intraradices* performed significantly better than *G. etunicatum* in terms of spore germination and external hyphal growth.

Effects of the Propagated Field Isolates on Root Dry Weight

Statistical analyses showed that the means of root dry weights were not significantly influenced by the presence of AM fungi in the roots of the three 10-wk-old soybean cultivars. There were significant differences (P<0.001) in root dry weight among the three cultivars, however, and they were somewhat correlated to the shoot dry weights. The average root dry weights for the three cultivars were 1.57 and 1.61 g pot^{-1} for Peking, 1.35 and 1.22 g pot^{-1} for BSR201, and 1.00 and 0.89 g pot^{-1} for Iowa2052 for control and inoculated plants, respectively (Table 5.2). The Peking plants and some of the BSR201 plants produced more roots with AM fungal inoculation than without it, but the reverse was true for most of the BSR201 plants and for all of the Iowa2052 plants.

Shoot-to-Root Ratio Growth Parameters

Although the influence of mycorrhizal inoculation on the root growth of the host soybeans was insignificant, there was an apparent increase in the shoot-to-root (S/R) ratios of mycorrhizal plants over similar noninoculated plants (Table 5.2). The average S/R ratio for the BSR201 plants (6.60 to 7.87) grown in pots inoculated with G.clo.C1.BSR, G.clo.C4.BSR, G.clo.C4.Pek, G.etu.C1.BSR, or G.mos.W4.Man was approximately twice that of their equivalent nonmycorrhizal plants (mean of 3.49). Lower values were recorded for the remaining three isolates, which ranged from an average of 4.23 for plants inoculated with G.etu.W4.Man to 5.35 for plants associated with G.clo.W4.Man. Similarly, the average S/R ratio (7.64) for G.mos.W4.Man-inoculated Peking plants nearly tripled that of AM fungal inoculum-free Peking plants (2.38). The corresponding values for the Peking plants inoculated with the remaining strains varied from 4.18 when the inoculum was G.clo.W1.Man to 5.85 for plants inoculated with G.clo.C4.BSR. The pots S/R ratio for mycorrhizal Iowa2052 plants was 6.81 on average and represented a smaller increase over the average ratio of control plants (5.06), as shown in Table 5.2.

Table 5.2. Shoot-to-root (S/R) ratios of BSR201, Iowa2052, and Peking soybean cultivars grown in pots in a greenhouse with inoculum of *Glomus claroideum* (G.clo), *G. etunicatum* (G.etu), or *G. mosseae* (G.mos) derived from BSR, Peking, or Mandarin soybean grown in Clarion (C) or Webster (W) soil in field 1 or 4.

AM species	Soil and field	Soybean source	Shoot wt., g			Root wt., g			S/R ratio		
			BSR	Iowa	Pek	BSR	Iowa	Pek	BSR	Iowa	Pek
			--------------------g pot^{-1}--------------------								
None	---	---	4.71	5.19	3.73	1.35	1.00	1.57	3.49	5.06	2.38
G.clo	C 1	BSR	6.85	5.54	9.25	0.87	0.84	1.66	7.87	6.58	5.57
G.clo	C 4	BSR	6.86	6.27	9.86	0.94	0.95	1.69	7.27	6.63	5.85
G.clo	C 4	Pek	6.42	6.45	9.31	0.96	0.92	1.64	6.67	7.03	5.68
G.clo	W 1	Man	8.33	5.72	6.81	1.69	0.89	1.63	4.94	6.40	4.18
G.clo	W 4	Man	9.60	6.76	8.47	1.79	0.95	1.78	5.35	7.12	4.76
G.etu	C 1	BSR	6.23	6.20	9.17	0.87	0.99	1.70	7.13	6.25	5.40
G.etu	W 4	Man	7.37	4.21	7.17	1.74	0.66	1.70	4.23	6.42	4.22
G.mos	W 4	Man	5.98	7.36	8.57	0.91	0.94	1.12	6.60	7.81	7.64
Average with AM			7.21	6.06	8.57	1.22	0.89	1.61	5.91	6.81	5.32

The AM fungal strains in this study may have stimulated their host plants to shift larger amounts of photosynthates from root production to the shoots or to invest more carbohydrates in extending the AM fungal mycelial structures that exploit the soil for nutrients.

Smaller S/R dry weight ratios were recorded for the Iowa2052 soybean cultivar compared to the other two cultivars, possibly because of the difference in growth stages of the three cultivars. The Iowa2052 plants had produced maturing seeds, while some BSR201 plants were still developing pods and some Peking plants were still flowering and just beginning to form pods. Thus, Iowa2052 plants would have been translocating or have already translocated photosynthates to the maturing seeds, thus increasing the S/R dry weight ratios.

Pearson correlation analysis indicated that most of the AM fungal isolates used in this study resulted in significant positive correlations between AM colonization, shoot dry weight, root dry weight, and plant height.

A greenhouse study (Augé et al., 1986) was conducted to test the effect of increased P on several growth parameters such as shoot and root weights and root colonization levels of rose plants (*Rosa hybrida* L. sv. 'Samantha") inoculated with *G. intraradices* and *G. deserticola*. The S/R ratio increased and the surface areas of the roots increased by amounts that depended on the myorrhizal species, but the differences were not statistically significant. Berta et al. (1990) reported that nonmycorrhizal plants developed longer and more adventitious roots than the mycorrhizal plants, contributing to a significant increase in S/R dry weight ratios in leek plants with mycorrhizal colonization.

Increase in Shoot Dry Weight of Mycorrhizal Plants

Isolates of *G. claroideum* from Clarion soils of either field were generally more efficient in increasing shoot dry weight of Peking plants. Average increases ranged from 76 to 90% for Peking plants (Table 5.3). In Webster soils, *G. claroideum* isolates generated the highest shoot dry weights for BSR201 plants with an average increase over control plants of 77 and 104% for BSR201 plants inoculated with G.clo.W1.Man and G.clo.W1.Man, respectively. With minor exceptions, Iowa2052 plants responded to individual AM fungal isolates in a pattern similar to Peking plants, but with *G. mosseae* outperforming the other isolates. The overall lowest increase (13%) was recorded in Iowa2052 plants inoculated with

G.etu.W.Man, the isolate that also had the lowest level of root colonization in these plants. Although certain isolates performed well with all three cultivars, this study emphasized that choosing the right combination of host plant and AM fungal isolate is crucial for optimum benefit to the host plant. There were strong interactions between AM fungal strains of the three *Glomus* species used this study and the three host soybean cultivars that responded differently to different strains in terms of the measured parameters, root colonization, shoot and root dry weights, and seed production.

Table 5.3. Increases in shoot dry weight above controls of soybean cultivars grown in pots in a greenhouse with inoculum of *Glomus claroideum* (G.clo), *G. etunicatum* (G.etu), or *G. mosseae* (G.mos) derived from BSR, Peking, or Mandarin soybean grown in Clarion (C) or Webster (W) soil in field 1 or 4.

Am fungal	BSR		Iowa		Peking	
species	Wt., g	Increase, %	Wt., g	Increase, %	Wt., g	Increase, %
Control	4.71		3.73		5.19	
G.clo.C1.BSR	6.85	45.4	5.54	48.5	9.25	78.2
G.clo.C4.BSR	6.86	45.6	6.27	68.1	9.86	90.0
G.clo.C4.Pek	6.42	36.3	6.45	72.9	9.31	79.4
G.clo.W1.Man	8.33	76.9	5.72	53.4	6.81	31.2
G.clo.W4.Man	9.60	103.8	6.76	81.2	8.47	63.2
G.etu.C1.BSR	6.23	32.3	6.20	66.2	9.17	76.6
G.etu.W4.Man	7.37	56.5	4.21	12.9	7.17	38.2
G.mos.W4.Man	5.98	27.0	7.36	97.3	8.57	65.1
Average	7.21	53.0	6.06	62.6	8.57	65.2

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CONCLUSIONS

To our knowledge this is the first study that evaluated the efficacy of individual AM fungal field isolates on soybean growth. The efficacy of mycorrhizal fungi is determined by a myriad of factors, including the host plant, the AM fungal species, soil properties, and the interactions among existing soil organisms. This study illustrated the effects of the host soybean cultivars, of soil P level and/or soil series, and that of the individual AM fungal isolates on the outcome of AM fungal associations on plant growth.

Generally, mycorrhizal plants exhibited higher shoot dry weights and produced larger average numbers of seeds (for those that matured early enough to produce seeds) compared to their equivalent nonmycorrhizal plants. The eight isolates used differed in their effects on the growth of the three test soybean cultivars, and the effects of each AM fungal isolate depended greatly on the host plant. Moreover, growth response, expressed mainly in shoot and root dry weights, differed sometimes significantly, among isolates of the same species. The isolates within the same species originated from different locations, one with a very high available P soil and the other with a lower available P soil.

Highly efficient strains increased the shoot dry weights of their host by an average ranging from 75 to 104% over that of the nonmycorrhizal plants. The increase depended on both the host plant and the inoculating strain. The efficacy of the strains of the same species varied according to their soil of origin and the plant host whose roots they colonized.

FUTURE WORK

The quality and quantity of benefits conferred to plants by mycorrhizal fungi are determined by complex interactions that exist among the host plant, the mycosymbiont, and the surrounding soil environment. The soil is a very heterogeneous environment where AM fungal species and their hosts face stiff competition with coexisting organisms for energy sources and a protected habitat. This research suggested that a single AM fungal strain may affect different host species in different ways, and that plant responses to more than one strain (single inoculation) are likely to differ according to the introduced AM fungal isolate. Therefore, it remains important to allocate more resources to understand the ecology of individual AM fungal species in the field.

The isolates used in this study were selected basically because of their high spore proliferation and population produced with three cultivars grown in trap-culture pots. The previous work indicated that at least a dozen other species existed in our field sites. Understanding the efficacy of other organisms is also important. Only single inoculations were practiced in this study, where the coexisting microbes in filtered soil suspensions were reintroduced to the potting soil. The interactive effects of two or more field isolates could turn out to be more efficient because of the possible complementary nature of the functions of the participating isolates. The use of a larger number of host plants could further expand the information about these isolates.

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CHAPTER 6. GENERAL CONCLUSIONS

This research evaluated the population composition and relative distribution of AM fungal spores in the Clarion and Webster soils of four soybean fields in Story County located in central Iowa. In addition, four soybean cultivars were assessed for their selection for or against fungal isolates native to these fields. Isolates of three AM fungal species that dominated the pots of the trap cultures of AM fungi from fields 1 and 4 were tested for their efficacy on growth promotion of three soybean cultivars.

Soil tests for available P ranged from 14 (low) to 40 (very high) mg P kg^{-1} soil in Clarion soils, of fields 1 and 3, but those from fields 2 and 4 tested much higher (130 to 143 mg P kg^{-1} soil). Tests for Webster soils ranged from 38 to 69 in fields 1 and 3 and from 178 to 203 mg P kg^{-1} soil in fields 2 and 4. Average AM fungal colonization of field-collected soybean roots varied from 53 to 72% for most soil locations. Spore counts were <1 spore g^{-1} soil and significantly decreased in very high P soils.

A total of at least twelve AM fungal species were identified in the surveyed field soils. The identification of AM fungi was determined based on similarities and differences in morphological characteristics of the spore directly collected from the fields or after trap cultures. For examining the variability within the fields, spores isolated directly from the field and spores derived from pot soils inoculated from individual soil samples from Webster soils of fields 1 and 4 and sown to BSR201 or Iowa2052 were identified. For the variability assessment among fields, the AM fungal composition was also assessed in trap cultures by growing four different soybean cultivars in pots inoculated with composite samples from Clarion and Webster soils of the four fields.

PCR-based rDNA fingerprinting using working published primers was used either to confirm the identity of spore morphotypes that were assigned to a specific species based on the study of the microscopy-based spore morphology or to the presence of certain species in the roots of certain host plants. The prevailing species were *G. claroideum*, *G. etunicatum*, *G. mosseae*, *G. viscosum*, and *P. occultum*, as their spores represented high proportions (greater than 10%) of the total spore populations in the original field soils and were found in 38 to 63% of the soil samples. Among the remaining identified fungi were species of the other genera, including *Acaulospora*, *Entrophopora*, and *Gigaspora*. Their distribution and

abundance exhibited greater variability within fields than that among the fields. Soil P level, soil type, and field location seemed to play a role in the distribution and abundance of certain AM fungal species in this study. For instance, *G. mosseae* and certain species of *Acaulospora* species were found to dominate in Webster soil of field 4 that had the highest P content (203 mg kg^{-1}). *G. viscosum* on the other hand had greater spore representation in Clarion than in Webster soils.

To our knowledge, this is the first study that used more than one host type to trap AM fungi in a single study. That allowed for greater number of AM fungal species to sporulate and be seen in the form of spores. The molecular work improves the credibility of the morphological work, and the two should be combined in identity and diversity studies.

Investigation of the possible selection or preference of BSR201, Iowa2052, Mandarin, or Peking for AM fungal species from the four fields discussed in the previous study was the second subject of the research project. The results from that work indicated the soybean cultivars exhibited preference, expressed in the difference in abundance of certain AM fungal species when associated with different cultivars. It was also suggested from the data that there was a degree of selectivity of the soybean cultivars for certain AM fungal species. As some AM fungal species, such as *G. constictum* and *G. intraradices*, were repeatedly detected in the pots of certain cultivars while they were not in other pots. The most conspicuous selective event in this experiment was the unique association of *E. infrequens* with the Iowa2052 cultivar. In conclusion, this study suggested that plants of the same species exhibited both preference and some selection for certain colonizing AM fungi.

The average shoot dry weight of the BSR201, Iowa2052, and Peking plants was significantly greater when they were inoculated with one of three AM fungal species, *G. claroideum*, *G. etunicatum*, and *G. mosseae*, compared to nonmycorrhizal plants, and the efficacy of the strains belonging to the same species on promoting plant growth differed according to both host and the soil type where the strain originated. Peking plants inoculated with *G. claroideum* isolated from Clarion soils of fields 1 or 4 produced higher shoot dry weights than when inoculated with the isolates of this species from Webster soils of fields 1 or 4. The opposite was true for BSR201.

The variability in field isolates of AM fungi active on soybean appears to be large. Much remains unknown about controlling factors leading to effective symbioses between host and fungi.

FUTURE WORK

The first and the second studies involved the identification of AM fungal species based on characterization of spores that were derived from both original field soil samples and AM fungi trapped in pot cultures. About 12 species were morphologically identified, with the identity of four *Glomus* species being confirmed by the amplification of the portion of their rDNA gene using workable species-specific primers in both spore morphotypes that were assigned to a particular species and in the roots of the host plant. The work should continue with adjusting the PCR molecular methods to verify the identity of the remaining morphotypes, and to confirm or reject their presence in the roots of the pots that contained their spores. Not detecting spores of certain species in a few pots does not necessarily prove the absence of those species in these pot soils. The species could still be present in hyphal structures inside and outside the roots perhaps because the growth conditions (biotic or abiotic) did not favor their sporulation or the host was not suitable. It is nearly impossible to provide conditions that would allow for all AM fungal species to sporulate. To avoid this time consuming work and minimize uncertainty, it will be best to resort to molecular techniques for AM fungal identification.

It has been established that sporulation of certain AM fungal species depended on the season of the year and sometimes on the stage of growth of the host plants. The first study identified AM fungal spores in the field soils from single soil sampling. We are aware that the single sampling might have limited the number of identified species, but we sampled in autumn when most AM fungal species tend to sporulate. In fact, we used two sets of trap cultures using four genetically different soybean cultivars, and that would certainly have provided some conditions that induced fungi to sporulate. It is not known what causes AM fungal species to sporulate at different times of the season, but several speculations have been made. It is likely that AM species that reach their peak in spore production at the end of the growing season do so in response to limited resources as the host plant tends to direct larger portions of its photosynthate to fruits (seeds, tubers, etc.). Sporulation of some AM fungal species occurs at early stages of development while that of others peaks at an advanced stage of development. It has been suggested that these two groups of species may require different types of C. Of course the compatibility between the fungus and the host

plant holds the key to any future association, and without it there would no spore or future AM fungus.

The recommendations for future work are the following: The identity of AM fungal morphotypes should be confirmed using PCR-based rDNA fingerprinting with the use of existing published species-specific primers. If the existing primers do not work, alternate specific primers could be designed from published sequences. The published sequences mostly for regions of rRNA genes of most AM fungal species are available in the GenBank, a database that displays DNA sequences received from researchers around the world. If this approach does not work, the investigator should resort to sequencing. Sequencing could be done directly from PCR amplicons or from amplified clones. Separation of a mixture of either clones or DNA fragments according to size has been conventionally achieved using gel electrophoresis. Fragments of DNA that migrate at the same pace and end up in the same position in the gel do not necessary have the same sequences. Thus, to narrow the list of clones to be sequenced and focus on the more likely group, a further processing is needed. For example, if the clones are subject to single-stranded conformational polymorphism treatment, the clones will be separated based on both size and sequence. If any of the above suggested approaches are successful, the study will be more complete by identifying the species whose identity certainty could be reached using spore morphology. The molecular work has the ability to identify the organisms directly inside the roots, and that would provide more meaningful results as they allow the identity of the AM fungal species in action, the root-colonizing species.

In the efficacy greenhouse study, selected strains of either *G. claroideum*, *G. etunicatum*, or G. *mosseae* increased the shoot dry weight of their hosts by about 70 to 100% over the control plants. The amount of increase depended on the fungus-host combination. For Peking the efficacy of isolates from Clarion soils was greater than that of isolates from Webster soil, but for BSR201 the increase from isolates originating from Webster soil was greater than that of AM fungal isolates from Clarion. The next logical step in this study would be to run the efficacy of these strains on the same soybean cultivars as before for at least one more generation to test for consistency in their efficacy. If the strains exhibit the same or greater efficacy, then the efficacy tests should be carried to a larger scale, the field. Testing the

efficacy of the other identified isolates should not be ignored. Even testing using a combination of organisms may be useful, as interactions are clearly possible.

APPENDIX A. AM SPORE POPULATION PERCENTAGES

		AM Species in Clarion Soil Samples									Total spores g^{-1} soil
		Glomus						*Acaulospora*	*Gigaspora*		
		G.clo	G.etu	G.geo	G.int	G.mos	G.vis	A.spp	Gi.spp	other	
	Sample	--% of total spores----------------------------------									
Site1	1	0	0	0	0	0	75.4	0	0	24.6	0.58
	2	1.1	0	0	0	2.1	60.4	0	0	36.4	0.68
	3	0	0	0	0	0	58.1	0	0	41.9	0.54
	4	0	0	0	0	4.5	70.4	0	0	25.1	0.70
	5	6.5	0	0	0	0	93.5	0	0	0	0.32
	Ave.	1.52	0	0	0	1.3	71.6	0	0	25.6	0.56
	St. Dev.	2.8	0	0	0	2	14.2	0	0	16.1	0.15
Site2	1	0	0	0	0	0	0	0	0	100	0.16
	2	0	0	0	0	0	46.2	0	0	53.8	0.26
	3	42.9	0	0	0	0	57.1	0	0	0	0.14
	4	0	0	0	0	0	100	0	0	0	0.06
	5	0	0	0	0	0	50	0	0	50	0.20
	Ave.	8.6	0	0	0	0	50.7	0	0	40.8	0.16
	St. Dev.	19.2	0	0	0	0	35.6	0	0	42.1	0.07
Site3	1	31.6	0	0	42.1	0	0	0	0	26.3	0.38
	2	50	0	0	0	0	0	0	0	50	0.16
	3	36.4	18.2	0	0	0	0	0	0	45.4	0.22
	4	0	0	0	0	0	100	0	0	0	0.10
	5	0	0	0	0	50	50	0	0	0	0.12
	Ave.	23.6	3.6	0	8.4	10	30	0	0	24.3	0.20
	St. Dev.	22.6	8.1	0	18.8	22.4	44.7	0	0	23.9	0.11
Site4	1	0	0	0	0	0	33.3	22.3	0	44.4	0.18
	2	18.2	0	0	18.2	0	0	0	0	63.6	0.22
	3	14.3	0	0	57.1	0	0	0	0	28.6	0.14
	4	0	0	0	33.3	0	53.8	0	5.1	7.8	0.34
	5	0	0	0	29.4	0	41.2	0	0	29.4	0.32
	Ave.	6.5	0	0	27.6	0	25.7	4.6	1.0	34.8	0.24
	St. Dev.	9.0	0	0	21	0	24.5	10.0	2.3	20.7	0.09
Overall average		10.1	0.9	0	9.0	2.8	44.5	1.1	0.3	31.4	0.29

APPENDIX A (continued)

		AM Species in Webster Soil Samples									Total
		Glomus						*Acaulospora*	*Gigaspora*		spores
		G.clo	G.etu	G.geo	G.int	G.mos	G.vis	A.spp	Gi.spp	other	g^{-1} soil
	Sample	--% of total spores----------------------------------									
Site1	1	35.7	7.2	0	0	7.1	50	0	0	0	0.56
	2	0	0	0	0	7.7	30.7	15.4	0	46.2	0.52
	3	8.3	18.8	0	0	6.2	25	0	2.1	39.6	0.96
	4	20	28.6	0	0	5.7	25.7	0	0	20	0.70
	5	18	0	0	17	0	33	4	0	28	0.90
	Ave.	16.4	10.9	0	3.4	5.3	32.9	3.9	0.4	26.8	0.73
	St. Dev.	13.4	12.5	0	7.6	3.1	10.1	6.7	0.94	18.1	0.20
Site2	1	25	50	0	0	0	0	25	0	0	0.18
	2	55.6	0	0		0	33.3	11.1	0	0	0.10
	3										0.10
	4										0.04
	5										0.06
	Ave.	40.3	25	0	0	0	16.7	18.1	0	0	0.10
	St. Dev.	21.6	35.4	0	0	0	23.6	9.8	0	0	0.05
Site3	1	0	7.5	7.5	0	2	30.2	22.6	0	30.2	0.66
	2	0	7.4	5.6	0	7.4	44.5	11.1	0	24	0.68
	3	0	5.6	12.5	0	0	52.7	13.9	0	15.3	0.96
	4	0	4.2	0	0	6.9	55.5	19.5	0	13.9	0.96
	5	18.3	6.5	0	0	6.4	33.9	0	0	34.9	1.04
	Ave.	3.7	6.2	5.1	0	4.5	43.4	13.4	0	23.7	0.86
	St. Dev.	8.2	1.4	5.3	0	3.3	11.2	8.8	0	9.1	0.18
Site4	1	0	0	0	0	100	0	0	0	0	0.04
	2	0	25	0	0	50	0	25	0	0	0.08
	3	0	0	0	0	100	0	0	0	0	0.02
	4	0	0	0	0	40	0	20	0	40	0.10
	5	0	0	0	0	33.3	0	66.7	0	0	0.06
	Ave.	0	5	0	0	64.7	0	22.3	0	8	0.06
	St. Dev.	0	11.2	0	0	32.8	0	27.3	0	17.9	0.03
Overall average		15.1	11.8	1.3	0.9	18.6	23.2	14.4	0.1	14.6	0.44

APPENDIX B. SOYBEAN SELECTION DATA

Table B. 1. Spore percentages of identified AM fungal species in field soils in trap pot cultures on BSR201 soybean cultivar inoculated with Clarion soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g⁻¹ soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.9	6.5
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.2	6.5
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.4	6.5
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16.6	6.5
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.1	6.5
Field 1	1	56.0	0	0	26.7	0	0	7.1	5.7	14.1	0	0	0.5	0	16.2	78.6	15.4	7
	2	51.7	0	0	28.5	0	0	4.5	5.6	9.7	0	0	0	0	15.6	77.9	17.2	6.5
	3	48.8	0	0	29.2	0	0	3.4	3.4	15.2	0	0	0	0	16.2	77.9	16.0	6.5
	4	50.2	0	0	31.9	0	0	3.0	0	14.9	0	0	0	0	14.9	77.1	17.7	7
	5	42.7	0	0	31.5	0	0	4.5	5.6	15.7	0	0	0	0	17.3	79.7	13.7	6.5
Field 2	1	55.5	10.0	0	0	0	0	0	34.5	0	0	0	0	0	18.8	89.5	16.2	7
	2	59.0	18.8	0	0	0.6	0	0	21.6	0	0	0	0	0	29.8	87.1	13.3	6.5
	3	55.2	12.0	0	0	0	0	0	32.8	0	0	0	0	0	25.5	87.4	17.7	7
	4	54.0	11.6	0	0	0	0	0.5	25.1	0	0	0	0	8.7	28.9	84.4	18.7	7
	5	52.5	10.0	0	0	0	0	0.9	26.6	0	0	0	0	10.0	20.6	85.3	16.0	6.5
Field 3	1	41.1	0	0	41.6	0	0	2.2	0	0	6.0	0	0	9.2	29.5	80.0	16.7	7
	2	29.7	2.4	0	50.9	0	0	1.4	1.4	0	9.0	0	0	5.2	33.6	85.0	16.4	7
	3	30.0	2.5	0	47.8	0	0	0	10.0	0	6.3	0	0	3.4	35.0	82.7	16.2	6.5
	4	19.1	3.1	0	49.6	0	0	3.1	14.5	0	1.5	0	0	9.2	34.5	84.4	17.8	6.5
	5	25.7	4.7	0	44.8	0	0	0	19.6	0	2.8	0	0	2.4	35.2	83.1	15.3	7
Field 4	1	42.2	0	0	0	0	0		44.3	13.5	0	0	0	0	21.2	94.0	18.3	7
	2	47.8	0	0	0	1.6	0	1.6	30.9	18.0	0	0	0	0	20.6	86.6	17.2	7
	3	35.0	0	0	0	0	0	0	45.0	16.3	0	0	0	3.7	21.6	87.8	16.2	7
	4	29.7	0	0	0	0	0	0	50.0	20.3	0	0	0	0	23.1	90.0	16.0	6.5
	5	30.2	0	0	0	0	0	0	50.1	15.0	0	0	0	0	24.2	89.1	17.4	7

Table B. 2. Spore percentages of identified AM fungal species in field soils in trap pot cultures on BSR201 soybean cultivar inoculated with Webster soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g^{-1} soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.9	6.5
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.2	6.5
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.4	6.5
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16.6	6.5
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.1	6.5
Field 1	1	37.0	0	0	0	0	0	0	34.8	26.1	0	0	2.2	0	19.4	89.6	17.4	7
	2	38.6	6.8	0	15.9	0	0	6.8	29.5	0	0	0	2.3	0	20.8	90.2	16.7	6.5
	3	50.3	0	0	10.0	0	0	1.5	18.3	17.6	0	0	2.3	0	22.6	91.9	16.4	6.5
	4	47.2	0	0	15.0	0	0	0	26.9	0	0	0	3.4	7.6	23.2	90.2	15.5	6.5
	5	35.6	0	0	0	0	0	10.9	17.2	22.8	0	0	0	13.4	22.0	89.8	14.7	7
Field 2	1	30.0	0	0	0	0	10	0	50.0	0	10.0	0	0	0	12.2	90.2	17.9	7
	2	22.2	0	0	0	0	0	0	44.6	0	22.2	0	0	11.0	11.0	88.9	16.9	6.5
	3	21.3	22.7	0	0	0	0	0	38.7	0	0	0	0	17.3	13.9	84.7	15.3	7
	4	28.8	0	0	0	0	0	0	38.8	0	13.8	0	0	18.8	12.6	84.4	15.3	7
	5	30.0	0	0	15.4	11.6	0	23.1	20.0	0	0	0	0	0	11.4	88.3	16.8	7
Field 3	1	86.0	0	0	4.0	0	0	2	7.0	0	0	0	1.0	0	12.7	82.6	18.5	7
	2	80.4	0	0	3.6	0	0	0	16.0	0	0	0	0	0	11.6	81.4	18.7	7
	3	83.1	0	0	2.8	0	0	0	14.1	0	0	0	0	0	8.8	81.2	17.1	7
	4	65.8	0	0	0	0	0	0	17.0	0	0	0	0	17.2	11.1	82.8	17.8	7
	5	61.4	0	0	11.1	1.8	0	2.3	18.1	0	0	0	0	5.3	10.7	83.6	17.0	7
Field 4	1	13.0	0	0	0	0	0	79.5	7.5	0	0	0	0	0	10.4	80.6	19.4	7
	2	10.0	0	0	0	0	0	80.0	10.0	0	0	0	0	0	7.8	83.5	19.6	7
	3	12.0	0	0	0	0	0	88.0	0	0	0	0	0	0	8.6	80.2	16.9	7
	4	20.3	0	0	0	0	0	73.6	6.1	0	0	0	0	0	11.1	76.9	20.0	7
	5	23.2	0	0	0	0	0	72.0	4.9	0	0	0	0	0	6.2	82.6	17.3	7

Table B. 3. Spore percentages of identified AM fungal species in field soils in trap pot cultures on Iowa2052 cultivar inoculated with Clarion soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g^{-1} soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.5	6
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.2	5.5
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.2	6
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.6	6
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.1	6
Field 1	1	42.3	1.9	1.3	12.2	0	0	3.9	5.1	18.6	8.3	0	0.7	5.8	5.4	67.0	6.7	6
	2	47.7	4.6	0	11.4	0	0	0	6.8	18.2	2.3	0	0	9.1	5.5	70.3	8.8	6
	3	45.2	4.8	0	10.7	0	0	0	3.6	27.4	8.3	0	0	0	5.6	86.6	7.0	6
	4	44.8	2.9	1.9	11.4	0	0	0	2.9	26.7	2.0	0	0	9.5	5.1	64.5	7.4	6
	5	50.0	2.0	0	9.0	0	0	0	3.0	29.0	3.0	0	0	5.0	4.7	80.0	7.3	6
Field 2	1	13.7	70.9	0	12.2	0	0	0	6.8	0	7.9	0	0.7	0	20.5	40.0	8.3	6
	2	11.8	77.2	0	11.4	0	0	0	4.2	0	5.9	0	0.8	0	15.2	27.0	9.1	6
	3	11.3	77.5	0	10.7	0	0	0	7.0	0	4.2	0	0	0	12.8	38.0	6.9	6
	4	15.6	78.3	0	11.4	0	0	0	6.1	0	0	0	0	0	15.6	45.0	8.9	6
	5	13.3	77.3	0	7.0	0	0	0	6.7	0	2.7	0	0	0	19.1	38.0	7.5	6
Field 3	1	0	0	0	87.4	0	4.6	0	1.0	0	2.5	4.6	0	0	19.0	60.9	7.6	6.5
	2	4.0	0	0	91.1	0	0	0	1.8	0	0	2.7	0.5	0	23.7	69.9	9.4	6
	3	3.7	1.7	0.4	79.7	0.3	7.1	0	0	0	1.7	5.1	0.3	0	23.9	77.9	8.2	6
	4	3.2	0	0	75.6	0	2.2	1.0	2.5	2.9	2.2	7.3	0.3	0	20.2	76.5	8.7	6
	5	3.2	0	0	78.9	0	2.7	0	3.2	1.1	2.7	5.2	0	0	24.8	80.2	7.0	5.5
Field 4	1	69.4	7.2	0	0	0	10.4	0	9.0	0	1.8	0	2.3	0	12.0	57.0	7.6	6.5
	2	71.8	5.1	0	0	0	7.5	0	5.4	1.6	3.6	0	2.0	3.2	14.6	53.0	8.7	6
	3	66.9	9.9	0	0	0	12.4	0	0	5.7	2.0	0	2.0	0	9.4	68.0	6.3	6
	4	69.1	3.5	0	0	0	10.3	0	12.7	3.0	2.4	0	2.0	2.6	9.0	53.4	7.4	6
	5	64.8	5.0	0	0	0	6.2	0	15.5	5.3	4.1	0	2.1	0	8.3	70.0	7.0	5.5

Table B. 4. Spore percentages of identified AM fungal species in field soils in trap pot cultures on Iowa2052 soybean cultivar inoculated with Webster soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g^{-1} soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.5	6
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.2	5.5
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.2	6
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.6	6
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.1	6
Field 1	1	24.2	4.8	4.0	8.1	0	0	4.9	0	58.1	0	0	0	0	3.6	78.9	6.2	6
	2	21.7	10.0	0	0	1.7	0	6.7	0	60.0	0	0	0	0	3.7	88.2	9.1	6
	3	30.8	6.8	0	0	0	0	6.0	6.8	56.4	0	0	0	0	3.6	84.5	6.9	6
	4	27.2	5.0	12.0	0	0	0	6.9	0	49.0	0	0	0	0	2.1	85.9	7.2	6
	5	28.9	6.0	14.1	0	0	0	5.4	6.0	45.6	0	0	0	0	2.2	87.0	6.5	5.5
Field 2	1	39.2	0	21.0	0	0	0	2.5	0	31.7	0	0	0	0	12.2	74.7	8.6	6
	2	44.2	0	20.0	0	1.3	0	1.3	0	28.6	0	0	0	0	9.9	78.0	8.9	6
	3	45.4	0	6.3	0	0.9	0	3.7	0	36.1	0	0	0	0	13.9	74.0	7.9	6
	4	44.7	0	3.0	10.6	2.4	0	7.1	0	35.3	0	0	0	0	10.0	72.9	7.7	6
	5	45.1	0	11.0	17.1	2.4	0	0	12.2	24.4	0	0	0	0	15.4	76.5	7.9	5.5
Field 3	1	56.1	14.9	14.0	14.9	0	0	5.6	0	0	0	0	0	0	9.8	68.9	8.0	6
	2	51.0	18.5	13.7	18.5	2.0	0	9.8	0	0	0	0	0	0	10.8	72.3	10.0	6
	3	54.2	13.1	0	13.1	0	0	6.0	26.8	0	0	0	0	0	10.1	66.3	8.8	6.5
	4	48.4	24.7	0	24.7	0	0	14.0	12.9	0	0	0	0	0	14.8	68.3	8.8	6
	5	37.4	23.4	8.4	23.4	0	0	10.3	14.0	0	0	0	0	0	14.5	68.8	7.3	6
Field 4	1	9.0	0	0	0	0	33.7	11.2	5.6	34.83	4.5	0	0	0	4.7	53.4	7.5	6
	2	13.8	2.1	1.1	0	0	31.9	12.8	4.3	24.47	5.3	0	1.1	0	4.5	56.1	9.8	6
	3	11.3	0	0	0	0	36.6	7.0	5.6	33.8	2.8	0	0	0	3.0	57.1	8.3	6.5
	4	10.8	2.0	1.0	0	0	33.3	6.9	4.9	36.27	2.9	0	0	0	4.6	51.5	10.4	6
	5	10.3	0	3.5	0	0	27.6	10.3	10.4	31.03	6.9	0	0	0	3.3	61.4	7.9	6

Table B. 5. Spore percentages of identified AM fungal species in field soils in trap pot cultures on Mandarin soybean cultivar inoculated with Clarion soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g⁻¹ soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.9	6.5
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.2	6.5
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.4	6.5
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16.6	6.5
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.1	6.5
Field 1	1	56.0	0	0	26.7	0	0	7.1	5.7	14.1	0	0	0.5	0	16.2	78.6	15.4	7
	2	51.7	0	0	28.5	0	0	4.5	5.6	9.7	0	0	0	0	15.6	77.9	17.2	6.5
	3	48.8	0	0	29.2	0	0	3.4	3.4	15.2	0	0	0	0	16.2	77.9	16.0	6.5
	4	50.2	0	0	31.9	0	0	3.0	0	14.9	0	0	0	0	14.9	77.1	17.7	7
	5	42.7	0	0	31.5	0	0	4.5	5.6	15.7	0	0	0	0	17.3	79.7	13.7	6.5
Field 2	1	55.5	10.0	0	0	0	0	0	34.5	0	0	0	0	0	18.8	89.5	16.2	7
	2	59.0	18.8	0	0	0.6	0	0	21.6	0	0	0	0	0	29.8	87.1	13.3	6.5
	3	55.2	12.0	0	0	0	0	0	32.8	0	0	0	0	0	25.5	87.4	17.7	7
	4	54.0	11.6	0	0	0	0	0.5	25.1	0	0	0	0	8.7	28.9	84.4	18.7	7
	5	52.5	10.0	0	0	0	0	0.9	26.6	0	0	0	0	10.0	20.6	85.3	16.0	6.5
Field 3	1	41.1	0	0	41.6	0	0	2.2	0	0	6.0	0	0	9.2	29.5	80.0	16.7	7
	2	29.7	2.4	0	50.9	0	0	1.4	1.4	0	9.0	0	0	5.2	33.6	85.0	16.4	7
	3	30.0	2.5	0	47.8	0	0	0	10.0	0	6.3	0	0	3.4	35.0	82.7	16.2	6.5
	4	19.1	3.1	0	49.6	0	0	3.1	14.5	0	1.5	0	0	9.2	34.5	84.4	17.8	6.5
	5	25.7	4.7	0	44.8	0	0	0	19.6	0	2.8	0	0	2.4	35.2	83.1	15.3	7
Field 4	1	42.2	0	0	0	0	0		44.3	13.5	0	0	0	0	21.2	94.0	18.3	7
	2	47.8	0	0	0	1.6	0	1.6	30.9	18.0	0	0	0	0	20.6	86.6	17.2	7
	3	35.0	0	0	0	0	0	0	45.0	16.3	0	0	0	3.7	21.6	87.8	16.2	7
	4	29.7	0	0	0	0	0	0	50.0	20.3	0	0	0	0	23.1	90.0	16.0	6.5
	5	30.2	0	0	0	0	0	0	50.1	15.0	0	0	0	0	24.2	89.1	17.4	7

Table B. 6. Spore percentages of identified AM fungal species in field soils in trap pot cultures on Mandarin soybean cultivar inoculated with Webster soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g⁻¹ soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.9	6.5
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.2	6.5
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15.4	6.5
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16.6	6.5
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14.1	6.5
Field 1	1	0	0	9.0	48.0	0	0	0	43.0	0	0	0	0	0	9.8	91.7	7.5	6
	2	0	0	4.0	39.0	0	0	0	47.0	10.0	0	0	0	0	8.1	87.8	7.3	6
	3	0	0	10.0	35.0	0	0	7.0	48.0	0	0	0	0	0	10.2	89.3	6.4	5.5
	4	0	0	4.0	37.0	0	0	15.0	44.0	0	0	0	0	0	8.0	86.6	7.2	6.5
	5	0	0	3.0	24.0	0	0	20.0	53.0	0	0	0	0	0	7.5	85.4	6.7	6.5
Field 2	1	35.0	0	2.0	9.0	0	0	0	47.0	0	0	0	0	0	6.7	77.5	8.4	6
	2	25.0	0	4.0	13.0	0	0	0	53.0	0	0	0	0	0	10.5	77.6	9.6	6
	3	27.0	0	4.0	15.0	0	0	2.0	47.0	0	0	0	0	0	6.9	75.0	7.0	6.5
	4	23.0	0	1.0	10.0	0	0	2.0	51.0	0	0	0	0	0	9.3	78.9	8.8	6.5
	5	19.0	0	1.0	15.0	0	0	2.0	47.0	0	0	0	0	0	7.6	76.6	6.7	6.5
Field 3	1	11.0	40.0	2.0	0	0	0	6.0	41.0	0	0	0	0	0	10.6	72.7	9.4	6
	2	10.0	37.0	1.0	0	0	0	6.0	46.0	0	0	0	0	0	5.5	78.8	9.5	6
	3	11.0	39.0	1.0	0	0	0	7.0	42.0	0	0	0	0	0	8.0	78.4	7.4	6
	4	7.0	43.0	2.0	0	0	0	5.0	43.0	0	0	0	0	0	6.2	79.5	8.3	6.5
	5	9.0	41.0	1.0	0	0	0	4.0	45.0	0	0	0	0	0	7.8	81.1	7.2	6.5
Field 4	1	14.7	0	0.0	28.4	0	0	50.8	0	0	0	0	0	6.2	3.9	65.0	8.4	6
	2	13.3	0	1.3	20.7	0	0	64.7	0	0	0	0	0	0	7.8	66.4	7.4	6.5
	3	11.8	0	0	16.9	0	0	65.4	0	0	1.5	0	0	4.4	3.4	67.2	7.3	6
	4	13.2	0	0	21.3	0	0	65.4	0	0	0	0	0	0	4.1	60.4	10.7	6.5
	5	10	0	0	18.0	0	0	72.0	0	0	0	0	0	0	3.8	67.6	7.2	6.5

Table B. 7. Spore percentages of identified AM fungal species in field soils in trap pot cultures on Peking soybean cultivar inoculated with Clarion soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g^{-1} soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.1	6
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.3	6
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.1	6
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.8	6
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.8	6.5
Field 1	1	0	0	0	53.0	0	0	2.0	40.0	0	0	0	0	5.0	11.2	89.2	14.4	6
	2	0	0	0	53.0	0	0	5.0	40.0	0	0	0	0	2.0	18.3	89.4	14.9	6
	3	0	0	0	50.0	0	0	2.0	48.0	0	0	0	0	0	12.5	92.2	13.2	6
	4	0	0	0	50.0	0	0	3.0	43.0	0	4.0	0	0	0	19.4	91.1	14.1	6
	5	0	0	0	65.0	0	0	2.0	33.0	0	0	0	0	0	12.2	91.5	15.3	6
Field 2	1	10.0	0	0	50.0	0	0	0	40.0	0	0	0	0	0	32.5	94.2	17.4	6
	2	15.0	0	0	46.0	4.0	0	0	35.0	0	0	0	0	0	35.9	89.2	20.1	6
	3	8.3	0	0	36.7	8.3	0	0	46.8	0	0	0	0	0	27.8	93.9	18.0	6
	4	0	0	0	46.0	1.0	0	0	49.0	0	0	0	4.0	0	34.1	91.4	17.5	6
	5	0	0	0	55.0	2.0	0	0	39.0	0	1.0	0	3.0	0	26.7	92.9	14.0	5.5
Field 3	1	0	0	0	59.0	1.0	0	0	40.0	0	0	0	0	0	13.0	93.5	17.4	6
	2	0	0	0	50.0	1.0	0	0	40.0	9	0	0	0	0	20.1	93.5	18.1	6
	3	0	0	0	46.0	2.0	0	0	48.0	0	0	0	4.0	0	18.8	94.5	14.0	6
	4	0	0	0	51.0	3.0	0	0	43.0	0	0	0	3.0	0	14.0	90.1	17.3	6.5
	5	0	0	0	57.0	1.0	0	0	41.0	0	0	0	1.0	0	13.4	90.4	15.6	6.5
Field 4	1	68.0	0	0	0	0	0	9.0	23.0	0	0	0	0	0	40.3	90.3	16.5	6.5
	2	73.0	0	0	0	0	0	7.0	20.0	0	0	0	0	0	31.8	91.7	18.2	5.5
	3	63.0	0	0	0	0	0	0	35.0	0	0	0	0	2.0	33.3	92.1	19.0	6
	4	71.0	0	0	0	0	0	0	28.0	0	0	0	0	1.0	41.2	93.1	17.5	5.5
	5	71.0	0	0	0	3.0	0	3.0	23.0	0	0	0	0	0	31.0	91.4	16.8	6.5

Table B. 8. Spore percentages of identified AM fungal species in field soils in trap pot cultures on Peking soybean cultivar inoculated with Webster soils from field sites. Spore count and other plant parameters.

	Rep	G.clo	G.clu	G.con	G.etu	G.geo	G.int	G.mos	G.occ	G.vis	A.spp	E.spp	Gi.spp	Others	Spores g^{-1} soil	% AM colonized	Shoot dry wt, g	Stage of maturity
Control	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.1	6
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.3	6
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.1	6
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.8	6
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.8	6.5
Field 1	1	11.0	0	0	38.0	1.0	0	21.0	23.0	21.0	0	0	0	6.0	10.7	93.6	10.7	5.5
	2	7.5	0	0	45.0	2.0	0	19.0	22.0	19.0	0	0	0.5	4.0	10.6	94.4	11.4	5.5
	3	6.0	0	0	39.0	2.0	0	20.0	19.0	20.0	0	0	0	2.0	11.5	90.3	15.0	6
	4	15.0	0	0	43.0	0	0	5.0	25.0	5.0	0	0	2.0	0	12.2	94.1	13.0	6
	5	20.0	0	0	38.0	2.0	0	0	35.0	0	0	0	0	0	8.5	92.3	14.0	6
Field 2	1	13.0	0	0	38.0	0	0	0	35.0	0	10.0	0	0	4.0	10.2	90.5	10.4	6.5
	2	0	0	0	49.0	0	0	0	41.0	8.0	0	0	0	2.0	17.1	90.1	14.5	6
	3	0	0	0	30.0	0	0	0	33.0	18.0	18.0	0	0	1.0	18.7	90.0	9.6	6
	4	10.0	0	0	40.0	0	0	0	28.0	20.0	0	0	0	2.0	20.2	91.5	9.9	6
	5	0	0	0	42.0	0	0	0	23.0	10.0	25.0	0	0	0	14.2	89.9	11.7	6
Field 3	1	4.5	0	0	8.0	0	0	70.4	3.0	5.0	9.1	0	0	0	23.2	90.9	15.8	6
	2	0	0	0	0	0	0	99.0	0	0	0	0	0	1.0	16.6	91.9	14.8	6.5
	3	0	0	0	0	0	0	100.0	0	0	0	0	0	0	22.4	91.4	14.3	5.5
	4	0	0	0	0	2.0	0	98.0	0	0	0	0	0	0	16.7	92.9	15.4	5.5
	5	0	0	0	0	3.0	0	97.0	0	0	0	0	0	0	15.5	88.3	13.1	6
Field 4	1	1.0	0	4.0	3.0	0	0	85.0	4.0	3.0	0	0	0	0	4.4	82.1	19.3	6.5
	2	0	0	3.0	0	0	0	97.0	0	0	0	0	0	0	2.5	82.4	18.4	6
	3	0	0	1.0	0	0	0	99.0	0	0	0	0	0	0	3.1	69.0	17.8	5.5
	4	0	0	5.0	0	0	0	92.0	3.0	0	0	0	0	0	3.4	70.9	20.6	5.5
	5	0	0	1.0	0	0	0	99.0	0	0	0	0	0	0	1.2	85.8	16.9	5.5

APPENDIX C – EFFICACY STUDY DATA

Inoculum	Block	Colonization %	Shoot dry wt g pot^{-1}	Root dry wt g pot^{-1}	Growth stage or Number of pods with 1 seed	2 seeds	3 seeds	Number of seeds per pot	Height (cm) plant # 1	# 2
Clarion field 1 - BSR201										
Control	1	0	4.29	0.77	2	3	0	8	28	31
Control	2	0	4.33	0.83	1	4	0	9	24	23
Control	3	0	4.39	0.82	Early pod formation				36	38
Control	4	0	4.57	0.86	Early pod formation				35	36
Control	5	0	4.06	0.77	Early pod formation				30	19
BSR-S1	1	10	6.80	0.84	1	8	1	20	25	27
BSR-S1	2	10	7.03	0.86	2	7	0	16	26	28
BSR-S1	3	9	6.31	0.82	1	9	0	19	28	28
BSR-S1	4	13	6.51	0.87	2	7	0	16	23	28
BSR-S1	5	13	7.62	0.96	Late flowering				39	38
BSR-S2	1	43	5.91	0.82	6	4	3	23	21	22
BSR-S2	2	40	6.03	0.82	1	6	2	19	31	30
BSR-S2	3	42	6.27	0.86	3	6	1	2	27	29
BSR-S2	4	46	5.63	0.83	1	5	0	11	25	22
BSR-S2	5	47	6.61	1.04	Late flowering				34	34
Clarion field 1 - Peking										
Control	1	0	5.33	1.59	Flowering				37	39
Control	2	0	5.57	1.60	Flowering				38	30
Control	3	0	5.28	1.57	Flowering				36	27
Control	4	0	5.38	1.49	Flowering				34	30
Control	5	0	5.53	1.56	Flowering				22	23
BSR-S1	1	22	9.93	1.72	Flowering				39	42
BSR-S1	2	25	8.73	1.59	Flowering				35	39
BSR-S1	3	26	9.55	1.52	Flowering				35	36
BSR-S1	4	26	9.57	1.70	Flowering				39	38
BSR-S1	5	23	8.45	1.78	Flowering				31	31
BSR-S2	1	43	9.09	1.67	Flowering				25	39
BSR-S2	2	44	9.56	1.78	Flowering				43	44
BSR-S2	3	49	10.07	1.66	Flowering				42	42
BSR-S2	4	45	10.27	1.52	Flowering				40	42
BSR-S2	5	41	9.97	1.86	Flowering				37	40
Clarion field 1 - Iowa 2052										
Control	1	0	3.98	0.89	5	4	0	13	26	26
Control	2	0	4.23	0.96	3	3	1	12	28	30
Control	3	0	4.38	0.95	3	5	0	13	27	28
Control	4	0	3.90	0.89	4	4	0	12	31	31
Control	5	0	3.18	0.93	4	3	0	10	21	24

APPENDIX C – (continued)

Inoculum	Block	Colonization %	Shoot dry wt g pot^{-1}	Root dry wt g pot^{-1}	Growth stage or Number of pods with 1 seed	2 seeds	3 seeds	Number of seeds per pot	Height (cm) plant # 1	# 2
Clarion field 1 - Iowa 2052 (continued)										
BSR-S1	1	19	5.24	0.82	3	11	0	25	31	30
BSR-S1	2	14	5.17	0.81	1	11	1	26	22	24
BSR-S1	3	14	5.48	0.86	2	10	1	25	41	28
BSR-S1	4	13	5.49	0.86	1	12	0	25	27	30
BSR-S1	5	15	6.11	0.86					35	28
BSR-S2	1	38	6.34	1.01	5	9	2	29	22	26
BSR-S2	2	40	6.13	0.96	0	10	2	26	22	25
BSR-S2	3	37	5.66	0.93	2	6	1	17	21	22
BSR-S2	4	34	6.48	0.95	4	9	0	22	23	26
BSR-S2	5	39	6.38	1.11	1	10	1	24	20	20
Clarion field 4 - BSR201										
Control	1	0	4.84	1.52	1	0	0	1	34	18
Control	2	0	4.97	1.56	Pod formation				32	35
Control	3	0	5.57	1.68	Pod formation				32	37
Control	4	0	5.46	1.50	Pod formation				36	37
Control	5	0	5.11	1.86	Pod formation				34	35
BSR-S1	1	28	6.74	0.95	7	5	0	17	24	28
BSR-S1	2	30	6.43	0.89	1	7	0	15	33	25
BSR-S1	3	33	6.41	0.86	4	4	2	18	45	30
BSR-S1	4	30	7.13	0.99	4	3	4	22	32	26
BSR-S1	5	31	7.88	1.03	Late flowering				35	49
PEK-S1	1	30	6.20	0.99	2	8	0	18	25	26
PEK-S1	2	26	5.72	0.88	4	7	0	18	20	22
PEK-S1	3	28	6.00	0.89	4	4	1	15	21	29
PEK-S1	4	27	6.11	0.94	5	4	1	16	29	26
PEK-S1	5	31	7.08	1.11	Late flowering				44	42
Clarion field 4 - Peking										
Control	1	0	6.23	1.81	Flowering				31	35
Control	2	0	6.09	1.48	Flowering				33	32
Control	3	0	5.24	1.39	Flowering				28	27
Control	4	0	5.32	1.49	Flowering				26	36
Control	5	0	5.89	1.45	Flowering				26	25
BSR-S1	1	33	10.31	1.52	Early pod formation				54	57
BSR-S1	2	32	10.09	1.77	and late flowering				43	44
BSR-S1	3	32	10.18	1.88	Early pod formation				44	45
BSR-S1	4	34	10.21	1.63	and late flowering				42	43
BSR-S1	5	35	9.50	1.63	Early pod formation,				42	32

APPENDIX C – (continued)

Inoculum	Block	Colonization %	Shoot dry wt g pot^{-1}	Root dry wt g pot^{-1}	Growth stage or Number of pods with 1 seed	2 seeds	3 seeds	Number of seeds per pot	Height (cm) plant # 1	# 2
Clarion field 4 – Peking (continued)										
PEK-S1	1	26	9.61	1.63	Early pod formation,				37	34
PEK-S1	2	21	9.14	1.62	and late flowering				36	34
PEK-S1	3	24	9.69	1.61	Early pod formation,				34	37
PEK-S1	4	25	9.60	1.64	and late flowering				34	38
PEK-S1	5	26	8.53	1.70	Early pod formation,				32	33
Clarion field 4 - Iowa 2052										
Control	1	0	3.69	1.14	1	5	0	11	29	31
Control	2	0	4.00	1.20	1	4	2	15	23	30
Control	3	0	3.68	0.94	1	5	0	11	29	32
Control	4	0	3.80	0.98	1	6	0	13	34	29
Control	5	0	3.48	1.13	1	6	0	13	29	30
BSR-S1	1	32	5.97		4	5	0	14	25	23
BSR-S1				Data not available						
BSR-S1				Data not available						
BSR-S1				Data not available						
BSR-S1	5	33	6.57		4	5	1	17	26	27
PEK-S1	1	13	6.15	0.91	1	8	1	20	30	36
PEK-S1	2	18	6.47	0.90	2	6	4	26	35	36
PEK-S1	3	17	6.46	0.90	1	9	2	25	37	39
PEK-S1	4	16	6.33	0.93	1	11	1	26	34	39
PEK-S1	5	19	6.84	0.95	4	11	1	29	35	37
Webster field 1 - BSR201										
Control	1	0	4.73	1.31	Flowering				22	28
Control	2	0	5.10	1.43	Flowering				28	38
Control	3	0	5.64	1.74	Flowering				29	26
Control	4	0	4.70	1.49	Flowering				44	33
Control	5	0	5.16	1.50	Flowering				35	35
MAN-S1	1	49	7.88	1.71	Flowering				33	31
MAN-S1	2	45	7.97	1.66	Flowering				44	46
MAN-S1	3	45	8.20	1.69	Flowering				42	44
MAN-S1	4	43	8.08	1.67	Flowering				45	37
MAN-S1	5	41	9.31	1.70	Late flowering					
Webster field 1 - Peking										
Control	1	0	4.75	1.74	Flowering				30	27
Control	2	0	4.72	1.49	Flowering				27	28
Control	3	0	4.75	1.49	Flowering				34	30
Control	4	0	4.81	1.44	Flowering				28	26
Control	5	0	5.33	1.60	Flowering				26	36

APPENDIX C – (continued)

Inoculum	Block	Colonization %	Shoot dry wt g pot^{-1}	Root dry wt g pot^{-1}	Growth stage or Number of pods with			Number of seeds per pot	Height (cm) plant	
					1 seed	2 seeds	3 seeds		# 1	# 2
Webster field 1 – Peking (continued)										
MAN-S1	1	49	6.58	1.39	Flowering				32	33
MAN-S1	2	51	6.41	1.57	Flowering				33	31
MAN-S1	3	51	6.67	1.62	Flowering				37	34
MAN-S1	4	45	6.54	1.69	Flowering				37	35
MAN-S1	5	40	6.96	1.87	Flowering				39	42
Webster field 1 - Iowa2052										
Control	1	0	3.58	0.94	4	3	0	10	24	19
Control	2	0	3.64	0.94	4	4	0	12	19	28
Control	3	0	4.59	1.13	5	4	1	16	34	37
Control	4	0	4.06	1.02	2	3	2	14	20	25
Control	5	0	3.93	1.14	6	3	1	15	34	23
MAN-S1	1	21	5.40	0.83	3	6	2	21	17	17
MAN-S1	2	20	5.60	0.87	1	8	1	20	22	15
MAN-S1	3	24	5.68	0.88	3	7	2	23	21	26
MAN-S1	4	14	5.61	0.91	2	8	1	21	24	23
MAN-S1	5	19	6.83	0.98	3	11	1	28	28	24
Webster field 4 - BSR201										
Control	1	0	4.04	1.37	Flowering				27	25
Control	2	0	3.94	1.29	2	3	0	8	21	21
Control	3	0	4.38	1.55	Flowering				40	43
Control	4	0	4.34	1.68	Flowering				29	31
Control	5	0	4.68	1.58	Flowering				33	28
MAN-S1	1	29	9.33	1.88	Early pod fromation				40	40
MAN-S1	2	31	9.04	1.65	Early pod fromation				35	42
MAN-S1	3	27	9.85	1.96	Early pod fromation				45	40
MAN-S1	4	27	9.76	1.72	Early pod fromation				45	43
MAN-S1	5	27	10.40	1.76	Early pod fromation				42	40
MAN-S2	1	25	6.95	1.72	Flowering				37	38
MAN-S2	2	26	6.96	1.76	Flowering				41	35
MAN-S2	3	26	7.02	1.70	Early pod fromation				31	30
MAN-S2	4	24	7.08	1.71	Early pod fromation				38	31
MAN-S2	5	23	8.82	1.83	Early pod fromation				48	38
MAN-S3	1	27	5.98	0.85	6	2	1	15	27	25
MAN-S3	2	25	5.14	0.92	2	4	1	13	24	30
MAN-S3	3	26	5.72	0.94	1	4	3	18	23	32
MAN-S3	4	28	5.78	0.79	3	7	0	17	29	24
MAN-S3	5	27	7.26	1.03	Early pod formation				48	42

APPENDIX C – (continued)

Inoculum	Block	Colonization %	Shoot dry wt g pot^{-1}	Root dry wt g pot^{-1}	Growth stage or Number of pods with			Number of seeds per pot	Height (cm) plant	
					1 seed	2 seeds	3 seeds		# 1	# 2
Webster field 4 - Peking										
Control	1	0	4.68	1.46	Flowering				24	24
Control	2	0	5.02	1.82	Flowering				40	37
Control	3	0	4.66	1.43	Flowering				25	31
Control	4	0	4.90	1.73	Flowering				36	34
Control	5	0	4.45	1.71	Flowering				26	27
MAN-S1	1	34	8.32	1.87	Flowering				35	34
MAN-S1	2	36	8.63	1.83	Early pod fromation				40	38
MAN-S1	3	28	8.22	1.63	Flowering				39	40
MAN-S1	4	33	8.55	1.90	Early pod fromation				32	33
MAN-S1	5	36	8.65	1.67	Flowering				42	37
MAN-S2	1	32	6.96	1.82	Flowering				34	34
MAN-S2	2	28	6.87	1.61	Flowering				39	27
MAN-S2	3	23	7.05	1.57	Flowering				38	35
MAN-S2	4	17	7.03	1.65	Flowering				44	34
MAN-S2	5	25	7.96	1.85	Flowering				34	29
MAN-S3	1	37	8.59	1.16	Flowering				33	39
MAN-S3	2	38	8.98	1.19	Early pod fromation				41	45
MAN-S3	3	38	8.72	1.09	Flowering				41	47
MAN-S3	4	30	9.02	1.10	Early pod fromation				43	50
MAN-S3	5	29	7.55	1.07	Flowering				29	36
Webster field 4 - Iowa 2052										
Control	1	0	2.99	0.98	3	3	0	9	34	24
Control	2	0	3.03	0.97	1	4	0	9	25	27
Control	3	0	3.78	0.94	3	4	0	11	28	29
Control	4	0	3.87	1.00	4	4	0	12	34	33
Control	5	0	3.60	1.02	4	4	0	12	38	27
MAN-S1	1	23	6.50	0.92	5	7	1	22	27	20
MAN-S1	2	15	6.83	0.96	8	9	1	29	24	22
MAN-S1	3	25	6.10	0.94	1	6	1	16	29	22
MAN-S1	4	22	6.16	0.94	3	9	0	21	29	23
MAN-S1	5	26	7.20	0.99	6	12	1	33	37	40
MAN-S2	1	12	3.99	0.66	2	6	2	20	23	24
MAN-S2	2	13	3.50	0.60	0	8	0	16	22	14
MAN-S2	3	14	3.61	0.62	4	6	0	16	25	17
MAN-S2	4	16	4.03	0.62	2	5	1	15	23	23
MAN-S2	5	17	5.01	0.78	5	7	2	25	37	40

APPENDIX C – (continued)

Inoculum	Block	Colonization %	Shoot dry wt g pot^{-1}	Root dry wt g pot^{-1}	Growth stage or Number of pods with			Number of seeds per pot	Height (cm) plant	
					1 seed	2 seeds	3 seeds		# 1	# 2
Webster field 4 - Iowa 2052 (continued)										
MAN-S3	1	34	7.82	0.96	3	10	4	35	31	28
MAN-S3	2	26	7.05	0.99	2	8	3	27	28	45
MAN-S3	3	30	7.55	0.89	3	8	2	25	33	41
MAN-S3	4	33	7.01	0.90	2	10	2	28	31	36
MAN-S3	5	29	7.38	0.97	5	10	1	28	40	33