ALTERNATIVE TOMATO FARMING SYSTEM PRACTICES AND THEIR INFLUENCE ON THE PERSISTENCE AND INFECTIVITY OF ARBUSCULAR MYCORRHIZAL FUNGI AND OTHER FUNGAL ROOT ENDOPHYTES

By

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Farming practices are known to significantly impact arbuscular mycorrhizal (AM) fungi, as well as other soil microbial communities in agroecosystems. The effects of highly divergent land management strategies on the incidence and infectivity of AM fungi and other fungal endophytes were evaluated in a 5-year cropping systems study at the USDA ARS Header Canal farm site in Ft. Pierce, FL. Two treatments, the conventional and disk fallow systems, represented farming practices considered to be detrimental to AM fungal populations. The remaining three treatments, the organic, bahia pasture and weed fallow systems, used practices thought to be more conducive to the presence of AM fungi.

In December of year four and five of the study, tomato rhizosphere soil and fibrous roots of tomato were collected. Roots were cleared, stained and examined for colonization by AM fungi and other fungal endophytes. Rhizosphere soil was assayed

for infection potential of AM and other root endophytic fungi based on maize seedling colonization at the five-leaf stage. Tomato roots and rhizosphere soil from year five were also analyzed for the AM fungal fatty acid biomarker 16:1ω5*c*. Sudangrass trap cultures were set up to determine abundance and diversity of AM fungal spore morphotypes.

Of all investigated agricultural practices, phosphorus (P) fertilization and soil disturbance had the greatest influences on AM fungal infectivity and abundance. All plots had very high levels of available soil P, which resulted in low levels of colonization across treatments. The bahia pasture system was the only treatment without regular extensive soil mixing, and these plots had the highest level of field root colonization by AM fungi. Field roots were more heavily colonized by fungal endophytes other than AM fungi in all treatments; tomato plants from organic plots, though, were unique in encouraging colonization by *Microdochium bolleyi*. Populations of root-associated AM fungi and other fungal root endophytes were positively correlated in all studies.

Hurricane-associated flooding and a shortened growing season played a role in reducing the infection potential in all treatments except for the organic plots in year five compared to year four. The levels of primary inoculum were maintained in organic plots, apparently through improved soil quality which resulted in decreased resistance to root development. Areas under active disturbance in disk fallow plots had the lowest levels of primary inoculum, but recovery to levels comparable to less disturbed treatments occurred after a single season of host root growth.

Diversity of AM fungal morphotypes was typical of agricultural fields, with at least 11 species present across treatments. *Glomus* spp. were the dominant spore type recovered in all treatments.

CHAPTER 1
INTRODUCTION

Arbuscular Mycorrhizal and Other Fungal Root Endophytes in Agroecosystems: Context and Importance

Arbuscular mycorrhizal (AM) fungi form symbioses with most plants, are present widely in terrestrial environments, and have been identified as performing a variety of functions in their associations within natural ecosystems and agroecosystems (Douds & Millner 1999; Jeffries *et al*. 2003). These mycorrhizal associations are believed to be capable of making a significant positive contribution to plant health as well as positively affecting soil quality. Among the benefits arbuscular mycorrhizae may provide to plants include increasing nutrient uptake, particularly of P and Zn; improving plant access to water; positively modifying rhizosphere populations; and increasing both pest and soil borne disease resistance in plants (Jeffries *et al*. 2003; Johansson *et al*. 2004). Additional potential positive effects that have been described as a result of the symbiosis between mycorrhizal fungi and plants are the improvement and maintenance of soil structure and alleviation of plant stress in otherwise inhospitable environments, such as recently disturbed or polluted soils (Douds & Millner 1999; Jeffries *et al*. 2003; Johansson *et al*. 2004).

The AM fungi have a central role in ecosystems and experimental evidence shows that considerable functional diversity occurs among taxa within the phylum Glomeromycota (Munkvold *et al*. 2004; Gosling *et al*. 2006). This diversity, however, does not necessarily constitute benefits provided to all plants in all environments (Bever

et al. 2001; Jeffries *et al*. 2003; Munkvold *et al*. 2004). Based on experimental evidence, the ability of AM fungi to function and maintain the mycorrhizal condition under some field conditions common in industrialized agriculture appears limited (Kabir *et al.* 1999; Oehl *et al.* 2004; Gosling *et al.* 2006), and while the symbiosis is generally perceived as mutualistic, there are "occasional commensal and parasitic excursions from this norm" (Johnson *et al*. 1997, p. 583). Management practices - such as high P fertilizer applications; tillage-mediated soil disturbance; and the application of pesticides, including the use of soil fumigants - are considered potentially detrimental to populations of AM fungi. In fact, when considering the widespread use of and production benefits gained from practices that may be deleterious to AM fungal populations, as well as the carbon cost inherent to plants in interactions with these obligately biotrophic fungi, the question can be raised as to whether or not AM fungi should be considered when making management decisions in many agricultural systems (Ryan & Graham 2002).

The AM fungi are not the only organisms widely capable of occupying the root cortex; there are many other fungal endophytes, some of which may also exist within the stele (Yu *et al*. 2001; Barrow 2003). Several definitions for the term endophyte exist and different uses of the word can sometimes be points of controversy (Wennström 1994; Sieber 2002). When used in this work, a relatively narrow definition will be adopted. The endophytes referred to herein are those fungi observed existing within the roots of plants without causing apparent symptoms of disease. Recognizing that AM fungi fit this definition of an endophytic organism, when the term endophyte is used in this work it will be qualified to distinguish fungi not recognized as residing within the phylum Glomeromycota. The individuals that make up this group of root-occupying fungi may

function as obligate or facultative biotrophs and exist within a range of plant-microbe interactions from mutualism to, in some instances, parasitism or pathogenicity, as in the case of some *formae speciales* of *Fusarium oxysporum* (Morton 1998; Sylvia & Chellemi 2001; Sieber 2002). These forms are pathogenic on select taxa of plants, and are seemingly innocuous or beneficial when associated with most other plants.

The data for this study were collected from the USDA, ARS cropping systems project at Header Canal in Fort Pierce, Florida. The project provides, among other objectives, an opportunity to examine the effects varied farming management practices have upon the components that define soil quality. Part of determining the status of soil quality and health involves monitoring biological indicators; this process begins by determining the condition of resident plant health, and evaluating components such as soil microbial biomass, activity, and diversity (van Bruggen & Semenov 2000; Hill *et al.* 2000; Chellemi & Porter 2001). As discussed above, both the AM fungi and other root endophytes occur globally, and it is probable that all plants form intimate associations with at least one taxa of fungal endophyte, while in many cases plants may be more or less indiscriminate in their associations (Sieber 2002). The value of these organisms as biological indicators of soil quality and health may be highly significant, and their impact on ecosystem functioning needs to be better understood. An integral aspect of that understanding in the context of agroecosystems involves the elucidation of the capability of fungal root endophytes to persist and remain infective under intensive management practices.

Study Site

The 5-year cropping systems study at the Header Canal Farm in Fort Pierce, FL, has five land-management systems that are highly divergent, yet all use management

methods not uncommon in the region. The land which is being farmed in this project had been in conventional tomato production using raised plastic-mulched beds fumigated with methyl bromide plus chloropicrin (MeBr + CP) annually for 10 years prior to being leased for research. The entire research site has soil that has been classified as a Pineda fine sand (loamy, siliceous, hyperthermic, Arenic Glossaqualfs), with a sandy texture from the surface to a depth of 96 cm (Lewis *et al*. 2003; Watts & Stankey 1980). The average pH among system plots over years four and five ranged from 6.8 to 7.7. Soil phosphorus levels were extremely high due to the previous fertilization history and nutrient applications made subsequent to the initiation of the land management treatments. Mean Mehlich-1 extracted phosphorus ranged from 794 to 1831 kg ha^{-1} among systems. During the initiation of tomato crop production in year five of the study, two hurricanes (Frances and Jeanne) struck the area of Florida where the experimental farm site was located, causing significant damage to the farm and surrounding communities. Rainfall totals exceeded 50 cm during the month of September, resulting in widespread flooding. Tomato transplantation was delayed approximately a month due to the hurricanes and the resultant field conditions.

Systems were initiated in the summer of 2000 and, with the exception of one treatment, as will be explained below, were allowed 3 years to establish prior to resumption of tomato production. The five treatments were established in a randomized complete block design, with each treatment replicated six times for a total of 30 plots, occupying 0.16 ha individually (plots are 68.5 x 23 m). Tomatoes in all treatments, except the bahia pasture system, were grown in raised beds with plastic mulch. The five systems are described below.

Conventional Production

This system continued the management practices of the preceding farmer, with the exception being replacement of the original soil fumigant with Telone C-35, a 62:35 formulation of 1, 3-dichloropropene (1, 3-D) and chloropicrin (CP). Trifluralin (Treflan) and napropamide (Devrinol) were spread and incorporated for weed control. Tomatoes in this treatment received an initial fertilizer application during bed formation (20% of total N and K), with subsequent fertilization provided during the growing season via injection through drip irrigation. In this system the 3-year transition was not necessary, so fall tomato production continued throughout system plots. During the spring and summer of 2001 this treatment had a cover crop of Japanese Millet (*Echinochloa crusgalli* var *frumentace*a); it was weed fallowed in 2002, and disk fallowed in 2003.

Organic Production

During the first 3 years no tomato crop was raised on the plots in this treatment. Broiler litter and urban plant debris were broadcast and incorporated into the soil in July. In August, a cover crop of sunn hemp (*Crotalaria juncea*) was planted, while in the following spring, Japanese millet was grown. After the transition period, starting in the fourth year, the south half of each plot was used to produce a tomato crop following approximately 6 weeks of soil solarization of the beds, while the other half remained under a sunn hemp cover crop. During the fifth year, the entire plot was put into tomato production using the techniques just described.

The production plan for these plots was not certified organic due to their close proximity to treatments that use technology prohibited by certifying bodies. However, the practices used in this treatment are aligned with methods that are in favor with advocates for organic agriculture.

Disk Fallow

The entirety of the plots in this treatment were disked regularly year-round to keep them free of vegetation. Disking depth was about 25 cm. Tomatoes were not grown in years one through three. In August of year four, the south half of these plots received fertilizer and herbicides identical to those applied to the conventional system. After the final harvest, both halves were again disked regularly. In year five, the entire plot was put into production using the same methods to integrate the northern half as were used in the southern half the year before.

Bahia Pasture

A bahia grass (*Paspalum notatum* var. Argentine bahiagrass) pasture was established in these plots and managed through mowing and spring fertilization for the first 3 years. In year four, the southern halves of the plots were put into tomato production using a strip tillage production system (Chellemi *et al.* 1999). A 46-cm strip was tilled into the bahia grass for planting, while the grass on either side of the row was managed through mowing and use of the herbicide sethoxydim (Poast). The fifth year involved cropping both halves of these plots using the strip-till technique.

Weed Fallow

These plots were left undisturbed for the first 3 years of transition, and weed communities were allowed to establish. The southern halves of the treatment plots were put into tomato production beginning in year four after disking and using the same practices used in the conventional system. The entire plots were cultivated to tomato during the fifth year.

Reasoning and Objectives

The main priority of this study is to characterize the impacts that varied management practices have upon an important and highly influential group in the soil community, the AM fungi. Across one soil type, a field with a uniform history of intensive agricultural production using MeBr + CP was transitioned into five divergent management strategies. Two treatments represented farming practices considered to be highly detrimental to AM fungal populations, the conventional plots and the disk fallow plots. The remaining three treatments used practices that are thought to be more conducive to encouraging the presence of AM fungi. Organic plot management included an intensive cover-crop regimen and nutrient sources that are more slowly available than in the other systems, while weed fallow plots were left undisturbed during the transitional period, allowing for the unrestricted development of an endemic plant population, and were not exposed to the use of agrichemicals until the re-initiation of tomato production. In the bahia pasture treatment, a sod known to be highly mycotrophic was established during the transitional period, and was maintained after strip-tilled rows were generated for tomato transplants.

Specifically of interest in this project was whether systems encouraged or discouraged the relative abundance and infectivity of fungal root endophytes. In addition, while considerable literature exists detailing and speculating on the impacts of certain agricultural practices on AM fungi, there is little information regarding the ability and extent to which populations recover from adverse conditions once land management practices are changed. Little is known about the long-term repercussions, if any, that farming strategies such as repeated soil fumigation have on AM fungal presence and infectivity. The quantification of the AM fungal presence among the systems during year

four and five is important in that it can provide information about the effects management has upon these organisms with transition to production and on the differences in populations occurring among the systems during production.

While considerable interest is developing in enhancing understanding of the possible functional role of non-AM fungal root endophytes in agroecosystems, particularly those fungi that have been designated as dark septate endophytes (DSE), there is little information regarding their ability to persist under intensive agricultural management. Gathering information regarding structures associated with the occurrence of non-AM fungal root endophytes as they exist in roots can provide a provisional identification or classification. Investigating the degree to which field tomatoes and other host crops are colonized may help us determine the AM fungal and other root endophyte abundance and aggressiveness within the various treatments and help us understand how these have been affected by management strategies.

The infectivity of AM and other fungal root endophytes was quantified in end-of-season field tomato roots, and their infection potential in field soil was assayed in a greenhouse study. The non-AM fungal endophytes found existing in field roots were identified when possible based on their morphological features within the root association. Fatty acid methyl ester (FAME) analysis was performed on year five tomato roots and field soil, providing an independent measure of biomass by quantification of the AM fungal fatty acid biomarker, 16:1ω5*c*. Spore extractions from a greenhouse study in which trap crops were grown in field soil were completed, achieving a provisional accounting of AM fungal morphotypes occurring in the field. In year five, spore numbers from the harvested trap cultures were enumerated.

The above work was accomplished to elucidate the impact of industrialized agricultural practices, as represented by the history of the farm site at Header Canal and perpetuated in two of the five treatments, on the survival, infectivity and occurrence of AM and other fungal root endophytes. In addition, it was important to determine the degree to which farming strategies that have been considered more amenable to encouraging the existence and functioning of AM fungi were able to affect these populations after long-term exposure to intensive tomato production.

CHAPTER 2
LITERATURE REVIEW

Research on Arbuscular Mycorrhizal Fungi

Our knowledge of the existence of AM fungi is generally accepted to have begun with descriptions Nägeli made in 1842 (Koide & Mosse 2004). In the late 19th and early 20th century, detailed descriptions of the morphological results of the symbiosis were in place, including both of the most important diagnostic structures: vesicles (Janse 1897) and arbuscules (Gallaud 1905). While the relationship between plants and AM fungi has been known to exist for well over 100 years, its widespread occurance in ecosystems and intensity therein, although speculated upon in some earlier literature, has been established much more recently (Read *et al*. 1976). A partial list of ensuing work, occurring on a broad range of scales while spanning multiple disciplines, includes: attempting to develop a taxonomic system and understand the phylogeny of the AM fungi; ascertaining and characterizing the effects of the mycorrhizal relationship on the symbionts and on the broader environment, as well as investigating the natural and anthropogenically derived edaphic and environmental conditions that impact the partnership; understanding the regulating factors in and the physiology of mycorrhizae; and the attempt at development of AM fungi as an agricultural technology (Koide & Mosse 2004). Research that is of particular interest in the context of this project includes studies that relate to the ecology of mycorrhizae, especially in managed systems.

Studying Arbuscular Mycorrhizae

Accurate methods to detect, quantify and identify AM fungi are paramount to developing an understanding of mycorrhizae in any environment and context. A variety of obstacles exist in studying arbuscular mycorrhizae, however, stemming mostly from their obligately biotrophic nature and complex, not yet fully understood, genetic condition. Classical, biochemical and molecular tools have been developed and used with varying degrees of success by researchers.

While tools and techniques exist both for quantification and identification of AM fungi as well as for measuring their metabolic activity, linking identified active taxa to possible ecological roles is more complicated (Millner & Wright 2002). There appears, based on experimental evidence, to be considerable inter- and even intra-phylo and morphotypic functional diversity in the AM fungi. Munkvold *et al*. (2004) compared the hyphal growth of 24 isolates from four described morphotypes and their ability to influence the growth and P-nutrition of *Cucumis sativus*, and found considerable variability existing within morphotypes in their mycelial extention and in the ability to promote growth. Another study comparing the capabilities of 16 isolates of *Glomus intraradices* from a single field produced similar results, with five-fold differences in final hyphal lengths (Koch *et al*. 2004).

Further confounding understanding of the functional diversity of AM fungi is that when taken out of the microcosm of a controlled experiment, the fungal role in mycorrhizal functioning seems plastic, depending largely on its environment (Johnson *et al*. 1997). If functional richness abounds in the AM fungal genotype, and is shown to not be dependent upon species diversity, but rather on isolates as influenced by their environment, it would represent a shift in a standard view of microbial ecology as it

relates to this type of mycorrhiza. The same uniquely diverse genetic makeup of AM fungi that makes them difficult to characterize, and in some cases investigate, may serve to place the organism in position to ensure the perpetuity of the relationship between symbionts in the presence of adversity through functional redundancy in the larger population. This would be especially important to understand in the context of agricultural or other disturbed environments where alteration of habitat occurs.

There remains substantial debate as to whether maintaining taxonomic diversity is important if functional diversity is preserved and is active. Our understanding of these concepts with regards to AM fungi and the scale on which we judge them has changed as our tools for reseach have developed and improved. In the early stages of mycorrhizal research the AM fungi were considered functionally redundant; we now are beginning to better understand the influence that the edaphic and climatic environment as well as the host plant imparts on the functional phenotype of the fungi. Likewise, in natural systems, the phenotype of the fungal component in mycorrhizal associations can directly affect the structure of plant communities (van der Heijden *et al*. 1998; Klironomos *et al*. 2000; Graham & Miller 2005). Future research determining the extent to which functional diversity and resiliency exists among AM fungal species and isolates as well as the degree to which it is present in given populations will help clarify the association between these factors (Kirk *et al*. 2004). Furthermore, when considering ecological studies of the mycorrhizal relationship it is vital that, where possible, experimental results from controlled environments be investigated on the broadest scale possible to verify their real world significance (Graham & Miller 2005).

Prior to discussing research on AM fungi in ecosystems in greater depth, it is important to describe some of the more important and commonly used tools and methods available for the research of these organisms, including problems associated with their use.

Non-Molecular Tools

Quantification of fungal colonization in roots using microscopy

Assessing the levels of fungal infection in host plants is performed in most studies involving AM fungi. Barring the use of biochemical or molecular techniques, both of which are still often correlated with classical methods, examination of roots takes place under a microscope. In order to best prepare samples for observation, harvested roots should be processed rapidly after taking any necessary measurements from the fresh root system followed by drying the roots to be examined as soon as possible to stop metabolic activity. To visualize intra-cortical fungi when using non-vital stains, the plant's pigmentation and cellular constituents need to be clarified prior to staining.

Phillips and Hayman (1970) were among the first to use a procedure that is still commonly used today, due to its low cost, low technical requirements and consistent results; host cytoplasm is cleared through heating roots in 10% (w/v) potassium hydroxide (KOH) followed by rinsing, with roots undergoing subsequent staining using trypan blue. An exceptional alternative clearing agent is concentrated chloral hydrate, which renders the roots transparent, although its legal status makes it difficult to acquire. Several variations on the basic procedure have been developed. The length of time required to clear roots varies with the type and age of the root, the size of the root sample and the temperature at which clearing takes place. Calibration is essential when establishing clearing procedures, as too little clearing will reveal little in terms of fungal

structures, while too much will destroy roots, stripping the cortex from the vascular tissue. The root samples to be processed are typically best kept to < 2 g, with segment lengths cut to 1- 3 cm. Heating of samples in KOH generally is accomplished in an oven or hot water bath (typically 60-95°C), but is fastest when autoclaved. It can also be done at room temperature, if extending the process to hours or days is not problematic. Further clearing with acidified bleach (1.7% NaOCl with 1% diluted HCL v/v in water) or alkaline hydrogen peroxide (0.5% NH_4OH with 0.5% H_2O_2 v/v in water) at room temperature provides additional removal of pigments in root tissue that contain particularly recalcitrant obscuring compounds. This step is especially valuable when further clearing in KOH would lead to destruction of the sample, but, once again, in this procedure the sample should be observed frequently to achieve the best results for a given type of root. If bleaching is performed using alkaline H_2O_2, the sample should subsequently be acidified with diluted HCL to enhance the staining that follows. Roots should be thoroughly rinsed with water after each clearing and bleaching step.

Non-vital stains are still the most commonly used technology for enhancing visualization of AM fungi in roots. These stains are valuable in that they provide a means to view the entirety of colonization at the time of plant harvest, and are therefore useful for observation of both viable and non-viable fungal tissue. Trypan blue (0.05% w/v) was used in the study that popularized the hot KOH clearing technique and is still widely used today (Phillips & Hayman 1970). This, and similar stains such as 0.05% (w/v) cotton blue, 0.01% (w/v) acid fuschin and 0.03% (w/v) Chlorazol black E (CBE), are typically accomplished by placing the roots in dye that has been suspended in lacto-glycerol (lactic acid – glycerol – water, 1:1:1 or 1:2:2) solution and heating. Stained

roots can then be stored in lacto-glycerol or diluted glycerol without dye, a step that can also enhance visualization of fungal tissue by removing excess stain, especially important when using trypan blue or CBE. Stain concentrations and staining time should be assessed and adjusted according to the root type and the needs of a particular study. Higher concentrations and longer staining times are more likely to be required with cotton blue and acid fuschin, while over-staining can often occur when CBE is used. Trypan blue is particularly useful when examining roots under a dissecting microscope for determination of colonization levels, while a higher contrast stain such as CBE is probably more appropriate for morphological studies.

In addition to the destructive, non-vital staining techniques described above, non-destructive and vital staining methods have been developed. Technologies such as epifluorescence and confocal laser-scanning microscopy (CLSM) allow for the examination of living tissue, albeit with inherent limitations in the use of both. Epifluroescence microscopy appears to only reveal collapsed and therefore inactive arbuscules, providing information of select value, while CLSM requires specialized and expensive equipment and supplies. Vital staining using nitro blue tetrazolium chloride (NBT), diaminobenzidine (DAB) and phosphatase assay kits can also be useful to visualize tissue that was metabolically active at the time of plant harvest. These methods are considerably more expensive than non-vital stains, their use can be an involved process, and investigators may still require or benefit from the use of specialized equipment.

An excellent general review of clearing and staining techniques was recently written by Vierheilig *et al*. (2005). Brundrett *et al*. (1996) provides detailed guidance with regards to non-vital staining materials and procedures.

There are two general approaches to studying AM fungi colonization levels that have evolved and generally supplanted a multitude of other techniques, favored because they minimize compromises researchers need to make with regards to accuracy, ease of execution, and reproducibility. One approach was adapted from a procedure developed by Newman (1966) to improve the ability of researchers to estimate total sample root lengths, which he called the "line intersection method," now often called the grid-line intersect or just the line-intersect method. This procedure was devised to provide another quantitative evaluation besides root weights for assessing the amount of root tissue in a sample, and yields accurate, reproducible estimations. As commonly adapted for the study of AM fungi, this method was described, as well as tested for accuracy and compared to several other techniques by Giovannetti and Mosse (1980). In the line-intersect method, previously cleared and stained roots are spread on a Petri dish onto which a grid of straight lines are affixed, drawn or scored. The grid of lines is then used as a reference, where intersections between them and the root sample are tallied and observed for evidence of infection using a dissecting microscope. This will yield data representing both the total root intersections and the total infected intersections, from which the proportion of infected tissue can be derived. An appropriate volume of roots is chosen, not so many that individual roots can not be observed, but not so few that at least 100 intersections can not be tallied. This means that when fine roots are being observed, the dry weight of roots to be examined will rarely exceed 0.2 g.

The grid of straight lines is usually laid out so that 1.27- cm (0.5- in.) squares are formed. The line-intersect method can be used to not only estimate the proportion of infected root, but also the total root length, using the formula

$$R = \frac{\pi N A}{2 H}$$

where R = total root length, A = the area of the surface over which the roots are observed, N = the number of intersections between roots and lines, and H = the length of all straight lines. When 1.27- cm squares are used, the total root length in cm = the number of root-line intersections, or R = N. Giovannetti and Mosse (1980) determined that increasing the number of observations from 100 to 200 improved the standard error of percent infection measurements from ± 4 to ± 3 when one set of observations was performed, while increasing the number to 300 yields a smaller improvement. This number can therefore be adjusted according to a researcher's need for accuracy.

McGonigle *et al*. (1990) developed a method that not only allows the determination of colonization levels and separation according to structures encountered, it additionally assists with problems encountered in distinguishing AM fungal structures from other cortical objects by virtue of its reliance on the use of a compound microscope to examine intersections. The use of dissecting microscopes to study root – endophyte interactions is inherently limited due to the relatively lower magnifications available when compared to compound microscope. A root system, even when adequately cleared and stained, and with its cortex intact after processing, can be a confusing mess of colors and shapes due to root cell content staining as well as the coexistence of a diversity of microorganisms within the root. This problem can be multiplied when an investigator is

studying field roots or has limited experience differentiating among the varied components of the root and its inhabitants.

The technique described as the magnified intersections method (McGonigle *et al*. 1990) examines intersections between the eyepiece crosshairs and roots at a recommended magnification of x 200, higher than that capable of achievement using dissecting scopes. In using this procedure, between two to four slides of roots from a cleared and stained subsample are observed. The field of view is moved so four, six or eight passes are made across a slide along its short axis. Where the crosshair intersects with a root section, the field of view is adjusted so the vertical line is parallel with the long axis of the root. Intersections are counted and classified according to their AM fungal structural content.

Although the accuracy of individual intersection assessment increases with this method, there are drawbacks. The authors themselves noted that the preparation of sampling and subsequent observation is a much lengthier process using their technique when compared to the line-intersect method. Further, it remains unclear as to whether this represents an improvement in the assessment of the true colonization level of a root system. The additional information yielded in determination of the level of types of AM fungal structures present also seems to have limited value. Where these determinations are perceived to be important or useful, the magnified intersections method is clearly a superior investigative process to the line-intersect method; however, it requires more skill on the part of the microscopist.

Spore extraction for identification and enumeration

The separation of spores from soil or other plant growth media is required for classification, as AM fungal structures formed during the association with roots are not

suitable for differentiation among morphotypes except at the higher taxonomic levels. Spore extraction is important not only for the determination of the presence of morphotypes using classical techniques, but is also currently necessary for molecular studies in identifying phylotypes. This section will detail the technique of spore removal and subsequent procedures that relate to morphological classification and quantitative evaluations, while the subject of molecular studies will be dealt with subsequently. Spore extractions in ecological studies are typically accomplished for two reasons: 1) to quantify the level at which sporulation is occurring in the field at different plant phenological stages, or to check for fluctuations in production related to seasonal or plant successional factors; and 2) for identification purposes, either from field soil, or from greenhouse pot cultures.

The processes of spore extraction that are most commonly used are adaptations of a method derived to extract nematodes, first modified by Gerdemann (1955). The method varies among researchers, but the general process involves homogenization of a soil or root-soil complex in water followed by sieving, centrifugation and decanting. Some investigators additionally include steps involving separation in sucrose density gradients with additional centrifugation (Brundrett *et al*. 1996). The specific method used in this study will be detailed in the Materials and Methods section.

Enumeration of spores is generally associated with directly or recently sampled field soil, although investigators in some studies count spores concurrently or exclusively from trap cultures (Liu & Wang 2003). Spore enumeration data is best observed with reservation and understood with qualification in ecological research. It does not necessarily represent an accurate indication of AM fungal presence, since the degree to

which spores are present in field soil or in pot cultures depends on many variables. Individual isolates are known to sporulate more heavily than others, with some isolates rarely forming spores under any conditions. Sporulation generally appears to occur in predisposed isolates after reaching a threshold of colonization within a plant and can be affected by a multitude of environmental conditions. Additionally, spores are not the most important infective propagule in many systems (Clapp *et al*. 1995; Klironomos & Hart 2002), and are only a small fraction of the total AM fungal biomass in any system.

Identification based on spores directly harvested from the field can be a difficult ordeal, owing to the large proportion of parasitized spores and associated organic material that is generally present, complicating both morphological and molecular identification. This problem is commonly resolved by segregating healthy-looking morphotypes from field soil extractions, then inoculating greenhouse or potted field grown host crops with the individual morphotypes. Growth media in pot cultures is usually sterilized sandy or sandy loam soil fertilized during crop growth using a weak Hoagland's solution without phosphorus. The host crop is then harvested at a particular developmental stage or at senescence, and a second extraction is performed, yielding spores more suited for identification.

There are currently two systems of taxonomic classification broadly recognized for AM fungi, based primarily on morphological and genetic variation among groups, respectively. The revisions to taxonomy have been continuous during the long history of research of these organisms, so it is with great relief that most researchers note that considerable homology has occurred, and that effort has been made to maintain congruence where possible thus far between the systems (Koide & Mosse 2004).

The taxonomic system built on the foundation of the morphological variation of spores exists in descriptions associated with the International Culture Collection of VA Mycorrhizal Fungi (INVAM), currently held at West Virginia University and maintained by Joe Morton. The system is not isolated from advances in molecular research, and has accounted for the elevation of the AM fungi to the level of division Glomeromycota that occurred based based on SSU rDNA sequence analysis by Schüßler *et al*. (2001). Additionally, results of SSU rDNA analysis helped elucidate the existence of the two most recently recognized families within this taxonomic system: the Paraglomaceae and Archaeosporaceae (Morton & Redecker 2001).

The INVAM collection currently recognizes one order, Glomales, within the phylum Glomeromycota. This order is divided into two suborders: the Glomineae, which contains four families and five genera; and the Gigasporineae, with one family and two genera (INVAM 2006a). There are, indeed, many divergent anatomical and morphological characteristics independent of spores that have been identified among these groups, some of which have aided in the separation of taxa. Examples include the formation of auxiliary cells associated with the Gigasporineae, the inter- and intra-cellular vesicles formed by the Glomineae, as well as differences in the shape, size and staining intensity of hyphae and arbuscules in the cortex. The ability to use these characteristics for diagnostic purposes, though, is extremely limited and therefore they are generally only noted in morphotype descriptions from pure culture. They can, in certain circumstances, provide evidence of the existence of taxa that are otherwise not evident through the primary means of morphotype identification: spore wall characterization. The use of alternative characteristics as a means of identifying AM

fungal taxa would only be important when primary evidence was lacking, for example, when spores are not extracted but colonized roots are observed. Identification with the use of spores is preferred as morphotype descriptions are formed based on a variety of spore attributes including the number of walls, external and internal anatomical feature structure, size and staining characteristics, and spore ontogeny.

The system of classification based on SSU ribosomal gene analysis does not refer in its descriptions to the other morphological, biochemical, phenological or other sources of genetic variability that occur among and within identified taxa. This classification groups the AM fungi to the species level (Schüßler *et al*. 2001; Schüßler 2006), and has rearranged the taxonomic organization creating multiple orders, as well as adding and shifting family-level classifications. Phylotypes currently recognized by this classification include four orders: Glomerales, now divided into two groups; Diversisporales, a grouping that includes the Acaulosporaceae, the Gigasporaceae, as well as two new families, Diversisporaceae and Pacisporaceae; the Archaeosporales with Archaeosporaceae and the newly formed Geosiphonaceae; and the Paraglomerales containing the Paraglomeraceae. Some of the problems with speciating the AM fungi and associated difficulties with molecular analyses are discussed in the next section. The separation of AM fungi from Zycomycota into their own phylum, the Glomeromycota, is widely accepted; finer taxonomic levels, however, will ideally ultimately depend on a more comprehensive knowledge of morphological and molecular variability.

Molecular Tools and Studying the Species Concept and Genetic Condition of Arbuscular Mycorrhizal Fungi

While the knowledge of the existence of mycorrhizae is over a century old, and interest in studying these organisms has long been high due to their ubiquitous nature and

perceived potential as an agricultural technology, the concept of species has yet to be firmly established with consensus. As noted previously, spore morphology has historically been the most important factor in AM fungal species determination, but there are a number of problems associated with relying solely on spores for identification in ecological studies. Some practical difficulties include rapid sample degradation, presence of associated, but non-sporulating morphotypes, the lack of significant distinguishing characteristics among some taxa, and the extreme laboriousness involved in processing and examining numerous samples (Redecker *et al*. 2003). The use of molecular tools has emerged and developed as an option for identification, but there are a number of associated problems using molecular methods in trying to identify AM fungi. Heterogeneous nuclear material exists within individual AM fungi, and the reasons for this state remain yet to be definitively confirmed. Additionally, polymorphism occurs in typical marker genes adding to the complications of studying an organism that is already difficult to classify (Redecker *et al*. 2003).

Two important questions were raised by Landis *et al*. (2004) and also discussed by Sanders (2004): what is the best way to quantify AM fungal richness in a given ecosystem, the classical method that categorizes by spore morphotypes, a molecular method, or some combination of both? Also, is there a most relevant level on which to measure diversity when studying an organism which up to this point has had a continuously transitioning species concept? Satisfactory answers to these questions still elude us and will require a great deal of additional work.

Two published studies cited by Sanders (2004) highlight the problems associated with using spore morphotype as the sole method for speciation. The first involved

research investigating AM fungal diversity in understory vegetation under forest cover in England, and found that PCR amplification detected a *Glomus* spp. phylotype strongly, yet it was not found sporulating during long-term soil and plant sampling (Clapp *et al*. 1995). Spore morphology as a measure of diversity was also found lacking in a study by Rosendahl and Stukenbrock (2004), in which rDNA analysis of AM fungi on sand-dune-occupying grasses in Denmark was performed. Phylogenetic analysis revealed 11 groups of distinguishable AM fungi, but only four correlated with species identified by spore morphotype. In addition, those identified clades were only "patchily distributed in the grassland," suggesting that a significant presence and greater diversity could exist undetected in the form of the non-sporulating population (Rosendahl & Stukenbrock 2004, p. 3184). Sanders, in his review, speculates that using spore morphotype counts only give us the barest beginnings of the ecological story (2004).

While the complications related to morphotype characterization might seem daunting, they are at least a concrete standard providing guidance, irrespective of ecological significance. Molecular methods, despite years of attempts at development, are still in the germinal stage in terms of adequately answering key ecological questions conclusively. The main problem lies in the previously mentioned genetic variation which occurs in the phylum and even within individual spores (Pawlowska & Taylor 2004; Sanders 2004). Before delving into the problems associated with molecular characterization, I will discuss some of the published evidence regarding the nuclear condition and life history of AM fungi.

The vast majority of fungi exist vegetatively as haploids, and it has recently been advanced that at least one of the AM fungi, *Glomus intraradices*, is also haploid; the

ploidy of the larger group of AM fungi remains to be positively determined however, and may even be variable (Hijri & Sanders 2003). The DNA content of nuclei has been estimated for 11 AM fungi other than *G. intraradices*, and ranged from approximately 127 Mb to 1058 Mb (Hosny *et al*. 1998). Interestingly, both extremes came from morphotypes of the same genus, *Scutellospora*. In contrast, *G. intraradices*, the species used most extensively in molecular studies involving AM fungi, has a genome size estimated to be about 16.5 Mb (Hijri & Sanders 2003). Also, spores and hyphae have been determined to be multinucleate, with the number of nuclei in each spore ranging from 800 to 50,000 (Sanders 2002).

When Pawlowska and Taylor (2004) looked at the ploidy status of isolates of *Glomus etunicatum*, starkly different conclusions were derived compared to the study describing *G. intraradices* as an organism with a haploid genome. Their methods involved investigation of the transmission of polymorphic markers as well as examining the rDNA variant distribution among spore nuclei using PCR to clone the ITS1 region. That study looked at the variation within four field isolates of *G. etunicatum*, the results of which supported the case for the homokaryotic condition, and therefore the conclusion that high variability is most likely a product of polyploidy. One of the most intriguing aspects of this study is their additional investigation of a *G. intraradices* strain, the same morphotype described in the 2003 work of Hijri and Sanders as haploid, and using the same PCR amplification technique predicted it was polyploidic.

The AM fungi have defied almost every attempt at their culturing due to their obligately symbiotic nature, although developments in monoxenic culture using transformed root tissue have been successful (de la Providencia *et al*. 2004). Studies

involving fungal persistence in soil subsequent to spore germination generated results showing only an ephemeral existence in the soil in the absence of a host (Sanders 2002). There has been evidence, however, for longer-term survival in soil after initial germination, though with arrested development (Logi *et al*. 1998). Experimental evidence revealed that isolates of the morphotype *Glomus caledonium* germinated without a host, stopped growth after 15 days, and subsequently retracted its protoplasm from hyphal tips, and then isolated empty hyphae by forming septations. These AM fungal isolates retained infectivity for extended periods, with some remaining viable 6 months after initial germination (Logi *et al*. 1998).

The AM fungi have been assumed to exist as asexual organisms, although this has not yet been definitively proven, but is instead a result of the lack of any evidence for the occurrence of sexual processes. The origin of the high levels of genetic variation within individuals remains an object of speculation in the absence of sexual recombination, with anastomosis and subsequent nuclear exchange among different isolates remaining under investigation as a possibility. Anastomosis has been known to occur in AM fungi for almost 50 years (Godfrey 1957), and has been periodically observed and quantified since that time as occurring extensively in various AM fungi. The process of anastomosis has not been seen to occur between different morphotypes, nor to this date in isolates of the same morphotype from different geographical locations. This has led to speculation upon the existence, and nature of unidentified recognition factors that seem to exist, during *in vitro* studies, on a finer level than any current taxonomic system describes (de la Providencia *et al*. 2004; Sanders 2002). Both de la Providencia *et al*. (2004) and Giovannetti *et al*. (2001) have looked at the nature and levels of anastomosis in AM fungi

in isolates from familes of both described suborders. They produced contradictory data with regards to the number of anastomoses formed per unit length of hyphae; however, they agreed that Glomeraceae strains formed anastomoses between individual hyphae. In their work, de la Providencia *et al.* (2004) found Gigasporaceae strains formed bridges only upon the same hyphal strand. These studies, and another by Giovannetti *et al*. (2004), provided important information through the visualization of cytoplasmic movement and nuclear exchange between anastomosing hyphae in both symbiotic and presymbiotic growth (de la Providencia *et al*. 2004; Sanders 2002). This suggests a mechanism whereby resource and genetic sharing might exist, although not yet detected on a level higher than intra-isolate.

There are two widely accepted yet conceptually divergent schools of thought regarding the nuclear condition of AM fungi that can explain detected polymorphism in single spore rDNA and the genetic diversity in these apparently asexual multinucleate fungi. The first model involves a collection of coexisting genetically diverse nuclei existing within each fungal individual that is passed on to successive generations. Not every spore in the next generation would receive the same complement of nuclei with the same frequency as the parent generation. Anastomosis, in this heterokaryotic model, would result in the transfer of intact nuclei from any donating fungi. Karyogamy would not have to occur to change a phenotype, as the hundreds to thousands of nuclei present in AM fungal cells would gradually accumulate mutations during replication (Sanders 2002; Pawlowska & Taylor 2004). The other model argues for a genome that contains more variability within each nuclei, the homokaryotic model. This would involve an assemblage of similar nuclei with variance manifested through the presence of multiple

gene copies either on individual or, in the case of polyploidy, multiple chromosomes. Genetic diversity could arise from either errors in mitotic recombination and accumulation of other mutations, or through as yet undescribed genetic exchanges involving sexual recombination (Sanders 2002; Pawlowska & Taylor 2004).

Taking into account the complexity and our lack of understanding regarding the AM fungal genome, the use of molecular techniques for ecological studies of AM fungi can be perceived as tools under development. Identification in such research almost exclusively targets the SSU rDNA, therefore revealing an inherently limited picture of diversity. Considering the high degree of polymorphism of rDNA that exists in these fungi, and the possibility that considerable variation may even occur within individuals, interpretation of results can prove problematic. Reliance on a single genetic locus for identification may prove less popular as our understanding of the source of the incredible diversity within this organism increases.

Quantification using molecular techniques in ecological studies of arbuscular mycorrrhizae is no less difficult than identification. While quantitative techniques like Real-Time PCR (RT-PCR) have proved invaluable in the study of other organisms, it probably has the most potential for the study of AM fungi when the presence of only one isolate is being measured. So far, quantitative measures with this technology have been limited to root-associated AM fungi, where it shows some promise for specialized studies.

Alkan *et al.* (2004) describe the use of RT-PCR to achieve quantitative results in detecting *Glomus intraradices* in tomato and alfalfa. Their RT-PCR data was calibrated with microscopic observations, thus enabling them to subsequently screen large numbers

of samples with reduced labor. They suggested that a similar application of using calibration curves to allow for high-throughput colonization assays using RT-PCR. This method appears likely to be useful for rapid identification and quantification of individual fungi for which reliable, established primers exist, though less useful for quantification in field plants, where a complex of diverse AM fungi are present.

Ishii and Loynachan (2004) described two methods using nested-PCR, in which root segments infected with AM fungi previously stained by trypan blue were used in the extraction of DNA. The four greenhouse-grown plants inoculated with an isolate of *Glomus mosseae* acquired from INVAM were maize (*Zea mays*), alfalfa (*Medicago sativa*), soybean (*Glycine max*) and sudangrass (*Sorghum sudanense*). Both methods used bead beating to expedite sample processing. The use of this technique would allow correlation with microscopic observations of the same samples, and could confirm colonization levels if phylum-specific primers were available and quantitative PCR was used.

Further results from the use of molecular and other investigative tools will be presented in a subsequent section where ecological research is discussed.

Fatty Acid Methyl Ester Analysis

Fatty acids occur at a reasonably constant proportion of cell biomass as they exist largely as components of membrane lipids. Signature fatty acids are biomarkers that can differentiate groups of organisms within studied communities and can provide quantitative estimates of their microbial biomass. These compounds are represented in literature by the number of carbon atoms with the degree of their unsaturation separated from the carbon chain length by a colon. The position of a double bond is indicated next, following the ω symbol indicating the number of carbon atoms from the terminal methyl

end of the molecule to the first occurrence of unsaturation (Zelles 1999). The suffixes *c* and *t* stand for the *cis* and *trans* isomers of the unsaturation, respectively.

Fatty acid analysis using gas chromatography (GC) has proven to be a useful tool for structural analysis of soil and plant-associated microbial communities. Fatty acid methyl ester (FAME) analysis has been developed into an automated process by MIDI Inc. (Newark, Del.), whereby whole cell fatty acids are extracted, then analyzed using appropriate software. Analysis of whole cell fatty acids to study broad microbial communities that occur within varying agricultural management strategies has been well established (Cavigelli *et al*. 1995; Buyer & Drinkwater 1997). The use of FAME extraction analyzed using the MIDI system is widespread due to the ease of sample processing; however, careful interpretation of the results is necessary, since both cellular and extracellular lipids are detected with this technique. Extracellular lipids can be derived predominantly from organic matter as opposed to the active fraction of the targeted organism, and therefore, data should be considered accordingly (Zelles 1999).

The fatty acid 16:1ω5*c* has been confirmed as a biomarker for AM fungi in studies investigating colonization of roots (Graham *et al*. 1995), in estimations of fungal biomass in the soil (Olsson *et al*. 1995), and in work that requires quantification of the AM fungal presence associated with both substrates (Thygesen *et al*. 2004). The AM morphotypes within the Glomineae are reported to have higher concentrations of 16:1ω5*c* compared with some members of the Gigasporineae, within which it appears to negligible. Therefore, the use of this particular fatty acid will not detect comprehensively at the phylum level; however, it is capable of being used for detection and quantification of the AM fungal morphotypes that are considered the most important in agricultural

ecosystems. As a result, the use of 16:1ω5*c* has good potential for use in studies to reasonably quantify AM fungal abundance of members of the Glominaeae in roots and soil when finer levels of identification are not deemed necessary; either in lieu of using labor-intensive root, spore and hyphal quantification, or as a means of complementing classical techniques.

The fatty acids 18:1ω9c and 20:1ω9c have been identified as possible biomarkers for members of the suborder Gigasporineae, yielding useful results in studies on plant roots (Graham *et al*. 1995) and from soil (Madan *et al*. 2002), although their extensive use has yet to be undertaken. Perhaps complicating the matter of the use of fatty acids that appear to be produced in abundance other than 16:1ω5*c*, is the evidence that fatty acids seem to be differentially produced in the various AM fungal structures (Graham *et al*. 1995; Sakamoto *et al*. 2004). For example, some evidence shows that the external hyphae of *Gigaspora rosea* seems to lack 20:1ω9c, while it has been shown to be abundant within spores (Sakamoto *et al*. 2004).

Other Tools

There are additional techniques for the characterization of AM fungi in soil and roots, including variations of the methods listed previously. Those described above, however, are the most commonly found in research currently. Undoubtedly, as our understanding of these organisms and the technology used to study them advances, a more distinct picture of the ecological role of mycorrhizae will similarly develop. A good introductory overview of some of the other techniques used in AM fungal research, such as the quantification of extraradical hyphae, assays for functional activity, and identification using immunological methods is given in Millner and Wright (2002), and references contained therein.

Arbuscular Mycorrhizal Fungi in Agroecosystems

There has been an abundance of work published focusing on the existence, aggressiveness and state of functioning of AM fungi within ecosystems, including a substantial number of reviews and even meta-analyses of other researchers' results. In a review of the diversity of AM fungi in a large variety of managed and natural ecosystems, the morphotype numbers found among the various studies ranged from 3 to 46 per site (Douds & Millner, 1999). Other studies that were cited in that work have shown, as was discussed in the first chapter, that the choice of crop rotation (or lack thereof), tillage and fertilization strategies all can profoundly affect populations of AM fungi. A meta-analysis of data from independently published field studies was accomplished by Treseder (2004), looking at the impacts that fertilization and CO_2 enrichment have on the mycorrhizal state. The author found that across studies, mycorrhizal abundance was reduced by 15% due to N fertilization, 32% by P fertilization and increased 47% as a result of elevated CO_2 levels. While ectomycorrhizal fungi were included in the meta-analysis, 18 of 20 studies compared for impact of P fertilization focused on the AM fungi.

In the following sections, I will undertake a brief review of literature, and will concentrate on research that focuses on anthropogenically derived alterations to the environment under agricultural production and their impact on AM fungal biomass and infectivity. In addition, an emphasis will be placed on field conditions that were important in this study.

Phosphorus Fertilization.

If such a thing as common knowledge exists for AM fungi, it is related to their role in plant P nutrition and, to a lesser degree, the impact high P levels can have on the

organism. This does not mean accurate characterizations of these fungi occur in discourse as most, if not all, public awareness of mycorrhizae is due to the marketing of these fungi as an agricultural cure-all. Within the agricultural sciences it is widely accepted that they are capable of providing P to plants, especially, and perhaps exclusively, when soil availability of the nutrient is moderate to low (Schubert & Hayman 1986; Thingstrup *et al*. 1998), and that high levels of available soil P contribute to depressed colonization of roots by AM fungi (Daft & Nicholson 1969; Thingstrup *et al*. 1998).

The mechanism for increased absorption of soil P by mycorrhizal roots is likely twofold. The primary mechanism seems to be due to the increased soil exploration that roots with the association are capable of undertaking (Sanders & Tinker 1971; Koide 1991). There is also evidence that AM fungal hyphae may be able to excrete compounds increasing the solubility of the inorganic P (Joner *et al*. 2000; Tawaraya *et al*. 2006). The decrease in levels of the association when high levels of available soil P are present is apparently more a result of the P concentration in the plant than it is related to actual soil levels (Koide & Li 1990; Koide 1991), although the two are obviously connected. Additionally, sporulation has been shown to be related to a threshold of AM fungal root biomass and possibly plant P concentration (Douds & Schenk 1990), providing an explanation for why spore numbers are dramatically reduced when available soil P is high. This suppression of the mycorrhizal condition has been perceived as a self-regulatory process, whereby the plant reduces C allocation to the fungi when it is more P self-sufficient (Graham *et al*. 1981). The mechanisms through which plants inhibit infection by AM fungi are probably quite varied, considering the length of time the

organisms have had to coevolve and the diversity of the many plant and fungal genotypes involved. There is evidence, though, that both biochemical means and anatomical structures can be involved (Brundrett & Kendrick 1990; Fortin *et al*. 2002). The continuance of the mycorrhizal relationship when soil P is readily available can lead to a reduction in crop yield, through the misapplied C investment without a commensurate benefit in terms of plant P acquisition (Graham 2000; Kahiluoto *et al*. 2001; Ryan & Graham, 2002).

Soil Disturbance

The external hyphal network of AM fungi in soil is an important inoculum source for plant roots, so it comes as no surprise that disruption of soil through agricultural practices such as primary tillage and cultivation can reduce the overall biomass and root colonization levels. This has been shown in many studies with similar results; only a few will be mentioned.

A study by Drijber *et al*. (2000) considered changes in the broad soil microbial community in moldboard plowed, reduced tillage, no-tilled and sod plots. Using FAME analysis, AM fungal biomass was determined to have decreased significantly in the more heavily disturbed treatments compared to the no-till and sod plots. Jasper *et al*. (1991) investigated the infection potential of annual pasture, heathland and forest soil sown with clover plants after disturbance. There was a significant decrease in the AM fungal infection potential in heathland and forest soils after soil mixing, which led them to speculate that disturbance is probably most important where the initial propagule number is already low, a condition that was overcome in the pasture soil. More recently, an on-going tillage-rotation experiment in its sixth year in Chile compared different levels of soil disturbance on a variety of AM fungal-related parameters (Borie *et al*. 2006). The

less disturbed treatments had higher spore numbers and greater levels of root colonization, as well as higher total content of the AM fungal-derived glycoprotein, glomalin, when compared the more highly disturbed treatments.

Pesticides

One might anticipate that the use of pesticides, particularly broad range biocides and fungicides, would have a negative effect on mycorrhizal survival and functioning. While this is generally the case, the impact of many of these agrichemicals on AM fungal communities is often slight and usually appears to be only ephemeral in nature, and it can be difficult to predict the response to an individual pesticide. The range of responses has varied from deleterious to negligible to stimulatory (Koide & Mosse 2004). It has, in fact, proven difficult to control AM fungi with chemical methods for research purposes, a situation that has made the development of non-mycorrhizal mutants all the more welcome.

One of the fungicides considered most effective, benomyl, gives only partial control and requires frequent reapplication. In a study looking at the P nutrition of the perennial common bluebell (*Hyacinthoides non-scripta*), individual colonies were grown in 28-L cubic blocks of soil (Merryweather & Fitter, 1996). The colonies receiving fungicide were immersed monthly in a solution of benomyl (50% w.p.) for control of AM fungi. While a significant reduction occurred, colonization was still measurable, reaching approximately 40% at the final assessment.

In a recent work, six soil fumigants were investigated for their effects on the structure and functionality of the microbial community, including AM fungi, using FAME profile analysis (Klose *et al*. 2006). The sandy loam soil used in the microcosm study was fumigated for 24 hours, and then subjected to FAME extraction periodically

over the next 90 days. Four of the six fumigants had a significant depressive effect on AM fungi biomass for the duration of the experiment, while the other two did not differ significantly post-fumigation from control soil; neither methyl bromide with chloropicrin, nor methyl isothiocyanate had a significant impact. Although the authors imply a lack of community recovery in the case of the four effective fumigants, the absence of change in the AM fungal biomarker during the course of this study is not surprising, considering that no host was available for colonization in the microcosm.

A succinct review of the impact that some pesticides have on AM fungal populations is contained in a section from a publication by Gosling *et al*. (2006), while a more comprehensive, albeit dated, treatment is undertaken in Trappe *et al*. (1984).

Flooding

There has been less information published regarding the mycorrhizal status of agricultural crops during flooded conditions than there has of wetland plants. It has been demonstrated repeatedly that AM fungal associations can persist and benefit rice in wetland production (Solaiman & Hirata 1997; Solaiman & Hirata 1998), and it is well established that AM fungi colonize many wetland plants. While they are known to be aerobic organisms, there is an apparent means through which at least some taxa of these fungi are capable of surviving and functioning in flooded conditions. However, in native wetland plants, colonization levels have been reported to slow during periods of flooding, with substantial increases in mycorrhizal development during any extended periods between flooding (Miller 2000; Ray & Inouye 2006). Even when soils have been submerged for long stretches, resulting in little development of AM fungal colonization within roots, levels rebound in field plants and infection potential studies after drawdown of water levels (Miller 2000; Ray & Inouye 2006).

In a study of post flood syndrome in the Midwest and Great Plains, Ellis (1998) examined maize and soybean plants that were grown in fields that had been flooded for long periods the previous year. Plants were sampled three times during the growing season. Additionally, a greenhouse experiment was undertaken in which maize plants were grown in sterilized soil that was subsequently inoculated with *Glomus intraradices* spores and then flooded for either 0, 1 or 3 weeks. While flooded fields initially had low infection potentials the following year, colonization levels steadily increased as the season progressed. There was no significant difference among the levels of colonization in the greenhouse study, and overall levels were actually highest in the two flooded treatments. The author attributed the low levels of infection in fields during seasons subsequent to flooding episodes not to the extended periods of saturated soil, but rather to the absence of host tissue after the near complete crop death.

These and other (Sah *et al*. 2006) studies indicate that flooding conditions do not preclude the presence of a functional population of AM fungi. Mechanisms for coping with both long-term saturated conditions in wetlands and short-term flooding in agricultural fields and other land exist, and infective propagules persist after wet conditions subside. The reduced colonization during times of flooding is likely due to severely stressed plant root systems, which is relieved when drier conditions prevail and normal root growth can proceed.

Organic, Conventional, and Grassland Farming Management Strategies

In distinguishing among studies that focus on individual agricultural practices, it is easy to neglect research of a broader scope that compares pertinent management strategies. Of particular interest here is research that focuses on organic and grassland (or pasture) agriculture. Both of these represent types of farming that have been considered

to be relatively amenable to the encouragement of AM fungal populations in terms of biomass, functioning and diversity.

Organic agriculture is not a static management style and farmers incorporate a tremendous diversity of practices with yield goals, type of crop, certifying body, soil type and geographic location among some of the more important variables involved in deciding practices. Farming strategies in organic agriculture that are thought to have the potential to minimize disruption of AM fungal populations include crop rotations and cover cropping, the absence of highly soluble chemical fertilizers, and reduced reliance on pesticides with use restricted to botanically derived or mined sources. While not exclusive of either organic or conventional management, but rather an integral part of both in many instances, grassland or forage agriculture differs from agronomic or horticultural row crop production. Agroecosystems dedicated to or integrating long-term stands of native grasses or forage crops are generally distinguished by a lower relative agrichemical input, reduced traffic and soil disturbance and increased levels of plant-derived organic compounds in the soil when compared to row crops. It is important to bear in mind that there can be considerable overlap among farming management strategies and that what differentiates one from the other, in terms of what specifically influences AM fungal populations in a given work, may not be readily deduced.

Oehl *et al*. (2004) found 35 morphotypes of AM fungi in one field site in Switzerland where the systems trial had been in place for longer than 22 years. This large number of identified taxa was a product of their trapping techniques, in which cultures were kept for extended periods of time under different trap plant species. Morphotype richness, spore abundance and infection potential were all higher in two

organically managed systems when compared to two conventional systems. They attributed these differences, as well as the significantly higher colonization levels in bio-dynamic plots and the development of different morphotype populations in the organic treatments, to qualitative and quantitative variation in fertilizers.

Thirty-seven morphotypes of AM fungi were collected from a single one-hectare field under mixed grasses and forbs in North Carolina (Bever *et al*. 2001). The method used to extract so many species from a single field was a rigorous variation of trapping techniques; long, successive trapping periods, multiple trap plant species and other changes in environmental conditions were used in order to simulate the year-round field conditions from which the samples were taken. The authors speculated that when a mycorrhizal complex exists, as opposed to individual strains associating with one plant species, the host specificity of AM fungi is high with respect to fungal growth. That is, on certain plants, host specificity is manifested through more or less dominant AM fungal types developing. Similarly, the study lent credence to the idea that AM fungi exert an influence on plant community structure, but only in particular plant-fungal combinations.

A central philosophy of organic farming is that management practices should strive to maximize biological functioning and diversity in order to maintain the health of the broader ecosystem, and AM fungi are perceived as key players in these systems (Oehl *et al*. 2004; Harrier & Watson 2004). Most studies comparing organic and conventional management systems have shown higher relative colonization levels (Douds *et al*. 1993; Ryan *et al*. 2000), as well as spore number and morphotype diversity (Douds *et al*. 1993; Oehl *et al*. 2004) in organic farming. This is typically interpreted as a product of the lower levels of slowly available soil P that comes from the use of fertilizers acceptable in

organic agriculture (Ryan & Graham 2002). Where nutrient applications are in excess of crop needs and build to high levels in the soil, and when tillage is a frequent occurrence for seedbed preparation and cultivation purposes, this ideal of enhancing mycorrhizal relationships may be no more likely than in other high-input systems. Communities of AM fungi in the extensive root network in grassland and forage agriculture, on the other hand, can be expected to develop a considerable external hyphal network, undisturbed by tillage (except during periods of establishment) and with reduced occurrence of inhibition due to excessive nutrient levels.

Other Fungal Root Endophytes Known to Occur in Herbaceous Plant Roots

As non-AM fungal root endophytes represent a diverse range of fungal taxa, and probably exist within most plant species around the world, it is speculated that they contribute substantially to plant health and soil ecosystem functioning (Sylvia & Chellemi 2001; Sieber 2002). However, as mentioned in the introduction, the ecological roles of these endophytes can not be ascribed as being clear cut or uniform. In order to begin to understand them, a great deal of further work needs to be undertaken to ascertain both the phylogenetic status of many of these organisms and their host ranges (Jumpponen & Trappe 1998; Sieber 2002). Some of the non-AM fungal endophytes identified as commonly occurring in herbaceous plant roots include *Philophora* spp. and their teleomorphs, as well as species of *Fusarium*, *Microdochium*, *Trichoderma*, and the loosely described group of anamorphic fungi currently known as DSE's (Sieber *et al*. 1988; Skipp & Christensen 1989; Jumpponen 2001; Sylvia & Chellemi 2001; Sieber 2002). *Phialophora* spp. are some of the few endophytes commonly inhabiting herbaceous plants that have been identified and classified as belonging in that group.

The DSE are an informal grouping that comprises Melin's (Melin 1922; Wang & Wilcox 1985; Jumpponen & Trappe 1998) form taxon *Mycelium radicis atrovirens* (MRA) and other root-occupying fungi that meet a morphological standard and are apparently non-deleterious to the host plant. Melin described a fungus isolated from *Pinus sylvestris* and *Picea abies* roots that did not sporulate, which he believed was non-mycorrhizal and possibly harmful to the plant. Further, it grew on media, forming "grey air mycelium of tenuous hyphae" and produced a "dark olive-green pigment" when cultured (Melin 1922, p. 256; Wang & Wilcox 1985). The term MRA has continued to be used by some researchers for sterile fungi with darkly pigmented, septate hyphae found both growing in root cells and isolated from the soil (Wang & Wilcox 1985; Sieber *et al*. 1988; Jumpponen & Trappe 1998).

In a study examining mycorrhizal colonization of plant communities at different altitudes in Austria, Read and Haselwandter (1981) noted the existence of these endophytic fungi throughout the range of their survey and referred to the group as DS fungi. Thereafter, the term DSE has come into favor for dark septate endophytes and is used most frequently, although the exact meaning and appropriateness of this term is sometimes questioned. A commonly used description for DSE includes those root-occupying, biotrophic, mitosporic fungi with septate, melanized hyphae that typically also produce melanized microsclerotia within the root (Treu *et al*. 1996; Jumpponen & Trappe 1998; Sieber 2002). The search for a universally accepted name, or perhaps the dissolution of the need for a group, should come as taxonomic affinities are determined. It is interesting to take note of Barrow's (2003) suggested refining of the designation DSE to systemic endophytic fungi (SEF). His and others' work take note of the difficulty

in quantifying colonization by these endophytes due to their extensive network of non-staining, hyaline hyphae (Yu *et al*. 2001; Barrow 2003). In the present study a great deal of hyaline hyphae was encountered while observing samples; the abundance of hyaline hyphae with lesser amounts of dark, septate hyphae occurring, as well as the existence of other poorly defined structures made grouping into classes difficult. Therefore, these non-AM fungal endophytes were not otherwise designated in this study, except where species or other taxonomic units could be assigned. It is unclear whether changing the designation to SEF would truly be a refinement or if there is instead an overlap of two or more groups of endophytes. Additionally, it would seem such a change would likely open the door to greatly expanding the number of fungi included, potentially leading to more confusion.

CHAPTER 3
MATERIALS AND METHODS

Root and soil sampling. Soil samples were collected twice for use in infection potential and trap culture studies. Soil and root sampling for this study took place in years four and five of the Header Canal cropping systems project, and occurred in December of 2003 and 2004. While plant and soil sampling were completed on the same day in both years, plots in year five were planted 36 days later than in year four (October 7, 2004 compared to September 2, 2003) due to the severe weather. Four beds of tomato plants were in each half plot under production in 2003, but the hurricanes of 2004 destroyed a substantial portion of the beds that had been formed in September; only two beds were established in each plot half when tomatoes were planted in October.

To approximate the rhizosphere of the plant, for each sample that was taken, the probe entered at the base of a plant and was angled slightly to capture a substantial portion of the root system along with the soil. Soil cores were taken from beneath plants in the middle beds and from within the middle 100 plants in each row. Thirty cores from each plot were taken to a depth of about 20 cm, with 15 samples collected walking East to West in one center row, and the second 15 samples gathered walking West to East in the other center row. The cores from each plot were combined and homogenized in plastic bags, with the composite sample volume being approximately 2 L. Each of the five tomato production treatments was replicated six times, producing a total of 30 samples from the portion of the plots then used for tomato production in year four, and 60 samples in year five.

Soil sampling was also conducted within the transition areas in the organic, bahia pasture, weed fallow and disk fallow treatments in year four. Thirty cores were taken in each plot from the area parallel to the portion of the plots from which the tomato production soil samples were taken. Cores were taken from beneath the cover crop sunn hemp plants in the organic system, and from the bahia sod in the bahia system. Ragweed (*Ambrosia artemisiifolia*) was one of the most abundant and consistently present weeds across the weed fallow system plots, therefore, soil samples were taken to approximate this plant's rhizosphere in that treatment. Cores were also taken from the disk fallow plots in the areas being actively disked. There were 24 total composite samples taken from these transitional portions of the systems (four treatments x six replications).

In year five, all previously transitioning portions of the plots were put into production. However, in the bahia pasture and disk fallow treatments, a considerable portion of the plots were under sod or being actively disked, respectively. The section between each half plot remained free of tomato production throughout the experiment, and in these two systems this area approximated the continuance of the rotation. Therefore, soil samples were taken from this middle portion of the plots in these two systems using the same method described for sampling the transitional areas from the previous year, generating a total of 12 composite samples.

Root samples were taken from all plots with tomato plants and treatment plots with cover crops and were used to assess colonization by AM fungi and other fungal endophytes. Four tomato plants from each plot were dug from the center 100 plants of the middle beds. Loose soil was removed by gently shaking roots prior to cutting the entire root system from the stem. A total of 120 tomato plants were removed in year four

(four plants x 30 plots), and 240 plants were collected in year five (both halves of plots, eight plants x 30 plots). In year four, root samples were also taken from cover crops from the plots with sunn hemp (organic), ragweed (weed fallow) and bahia plants. The bahia root samples were extracted from the paths between the tomato rows and not the tomato sod. The root systems in both year four and five were placed in paper bags and dried at 70 ºC. Fine roots were subsampled for clearing and staining, roots were dried and subsample weights were between 0.05 and 0.07 g.

Infection potential study sample preparation. Infection potential studies provide a method to evaluate the relative amounts of viable inoculum by growing a mycotrophic crop that forms abundant roots quickly in either diluted (with sterilized sand), or undiluted soil (as in this study). Quite different from full-season studies, infection potential results are typically timed to provide data regarding levels of primary infection on host crop roots. Harvested roots are examined for either the percentage colonization by AM fungi or total mycorrhizal root length as described in the section on quantification of fungal colonization in roots using microscopy. In preparation for this and other evaluations, each of the composite soil samples from individual plots was sieved to remove structural roots and to further homogenize soil. Fibrous roots were cut into small pieces (< 2 cm) and mixed with the soil. A portion of soil from each plot was removed for fertility and other analyses. The remaining soil and roots from individual plots were divided in half, with one half designated for the spore trap culture described below, and the rest used for the infection potential study. For the infection potential test, soil was distributed at a volume of 125 mL among five to seven 150-mL conetainers based upon the amount of soil in the initial composite sample. Two germinated maize seeds were

planted and watered into each tube, then later thinned, selecting the healthiest looking plant. Infection potential plants were not fertilized, but were watered as necessary. Trays holding the conetainers were randomly rearranged weekly. Maize plants were harvested approximately 30 to 40 days after planting, at the five-leaf stage of development. Soil was gently washed from the roots prior to their separation from the plant, and root systems were then each placed in paper bags and dried at 70 ºC. Dried roots were subsampled for clearing and staining, with subsample weight typically ranging from 0.03 to 0.05 g.

Trap crop sample preparation. Acquisition of fresh spores for morphotype identification through culturing diluted field soil or previously extracted spores using live plant roots is highly desirable, if not necessary, as the rapid degradation in quality of field collected spores can render them useless (Millner & Wright 2002; Redecker *et al*. 2003). The portion of soil not used in the infection potential study was used to grow trap plants for subsequent isolation of AM fungal spores. The soil designated for the trap culturing in each of the composite samples was divided between two 450-mL tubes. Five sudangrass (*Sorghum sudanense*) seeds were planted and then watered into the soil in each tube, later thinned to three plants. Trays holding the conetainers were randomly rearranged biweekly through the first 6 months of growth, and were fertilized weekly using 35 mL of a Hoagland solution with a very low concentration of P (0.25 mM) after nutrient deficiency symptoms first developed. The plants were allowed to naturally senesce, and were harvested approximately seven months after planting.

Trap culture processing, spore extraction and observation. The method used to extract spores from trap cultures was an adaptation of the process mentioned in the

previous chapter (Gerdemann 1955; Brundrett *et al*. 1996). After the sudangrass plants had senesced, the aboveground biomass was removed and the soil within each conetainer was allowed to air dry. After removal from the tube, the soil and root system from each pot was broken down, with roots cut into small (< 2 cm) fragments. A representative 100 cm^3 of soil and root mixture were suspended in 500 mL of DI water for 5 minutes with agitation. The soil-root suspension was then poured over a set of three sieves. The sieve openings, top to bottom were 2 mm, 1 mm, and 38 µm. The material on the top sieve was discarded, while the contents in the 1mm sieve were gently washed over the bottom sieve; the material remaining after rinsing was thrown away. The material in the bottom sieve was then distributed equally among six 50-mL centrifugation vials followed by the addition of equal parts DI water and 50% sucrose (w/v). The sucrose was injected into the soil and associated material at the bottom of each tube after adding water, using a syringe with a piece of tubing attached, creating two layers with a clear interface. The six tubes were put in an International model HN centrifuge and spun for 5 minutes at approximately 771 x g. After centrifugation, the more dense soil was pelleted at the bottom of each tube, while the remaining root tissue and other organic debris became suspended at the top. From each of the six tubes, the 20-mL of solution surrounding the interface between sucrose and DI water was removed individually using a syringe, taking equal proportions from above and below the interface to maximize spore recovery. This collected solution was drained from the spores in suspension using vacuum filtration. The filtration system consisted of 55-mm circles of Whatman 50 hardened filter paper within a Büchner funnel and flask attached to a KNF Laboport vacuum pump. During filtration, after spores from all six tubes were extracted, the syringe carrying the solution

with spores was rinsed several times by loading and ejecting DI water from the syringe onto the filter paper. This served several purposes; it was a method of clearing the contents of the syringe for the next sample, it ensured that spores remaining in the syringe after extraction were recovered, and it washed the sucrose from the spores and the filter paper, which improved the subsequent handling of spores.

The filter paper was then moved to a Petri dish and observed under an Olympus SZH10 stereo microscope for enumeration and identification. Enumeration was undertaken using year five trap cultures and was based on the method used by INVAM (2006b). The area of the ocular field at 4x magnification was determined to be 23.8 mm^2, and the area of the 55-mm diameter filter paper was 2375.8 mm^2, providing 100 possible fields of view for each spore extraction. The average number of spores counted in 40 randomly chosen fields was determined, yielding an approximation of the spores / cm^3 of soil (average number of spores found x total possible fields / 100 cm^3 of soil from which spores were extracted).

Spore morphotypes were segregated after enumeration using the sharpened end of a thin wooden dowel. Spores were transferred to a slide, and observed after covering them with the several drops of a semi-permanent mounting agent consisting of polyvinyl-lacto-glycerol (PVLG) with Melzers reagent (1:1 v/v). Spore morphotypes were observed and identified to the extent possible using an Olympus BX51 compound microscope with brightfield and differential interference contrast (DIC) optics. Joe Morton, the curator of INVAM at West Virginia University, provided assistance with morphotype delineations, and the INVAM collection descriptions were used for classification.

Clearing and staining roots. Roots were cleared and stained for examination of colonization levels both in the IP study and for field roots. The procedure used for clearing and staining was a modified version of that described by Brundett *et al*. (1996). Fibrous roots were subsampled and placed into scintillation vials. Roots were covered with 10% KOH (w/v) and placed in an oven at 95 ºC until cellular contents and pigments were largely removed, and before significant damage occurred to the root system. This time period varied among the different plant roots. Maize roots from the IP study were heated for 15 minutes, while roots of tomato, sunn hemp, and rag weed were cleared for 25 minutes, and bahia roots were removed after 30 minutes of heating.

After rinsing several times with tap water, all roots, with the exception of those from maize plants, received an additional bleaching step. An acidified bleach solution was prepared using diluted Ultra bleach (1.7 % NaOCl) and 1 mL of diluted HCL per 100 mL of bleach. The bleach was added until the sample was covered, and then the sample was swirled in the solution for up to 30 seconds. Total bleaching time varied among samples with additional applications initiated until the roots became noticeably lighter in color; however, bleaching never exceeded 90 seconds. Roots were rinsed repeatedly after bleaching, then soaked in water for 5 minutes before staining.

Staining was with 0.05% (w/v) trypan blue in lacto-glycerol (1:2:2 lactic acid - glycerol - water). Maize and tomato samples were covered with stain and placed in a 95 ºC oven for 10 minutes, while the rest of the root samples were heated with stain for 12 minutes. Stained roots were rinsed and then destained in lacto-glycerol for at least 24 hours before examination.

Determination of % colonized root-length. Colonization levels were determined using the line-intersect method described in the section on quantification of fungal colonization in roots using microscopy, and derived from Giovannetti and Mosse (1980). Roots from both the infection potential study and field plants were observed using this method. Examination was with an Olympus SZH10 stereo microscope using dark-field illumination. Intersections were scored for presence of AM fungi and other endophytes. When structures of questionable origin were encountered, root sections were removed from the Petri dish, mounted on a slide, and observed under an Olympus BX51 compound microscope using brightfield and differential interference contrast (DIC) optics.

Soil fertility and fatty acid analysis. Soil from each plot was sent to Waters Agricultural Laboratories in Camille, GA for fertility analysis in both year four and five. The Mehlich-1 extraction procedure was used to determine levels of available P, as well as other nutrients.

Subsamples from composite soil and tomato roots were sent to John Larsen at the Danish Institute of Agricultural Sciences, for FAME analysis in his lab. Approximately 0.2 g of tomato root tissue from each plot, a portion of fine roots from each of the four tomato plants sampled, was stored in 2% (v/v) acetic acid, and a 10-g subsample of soil from each plot composite was subjected to lyophilization for preservation during transport to Denmark. Whole cell fatty acids were extracted and methyl esters separated using gas chromatography and subsequently identified using the automated procedure developed by MIDI Inc. (Newark, DE).

Statistical analysis. One-way analysis of variance was used to test for differences among treatments with regards to colonization levels. Means determined to be significantly different ($P < 0.05$) were separated using Tukey's HSD test. Data were transformed where necessary to achieve normal distribution. Pearson's correlations were used to examine relationships between FAME results from soil and roots and colonization levels in the IP and field plants, respectively, as well as interactions among endophytes and between endophytes and edaphic factors. Statistical analyses were performed using SAS release 8.02 for Windows.

CHAPTER 4
RESULTS AND DISCUSSION

Agricultural and Environmental Influences Impacting Edaphic Conditions at the Header Canal Study Site

Over the production history of this field, including the period during this research project, multiple applications of fertilizer have resulted in extremely high P levels (Table 4-1). The relatively higher levels of this nutrient that occur in the organic treatment are due to the annual addition of broiler litter in these plots, at the rate of approximately 22,400 kg ha^{-1}, which should result in about 800 kg of applied P_2O_5 ha^{-1}, or 350 kg of applied P ha^{-1}. The increase of soil P levels by 600 kg ha^{-1} between years four and five in the organic plots as revealed by soil testing indicate higher P concentration in the manure, greater relative mineralization leading up to the time of sampling, or a combination of both of these factors. The over-application of P compared to plant need when using broiler litter as a nutrient source is a common problem, resulting from the low N:P ratio of the material, because fertilizer rates are often determined based on the N needs of a crop. The plots in other treatments, during seasons of tomato production, received a comparatively conservative 56 kg ha^{-1} of applied P_2O_5. As discussed in the literature review, high levels of available P in the soil result in plant P sufficiency with accompanying higher tissue concentrations and correlative lower levels of plant-associated AM fungal biomass and spore production. All treatments had very high levels of soil P, and this was exacerbated by applications that exceeded plant uptake, with the most extreme example occurring in the organic plots.

Table 4-1. Results of soil analyses from year four and five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL.

Year	Treatment	%OM	P ($kg\ ha^{-1}$)	Bulk density ($g\ cm^{-3}$) [a]	pH	S ($kg\ ha^{-1}$)	Mn ($kg\ ha^{-1}$)
4	Bahia pasture	0.75 (± 0.04)	795 (± 55)	Not measured	7.14 (± 0.09)	77.90 (± 7.86)	23.15 (± 1.18)
	Conventional	0.60 (± 0.04)	821 (± 51)	Not measured	7.60 (± 0.16)	67.95 (± 18.35)	23.52 (± 2.09)
	Disk fallow	0.53 (± 0.03)	819 (± 58)	Not measured	7.70 (± 0.07)	44.20 (± 5.58)	25.59 (± 1.08)
	Organic	1.70 (± 0.14)	1220 (± 90)	Not measured	6.88 (± 0.07)	78.03 (± 11.40)	37.43 (± 2.61)
	Weed fallow	0.82 (± 0.02)	884 (± 51)	Not measured	7.40 (± 0.06)	58.24 (± 6.12)	26.76 (± 1.43)
5	Bahia pasture	0.73 (± 0.05)	794 (± 36)	1.43 (± 0.02)	7.04 (± 0.10)	24.22 (± 7.19)	39.48 (± 2.28)
	Conventional	0.55 (± 0.03)	849 (± 35)	1.55 (± 0.02)	7.47 (± 0.10)	42.28 (± 9.02)	11.29 (± 2.96)
	Disk fallow	0.44 (± 0.02)	899 (± 31)	1.55 (± 0.02)	7.65 (± 0.04)	27.60 (± 4.83)	40.25 (± 1.22)
	Organic	2.36 (± 0.25)	1831 (± 80)	1.24 (± 0.02)	7.10 (± 0.03)	13.64 (± 10.28)	76.26 (± 2.68)
	Weed fallow	0.69 (± 0.02)	859 (± 52)	1.47 (± 0.02)	7.34 (± 0.07)	17.45 (± 8.65)	47.88 (± 2.79)

Values are treatment means ± SE.
[a] Bulk density was not determined in year four plots.

Organic matter levels were highest in the organic plots (Table 4-1), due to the broadcast of urban plant debris at an approximate rate of 70,000 kg ha^{-1}, spread concurrently with the broiler litter. The bahia pasture and weed fallow treatments both had long-term stands of vegetation, resulting in substantial carbon input through root-derived deposition, and therefore had higher organic matter levels than either the conventional or disk fallow plots. As would be expected on a field with a homogeneous soil type, the addition of ample organic matter had the corresponding effect of decreasing soil bulk density in individual treatments, as was determined through soil testing in year five. While bulk densities were high for a plow layer, they were not uncharacteristically so for sandy soils.

Additions of organic matter are known to have a number of positive effects on soil structure. Water-stable aggregates are formed as organic matter is added from external inputs or directly from plant and microbial deposition and decomposition. Many physical, chemical and biological factors interact in forming soil aggregates, including a substantial contribution derived from the activity of AM fungi (Rillig & Mummey 2006). The benefits that organic matter lend to the development and maintenance of soil structure were evident after two hurricanes swept over the experimental farm site in 2004. While most treatment plots had their raised beds destroyed (Figure 4-1A), many of the organic plots largely retained their bed structure (Figure 4-1B), despite substantial flooding.

Soil fertility test results additionally indicated that flooding conditions persisted long enough to have a substantial impact on nutrient availability, revealing the extent of

Figure 4-1. Header Canal experimental farm site plots after the hurricanes of 2004. Photo taken prior to final tomato transplantation in year five of a 5-year cropping systems study in Fort Pierce, FL. A) Remnants of raised beds for tomato production in conventional system. B) Raised beds still largely intact in organic plots.

waterlogging. Manganese (Mn) is extremely soluble at the low redox potentials that occur in soil with flooding (Tisdale *et al*. 1993a); this effect is evident at the farm site when comparing extractable Mn^{2+} between year four, a season with normal rainfall, and year five, a season with abnormally high rainfall. The status of sulfur (S) was also altered in the field between the two seasons. The form of S absorbed by roots is SO_4^{2-}, and is easily leached from soils. Additionally, when soil moisture is high, mineralization from organic matter and oxidation of inorganic S is decreased (Tisdale *et al*. 1993b). Reduced amounts of S were extracted in treatments during year five as a result of these processes.

Colonization of Roots of Tomato and Cover Crops by Arbuscular Mycorrhizal Fungi and Other Fungal Endophytes

Arbuscular Mycorrhizal Fungi in Tomato and Cover Crop Roots

Tomato root colonization by AM fungi was very low to moderately low across treatments, with significantly higher levels occurring in the bahia pasture plot in both years (Table 4-2; Figure 4-2). The organic system had significantly lower levels of colonization compared to all other treatments in year five. The colonization levels observed in year five tomato roots were corroborated by FAME analysis (Figure 4-3). There was a strong correlation between colonization levels and 16:1ω5*c* content measured from root samples on the plot level.

The overall pattern and levels of colonization in tomatoes among treatments did not vary much between years (Table 4-2). There are two main distinguishing characteristics that separate the bahia treatment, which had the highest levels of colonization, from the others: the bahia pasture plots had neither extensive soil mixing nor raised bed formation prior to tomato production. Instead, maintenance of a highly mycotrophic sod was preserved throughout the 5-year experiment, except where narrow strips were tilled for

Table 4-2. Field tomato root colonization by arbuscular mycorrhizal (AM) fungi and other fungal root endophytes from year four and five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL.

Year	Orientation	Treatment	AM fungal % colonization [a]	Other fungal root endophyte % colonization [ab]	*M. bolleyi* % colonization [acd]
4	South	Bahia pasture	24.1 (± 3.5)	Was not measured	Was not measured
		Conventional	8.2 (± 3.4)		
		Disk fallow	8.8 (± 1.8)		
		Organic	8.2 (± 2.3)		
		Weed fallow	14.4 (± 3.5)		
P values					
Treatment			0.0039		
5	North	Bahia pasture	32.6 (± 4.6)	52.1 (± 2.0)	0.6 (± 0.4)
		Conventional	9.2 (±1.6)	39.6 (± 1.1)	0.1 (±0.1)
		Disk fallow	9.9 (±2.5)	47.9 (±5.7)	ND
		Organic	6.7 (±1.7)	44 (± 1.4)	12.8 (± 2.2)
		Weed fallow	12.7 (± 1.8)	37.9 (± 5.0)	ND
	South	Bahia pasture	30.3 (± 2.6)	58.4 (± 4.8)	0.5 (± 0.4)
		Conventional	15.2 (± 2.4)	47.8 (±5.4)	ND
		Disk fallow	11.7 (± 1.4)	48.8 (± 6.3)	ND
		Organic	4.3 (± 0.9)	37.5 (±1.6)	13.0 (±3.1)
		Weed fallow	13.0 (± 2.0)	36.2 (±3.3)	ND
P values					
Treatment			<0.0001	0.0083	<0.0001
Orientation			0.4011	0.5263	0.7934
Interaction			0.1541	0.2828	0.9856

[a]Proportion data were transformed to the square root of acrsine prior to performing ANOVA; values in table are actual mean proportions of root colonization of treatments ± SE.

[b] Colonization levels of non –arbuscular mycorrhizal fungal root endophytes were not measured in year four tomato roots.

[c] Colonization levels of *Microdochium bolleyi* were not measured in year four roots.

[d] ND indicates treatments in year five where *Microdochium bolleyi* was not detected.

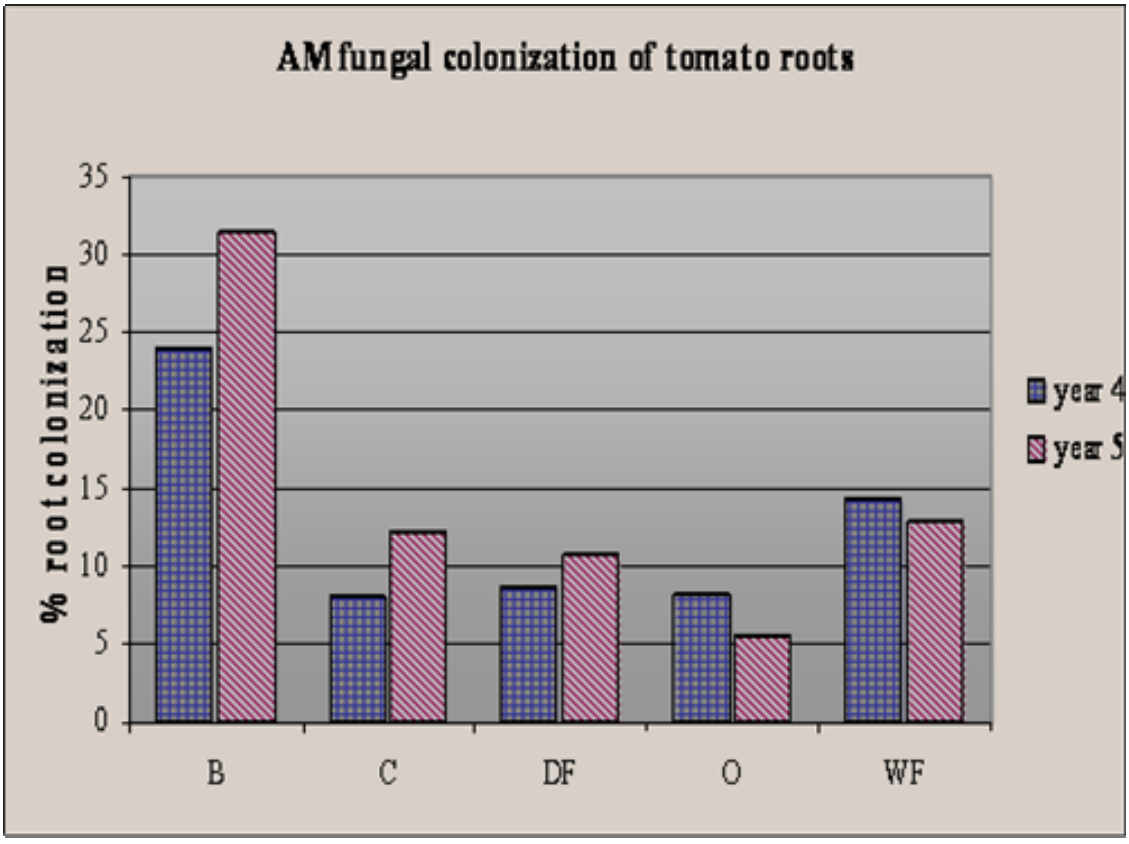

Figure 4-2. Field tomato root colonization by arbuscular mycorrhiza (AM) fungi in years four and five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL. The treatments in the study were bahia pasture (B), conventional (C), disk fallow (DF), organic (O), and weed fallow (WF).

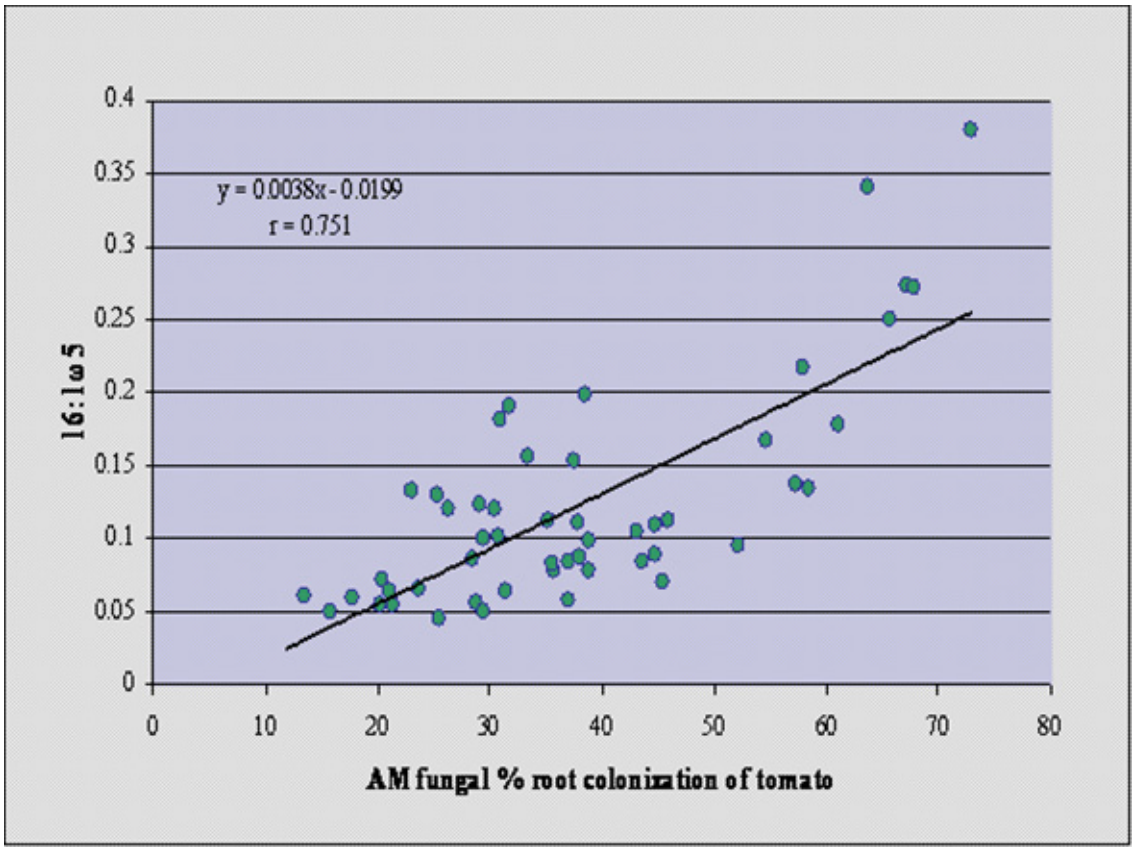

Figure 4-3. Correlation with least squares regression of the fatty acid 16:1ω5 content in tomato roots to the observed proportion of colonization of tomato roots in field plots by arbuscular mycorrhizal (AM) fungi. Results are from year five of a 5-year cropping study at the Header Canal farm site in Fort Pierce, FL. Data were normalized by transformation to square roots.

tomato transplantation (Chellemi *et al.* 1999) (Figure 4-4). The minimal levels of disturbance creating conditions to sustain preservation of the external hyphal network associated with bahia roots provided transplants with a directly infective, thoroughly distributed and effective source of inoculum.

The other treatments all had soil mixing to different degrees prior to tomato transplantation, and, with the exception of organic plots in year five, this resulted in full season colonization levels that were not statistically distinguishable from each other (Table 4-2). The significantly lower levels of colonization in year five organic tomatoes may be attributed to soil-related factors, including conditions that both enhance root growth and reduce dependency on mycorrhizal fungi. It has already been established that the P status in the field was well in excess of plant need; levels in the organic plots were so high that it is surprising that AM fungi were able to consistently gain ingress. Additionally, the lower bulk density and improved aggregate formation that develops from organic matter additions in these plots would improve the physical environment for root growth by reducing resistance for root extension and improving drainage after waterlogging. Reduced relative colonization levels in these conditions may not be a result of less inoculum or decreased infectivity of the inoculum present, but rather may be derived from conditions that allowed roots to grow in the organic treatment at a rate that outpaced the ability of AM fungi to develop; a faster rate than occurred in the other treatments with regularly homogenized soil. This possibility is supported by the results of the infection potential study that show that the levels of primary infection were not comparatively depressed in the organic treatments (Table 4-3).

Figure 4-4. Bahia pasture plots with rows of tomato plants. In this treatment, the only soil disturbance after establishment of the sod occurred in years four and five of the project, when 46-cm rows were tilled for tomato transplants. Picture taken during year five of a 5-year cropping study at the Header Canal farm site in Fort Pierce, FL.

Table 4-3. Field soil infection potential of arbuscular mycorrhizal fungi and other fungal root endophytes on maize from year four and five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL.

Year	Orientation	Treatment	AM fungal % colonization[a]	Non-AM fungal root endophyte % colonization[a]
4	North	Bahia pasture	23.4 (±1.7)	52.2 (± 1.2)
		Disk fallow	4.3 (± 0.8)	29.6 (± 3.8)
		Organic	14.8 (±2.3)	46.6 (± 3.3)
		Weed fallow	21.7 (± 2.5)	53.1 (± 3.8)
	South	Bahia pasture	26.5 (± 2.3)	54.9 (± 3.3)
		Conventional	24.0 (± 1.5)	40.0 (± 3.1)
		Disk fallow	25.4 (± 5.7)	34.4 (± 4.5)
		Organic	18.0 (± 2.0)	28.4 (± 3.0)
		Weed fallow	21.2 (± 3.5)	45.4 (± 6.5)
P values				
Treatment			0.0040	0.0001
Orientation			0.0018	0.1668
Interaction			0.0010	0.0739
5	North	Bahia pasture	16.9 (± 2.5)	48.7 (± 2.9)
		Conventional	8.3 (± 1.8)	33.3 (± 6.9)
		Disk fallow	8.8 (± 0.7)	29.8 (± 3.1)
		Organic	17.1 (± 1.7)	19.7 (± 2.5)
		Weed fallow	8.8 (± 1.2)	34.2 (± 3.0)
	South	Bahia pasture	18.1 (± 4.4)	47.6 (± 4.7)
		Conventional	9.0 (± 1.5)	28.7 (± 6.1)
		Disk fallow	9.7 (± 1.4)	30.7 (± 3.1)
		Organic	22.7 (± 4.9)	17.6 (± 1.8)
		Weed fallow	7.6 (± 1.3)	38.9 (± 3.0)
	Middle	Bahia pasture	13.4 (± 1.5)	28.8 (± 2.9)
		Disk fallow	3.7 (± 1.6)	19.2 (± 1.4)
P values				
Treatment			< 0.0007	< 0.0001
Orientation			0.8685	0.8300
Interaction			0.1571	0.7773

[a]Proportion data were transformed to the square root of acrsine prior to performing ANOVA; values in table are actual mean proportions of root colonization of treatments ± SE.

Colonization levels of AM fungi were also determined for cover crop roots in the northern half of year-four plots, the portion that remained out of production until the following year. Mean root colonization levels were approximately 17.2 ± 2.5 and 21.8 ± 3.3 %, respectively, for bahia roots from the bahia pasture and ragweed roots from the weed fallow plots. Sunn hemp was colonized to a much lesser extent, with average levels among organic plots only reaching 3.4 ± 0.6 %. Again, it is likely that a lack of soil homogenization up to that point in the weed fallow plots probably allowed for the development of an extensive mycelial network as in the bahia pasture treatment. This consequently maximized the spatial distribution of AM fungal inoculum. Tillage was performed in the organic plots to establish cover crops, however, and soil P had been steadily building due to manure applications. Additionally, the mycorrhizal status of sunn hemp under conditions of P sufficiency may simply be negligible.

Tomato Root Colonization by Non-Arbuscular Mycorrhizal Fungal Endophytes

The extensive occurrence of other endophytic fungi and fungal-like organisms was noted in year-four tomato roots, and quantified in the year-five roots (Table 4-2; Figure 4-5). In addition, quantification of this group of organisms was determined through the infection potential study using soil from treatments in both year four and five (Table 4-3). When considered as a discrete group, the levels of infection of tomato roots in year five by these other fungal endophytes was considerably greater than that of the AM fungi in the same year.

The majority of non-AM fungal endophytes identifiable through the direct observation of tomato and other field roots, appeared to be *Phialophora* spp., as determined by the presence of distinctive, enlarged fungal cells within cortical tissue (Figures 4-6 and 4-7). These structures are often called microsclerotia in studies where

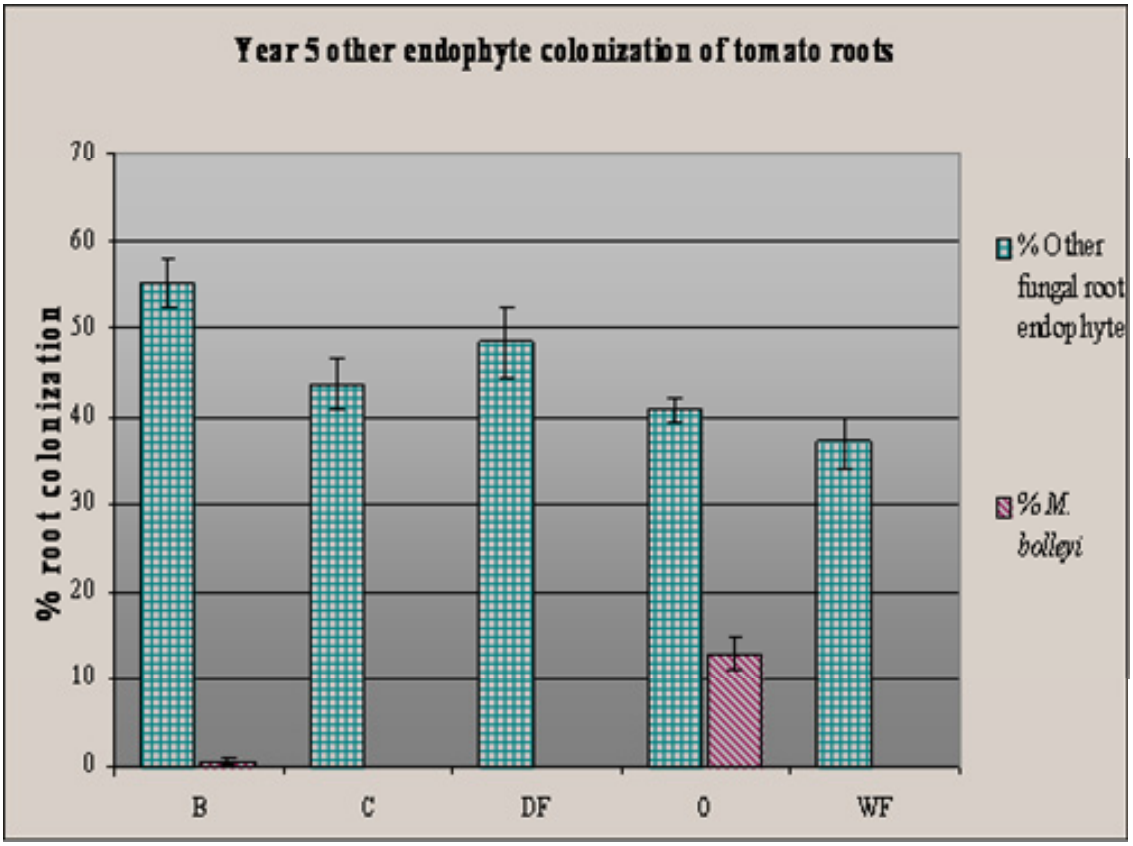

Figure 4-5. Field tomato root colonization by all non-arbuscular mycorrhizal fungal root endophytes in year five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL. The method used to determine colonization levels was identical to and concurrent with assessments for the presence of arbuscular mycorrhizal fungi. The treatments in the study were bahia pasture (B), conventional (C), disk fallow (DF), organic (O), and weed fallow (WF).

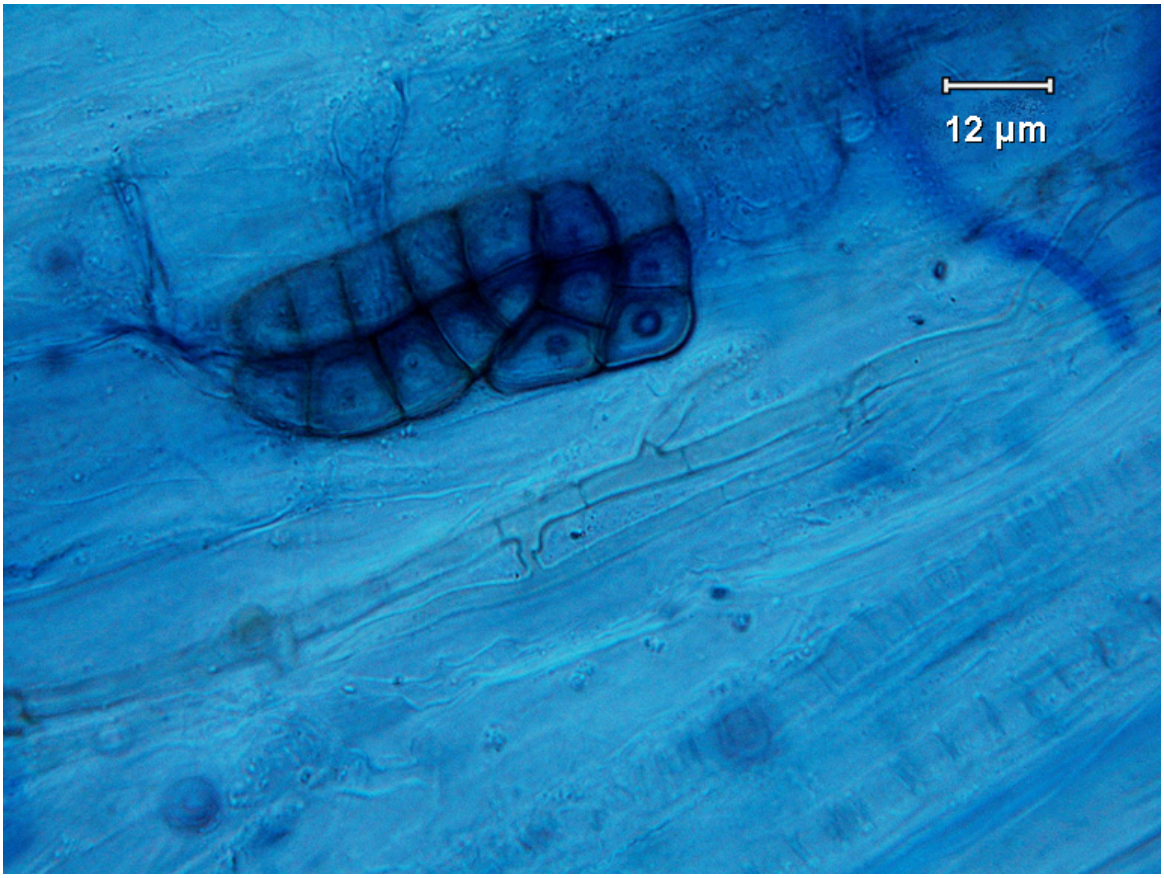

Figure 4-6. Mass of enlarged fungal cells produced by *Phialophora* spp. within the cortex of tomato roots; blue color is a result of staining with trypan blue.

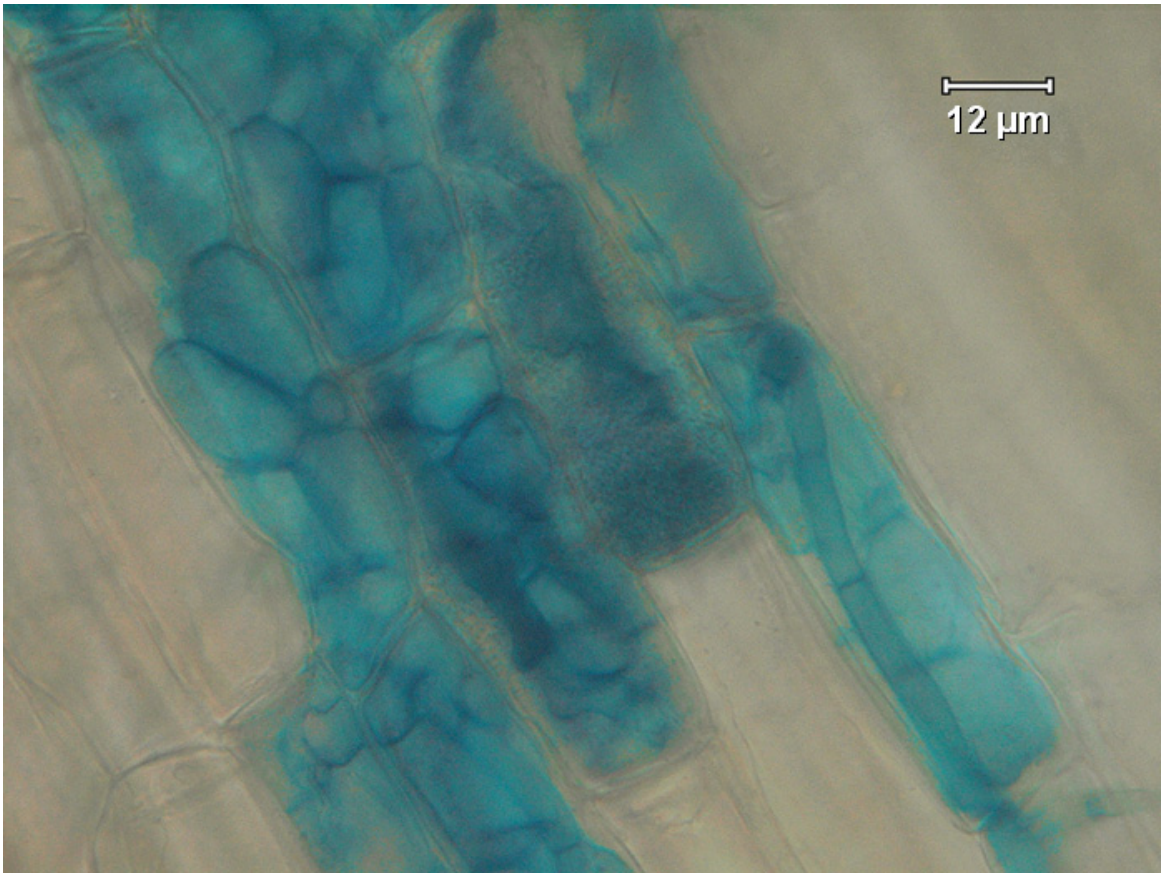

Figure 4-7. Mass of enlarged fungal cells and hyphae produced by *Phialophora* spp. within the cortex of maize roots; blue color is a result of staining with trypan blue.

the fungi are referred to as DSE. The hyphae associated with these structures were only occasionally pigmented, although in individual roots the extent of naturally pigmented hyphae could be considerable. These hyphae were often stained by procedures used to detect AM fungi. *Polymyxa* spp. are endophytes classified in the order Plasmodiophorales, and were found on several occasions in tomato roots; these organisms were easily recognized by the clusters of resting spores formed within the cortex (Figure 4-8). There was a diversity of other structures that were only occasionally observed in field roots, although identification was not always achieved. Unidentified structural features were often not positively related to taxa due to the lack of distinguishing characteristics (an example being intracortical hyphae without distinctive associated features), or the absence of confirming descriptions. Identifying taxa was not a priority when designating colonization levels of non-AM fungal root endophytes, although tissue had to clearly be fungal, or from fungal-like organisms, such as the plasmodiophorids, in order to be positively designated during colonization assessments.

It is important to note that none of these observed organisms were determined to be deleterious to the health of their host plants in any way, nor was there any obvious antagonistic relationship among studied organisms. In the season that both groups of fungi were quantified from field roots, their concurrence was positively correlated ($r = 0.472$, $P < 0.0005$). Similar observations were made in the infection potential study and will be discussed below.

Microdochium bolleyi in Tomato Roots from the Organic Treatment

The distinctive, corn-cob-like structures produced by *M. bolleyi* within tomato roots in this study were the stimulus for examining the occurrence of a broader range of

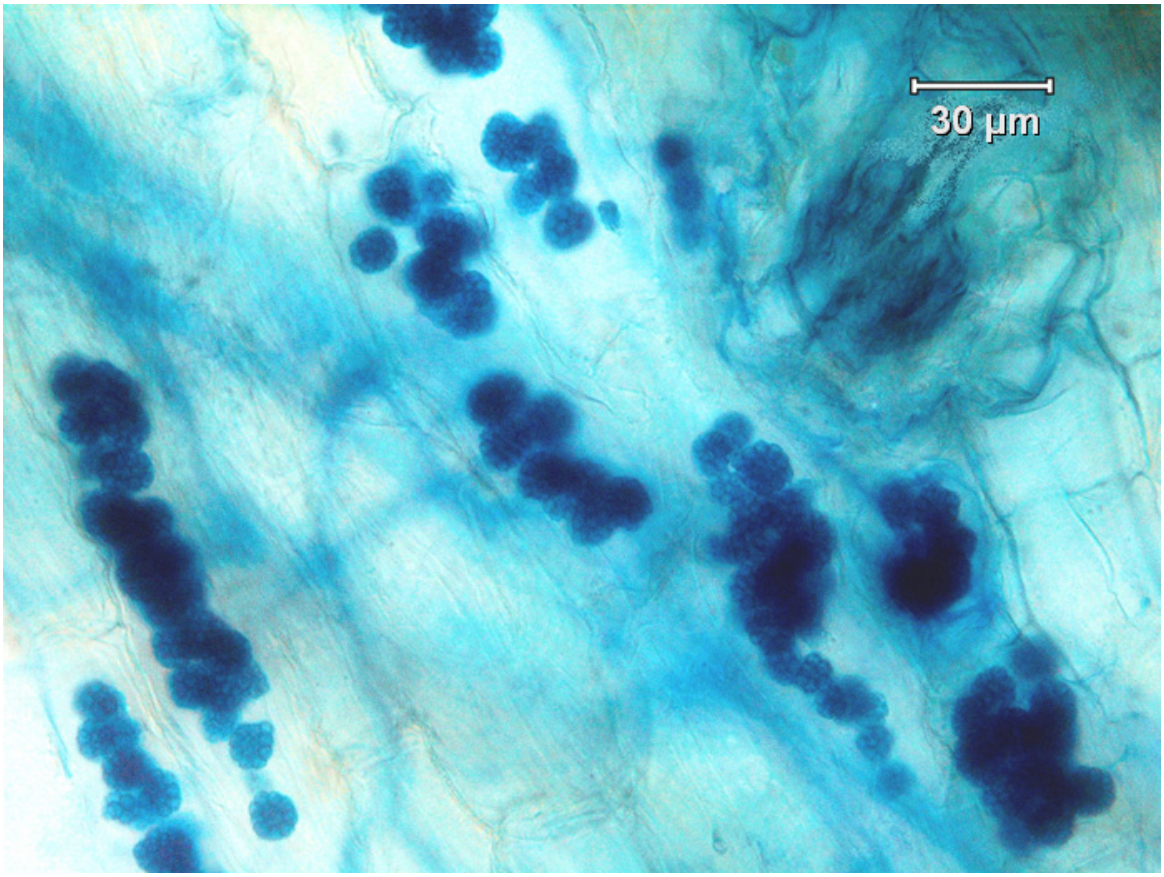

Figure 4-8. Clusters of resting spores (cystosori) formed by *Polymyxa* spp. within the cortex of a tomato root; blue color is a result of staining with trypan blue.

fungal endophytes than just the AM fungi (Figure 4-9 and 4-10). After clearing tomato roots for observation, these masses were the most clearly distinguishable structures where they occurred, irrespective of staining. These masses of fungal tissue did occasionally absorb trypan blue and stain blue (Figure 4-11), but this did not enhance visualization compared to naturally pigmented tissue. Tomato roots in the organic treatment were unique in that they were the only roots in this study in which significant levels of this organism were measured (Figure 4-5), a phenomena that occurred in both years, although it was only measured in year five.

The diagnostic structures for this organism as it develops within the plant cortex have been called alternatively, chlamydospores, dark cells, or microsclerotia, and are known to preferentially occupy the inner and outer cortex (Murray & Gadd 1981; Kirk & Deacon 1987; Sieber 2002). While these structures were obvious within roots when the organism produced them, they appeared to be a small portion of the total biomass produced by *M. bolleyi*, as extensive colonization occurred through hyaline hyphae that did not stain with trypan blue (Figure 4-10). In fact, extension of the transparent intracortical hyphae appeared to terminate with these pigmented structures, making their origins difficult to discern.

The ability of *M. bolleyi* to flourish in the organic plots was not due to the tomato plant host crop in which the organism was detected, as this was the production crop in all systems and the same variety was used across the field. However, the use of cover crops and organic matter applications in the organic treatment may provide the answer to its selectively prolific nature. *Microdochium bolleyi* is a saprophytic fungus that is known to occur widely within the roots of small grains, in which it has been reported to be a

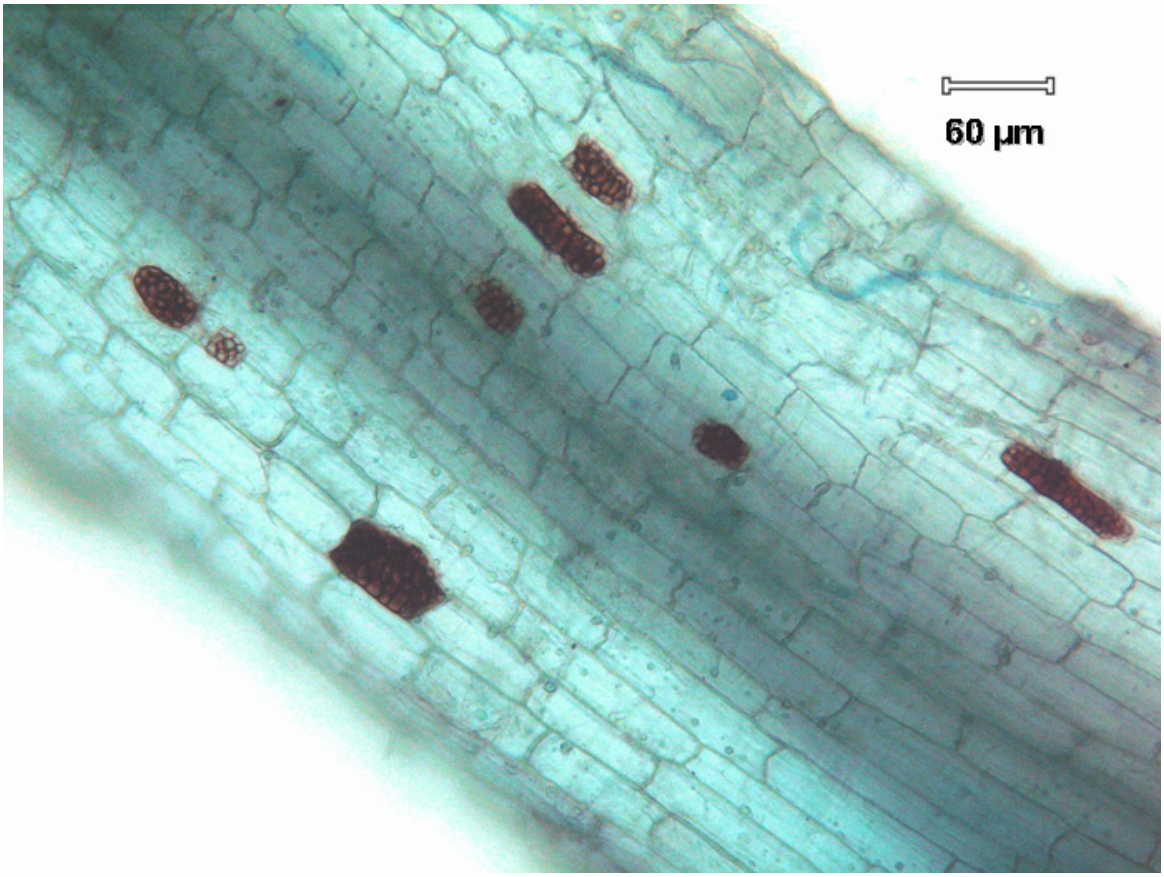

Figure 4-9. Pigmented cells formed by *Microdochium bolleyi* within tomato roots.

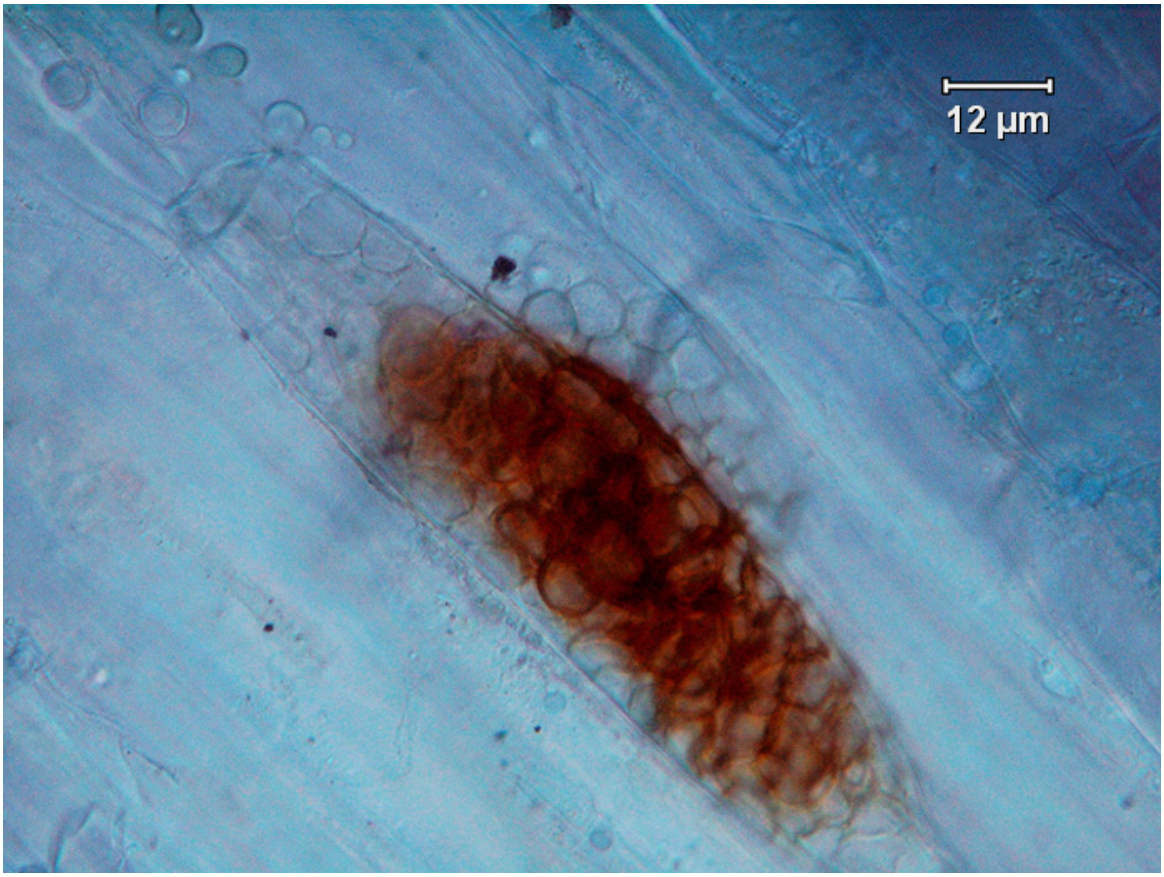

Figure 4-10. Close-up of pigmented cells formed by *Microdochium bolleyi* within tomato roots. Transparent cells and hyphae can be seen associated with the structure.

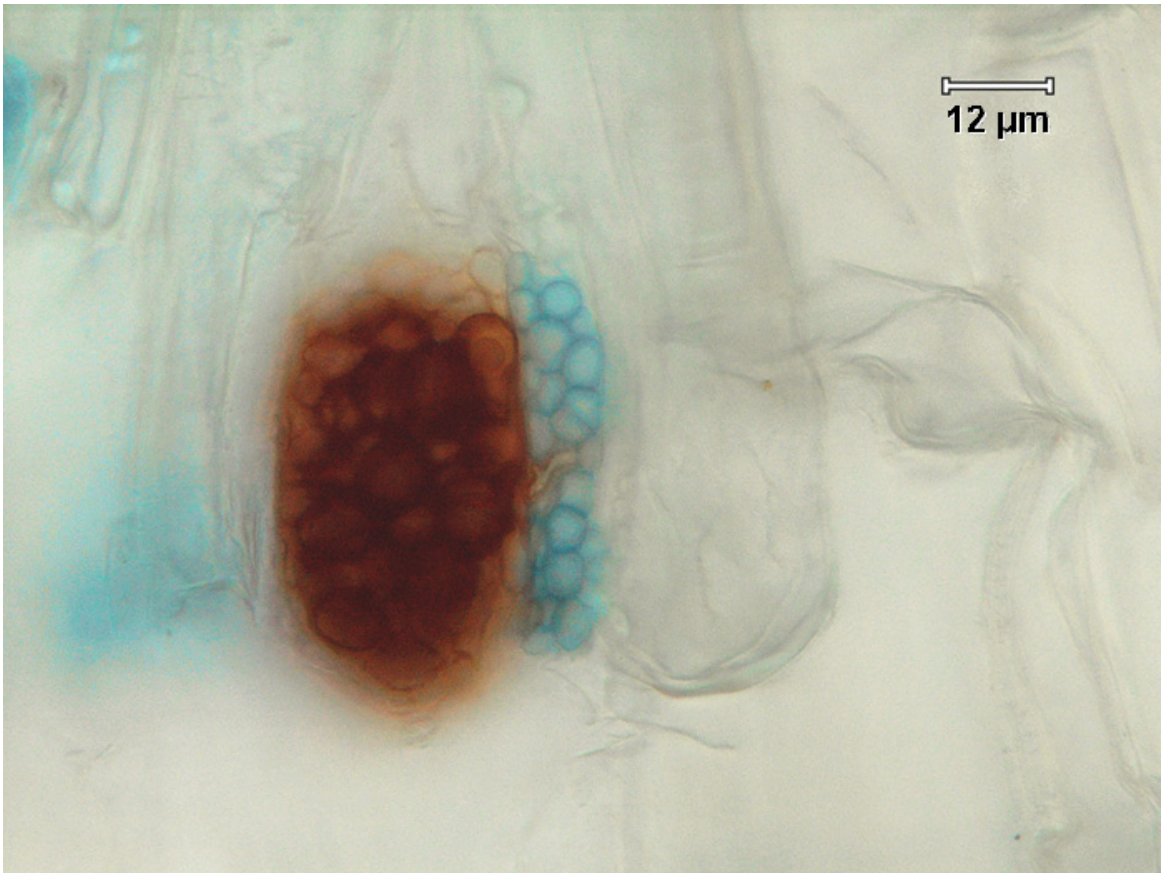

Figure 4-11. Close-up of pigmented and trypan blue-stained cells formed by *Microdochium bolleyi* within tomato roots.

weak parasite with a possible antagonistic interaction with pathogenic fungi and thus a potential biocontrol agent (Murray & Gadd 1981; Kirk & Deacon 1987). The organic treatment, with a spring crop of Japanese millet directly preceding tomato production, was the only system with a cover crop similar to the small grains. Extensive sampling of this cover crop did not occur, although an informal sampling found *M. bolleyi* to be very common within its roots.

Infection Potential of Field Soil on Greenhouse-Grown Maize

Arbuscular Mycorrhizal Fungal Infection Potential

Whereas determining the mycorrhizal status of end-of-season roots provides a measure of the ability of AM fungi to develop associations in the field over time, the infection potential bioassay is used to provide an indication of the infective inoculum available to create primary infections. In order to estimate this potential, it is crucial that host plants are harvested in a timely manner. It has been determined previously that secondary infection in maize roots can be minimized when plants are harvested at the five-leaf stage of development (approximately 30 days after seed germination) (Moorman & Reeves 1979).

The results from the study on the infection potential of AM fungi revealed relationships between these organisms and management tactics that were not evident from the full season tomato plants that were investigated (Table 4-3; Figures 4-12 through 4-14). Soil sampled from both halves of system plots produced generally uniform levels of colonization on maize roots in year four, with the exception being the northern halves of disk fallow plots which differed significantly from all other treatments regardless of management strategy (Figure 4-12). The northern portion of the disk fallow

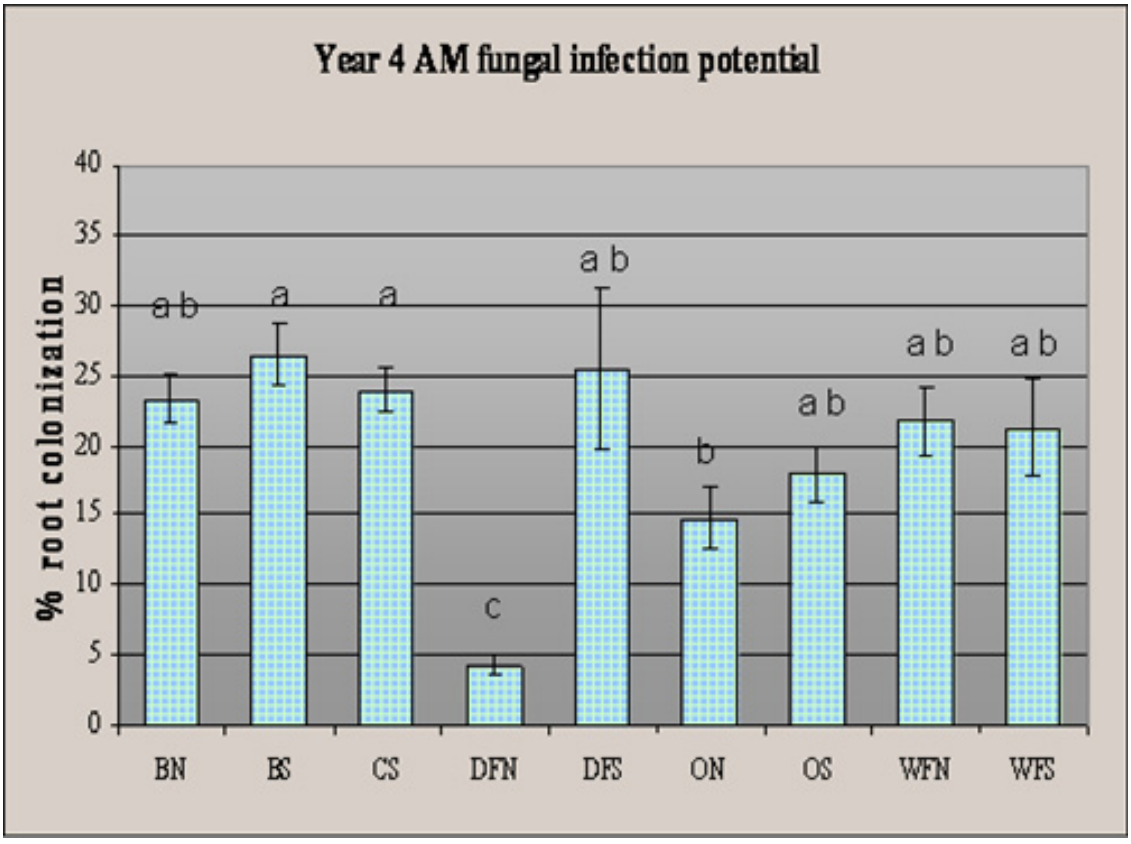

Figure 4-12. Infection potential of treatment soil sampled in year four of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL; infection potential was determined by colonization levels of arbuscular mycorrhizal (AM) fungi on greenhouse grown maize plant roots at the five-leaf stage. Columns with the same letter above SE bars indicate no significant difference (means separated at $\alpha = 0.05$ using Tukey's HSD). The treatments in the study were bahia pasture (B), conventional (C), disk fallow (DF), organic (O), and weed fallow (WF).Treatment codes are followed by an N or S, designating the plot half sampled (N = north, S = south). Conventional plot soil was not sampled in the northern half in year four.

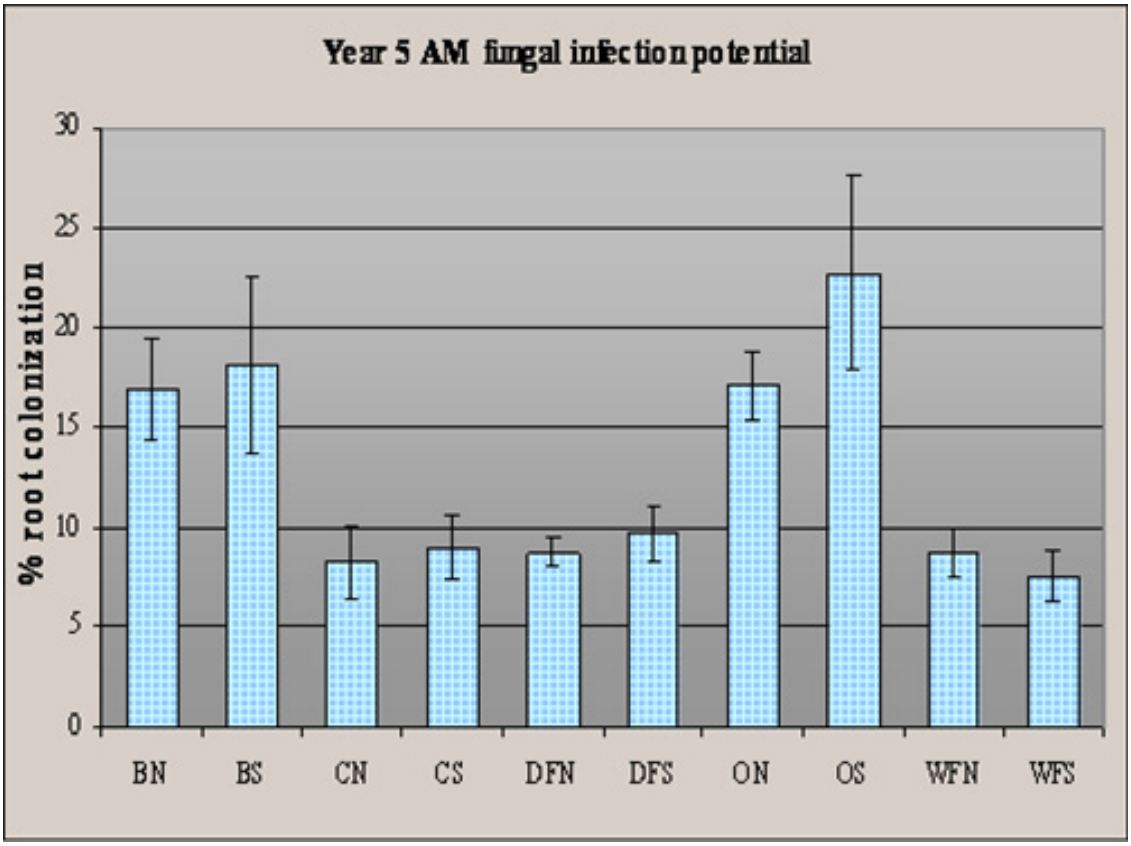

Figure 4-13. Infection potential of treatment soil sampled in year five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL; infection potential was determined by colonization levels of arbuscular mycorrhizal (AM) fungi on greenhouse grown maize plant roots at the five-leaf stage. The treatments in the study were bahia pasture (B), conventional (C), disk fallow (DF), organic (O), and weed fallow (WF).Treatment codes are followed by an N or S, designating the plot half sampled (N = north, S = south).

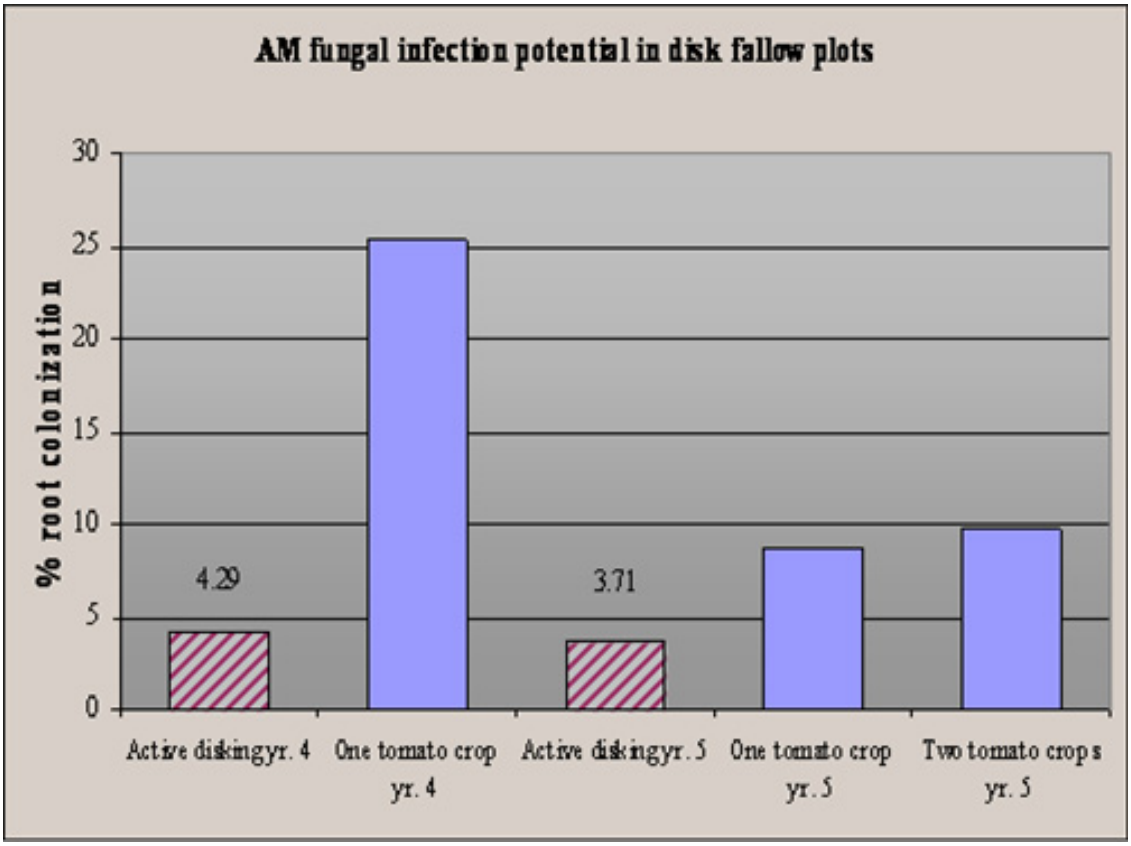

Figure 4-14. Infection potential of disk fallow treatment soil sampled in year four and five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL; infection potential was determined by colonization levels of arbuscular mycorrhizal (AM) fungi on greenhouse grown maize plant roots at the five-leaf stage. During this study, the southern half of disk fallow plots produced their first tomato crop in year four and finished a second year of production at the end of year five. Northern plot halves only produced one tomato crop, harvested at the end of year five. Actively disked areas were located in the northern half of plots in year four, and in between plot halves in year five.

treatment was still actively disked at the time of sampling and had been managed accordingly to eliminate plant growth for the four previous years. All other treatments were statistically similar, with the exception of slightly lower levels of colonization in the northern half of organic plots at the end of their first year of tomato production when compared to bahia pasture and conventional system plots. This difference in organic plots might be attributable to the impact of the high P levels in that treatment, although the results do not carry over to the following year.

While the extremely low levels of AM colonization occurring on infection potential hosts grown in actively disked soil is not surprising, especially considering the generally adverse conditions due to other agricultural management practices in the field, a direct comparison between the northern and southern halves of plots revealed an unexpected response (Figures 4-12 and 4-13). After a single full season of host growth, such as occurred in the southern half of disk fallow plots, the infection potential recovered to the extent that it was indistinguishable from all other treatments. The implication is that AM fungal inoculum density and infectivity following 14 years of intensive agricultural management, including three consecutive years of continuous disturbance, was not significantly impacted compared to the other farming practices investigated after host roots were allowed to persist for several months. This indicates that inoculum recovers from periods of intense disturbance in the absence of host tissue more rapidly than previously suspected (Gosling *et al*. 2006).

The intense flooding and reduction in exposure to host roots in year five appears to have altered treatment differences compared to the previous year (Table 4-3; Figure 4-13). Both halves of the disk fallow plots had been put into production and at the end-of-

season sampling time they both had similar levels of colonization, albeit reduced from colonization levels that occurred in disk fallow production plots from the year before. However, the infection potential of that portion of plots where disking continued, in the stretch of land between the northern and southern plot halves, colonization levels were almost identical to actively disked soil from the year before (Figure 4-14). This suggests a possible baseline level of viable inoculum in this field regardless of management, from which recovery to more extensive levels occur after host roots prime the soil. Because tomato roots were in the soil for 36 fewer days in year five compared to year four, it is possible to surmise the reduced recovery of infection potential in year five was partially a product of the shorter priming period.

The infection potential of all treatments was reduced in year five, with the exception of organic plots (Table 4-3). Determination of the 16:1ω5 content of field soil using FAME analysis helped confirm the infection potential study results in year five (Figure 4-15), and correlation at the treatment level was verified ($r = 0.9001$, $P = 0.037$). When comparing the colonization levels of full season field tomatoes to infection potential maize plants in the organic treatment, the discrepancy mentioned in the previous chapter is clear; despite low levels of colonization occurring on tomato plants, the organic plot had considerable active inoculum as evidenced by substantial primary infection on maize in infection potential assays. The likely reasons for the low levels of field tomato root colonization were discussed in the previous chapter, and are related to the clearest explanations for higher infection potentials in the organic treatment. The improved soil structure in these plots allowed for more rapid drainage from saturated conditions and also reduced resistance to root growth when compared to the other treatments with higher

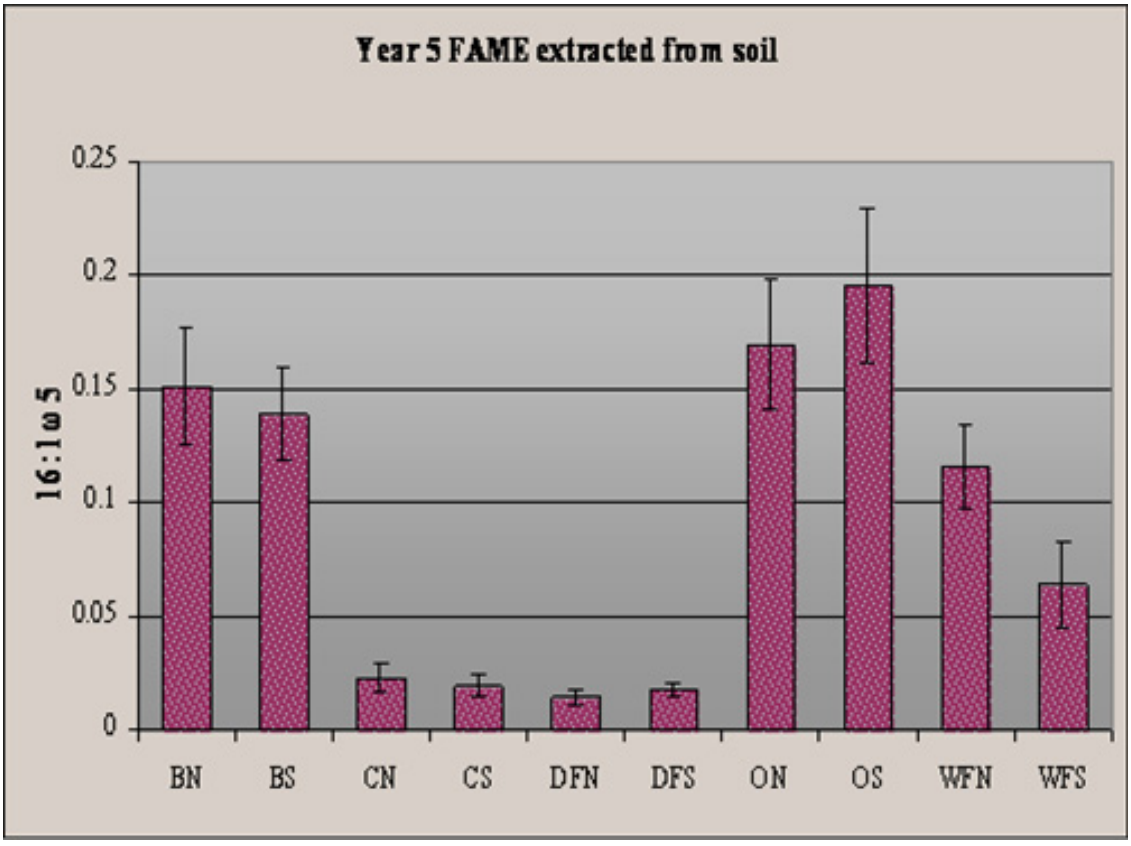

Figure 4-15. Results from Fatty Acid Methyl Ester (FAME) analysis of soil from year five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL. Results show the levels of the arbuscular mycorrhizal (AM) fungal biomarker 16:1ω5 within treatments. Content of treatment soil is relative to a fatty acid standard (19:0). The treatments in the study were bahia pasture (B), conventional (C), disk fallow (DF), organic (O), and weed fallow (WF).Treatment codes are followed by an N or S, designating the plot half sampled (N = north, S = south).

bulk densities. This would lead to a quicker recovery of tomato plant root growth from stressful conditions, and a generally more favorable habitat for roots in the organic plots. Concurrent with increased relative root growth is a greater extension of an active AM fungal mycelial soil network, and thus increased generation of inoculum. Where the pace of root growth exceeds the ability of the AM fungi to develop and advance in the cortex, lower overall colonization levels can be expected to occur. Additionally, extensive development within plant tissue is more likely to be retarded in plants that have access to abundant P, while the primary infection of newer fine roots may proceed unabated.

The bahia pasture plots had infection potential levels that were not clearly elevated compared to other systems as occurred in field roots, although colonization levels were significantly higher than conventional, disk fallow and weed fallow plots in year five (Figure 4-13). The moderation of bahia pasture infection potentials compared to full season field root colonization can be attributed to sample preparation for the greenhouse study. Tomato transplant colonization levels in bahia pasture plots benefited from a largely undisturbed, spatially dense mycelium in the soil; but after cores were sampled at the end of each growing season, soil samples from each plot were bulked and thoroughly homogenized. While colonization levels in infection potential maize plants were decreased compared to field plants, the bahia pasture plots consistently generated levels of inoculum that overcame the short-term disturbance with moderate levels of primary infection.

Non-Arbuscular Mycorrhizal Fungal Root Endophyte Infection Potential

As in the field roots, no negative interaction among these groups of fungi was detected. Instead, positive correlations occurred in year four ($r = 0.441$, $P = 0.002$) and year five ($r = 0.201$, $P = 0.117$) with respect to colonization levels of both groups of

endophytes within maize roots from the infection potential study. Levels of colonization by non-AM fungal endophytes in the field were comparable to the results of the infection potential study (Tables 4-2 and 4-3). Bahia pasture soils had the highest overall infection potential for non-AM endophytes, while organic plots had significantly lower levels of colonization in year five compared to other systems. In addition, there was a general decline in the colonization levels in the infection potential study in year five compared to the previous year, probably because of the widespread waterlogging of soils. There was no evidence of *M. bolleyi* in maize roots, although this does not preclude the presence of this endophyte. The maize roots were quite young at the time of harvest and *M. bolleyi* is known to preferentially occupy naturally senescing root cells, generally forming the darkly pigmented diagnostic structures where the plant forms papillae (Kirk & Deacon 1987), while hyphae are hyaline and do not stain. The morphological structures of fungal root endophytes observed in maize were similar to those seen in tomato roots, although extensive occurrence of the chytrid *Olpidium* spp. was observed. (Figure 4-16).

Making assessments on the status of such a broad group of organisms based on their ability to initiate primary infection would be difficult and probably be of limited value. However, it is clear from these data, in addition to that from full-season tomato roots, that considerable root colonization by a diverse set of fungal taxa occurred in this field and that colonization can develop quickly.

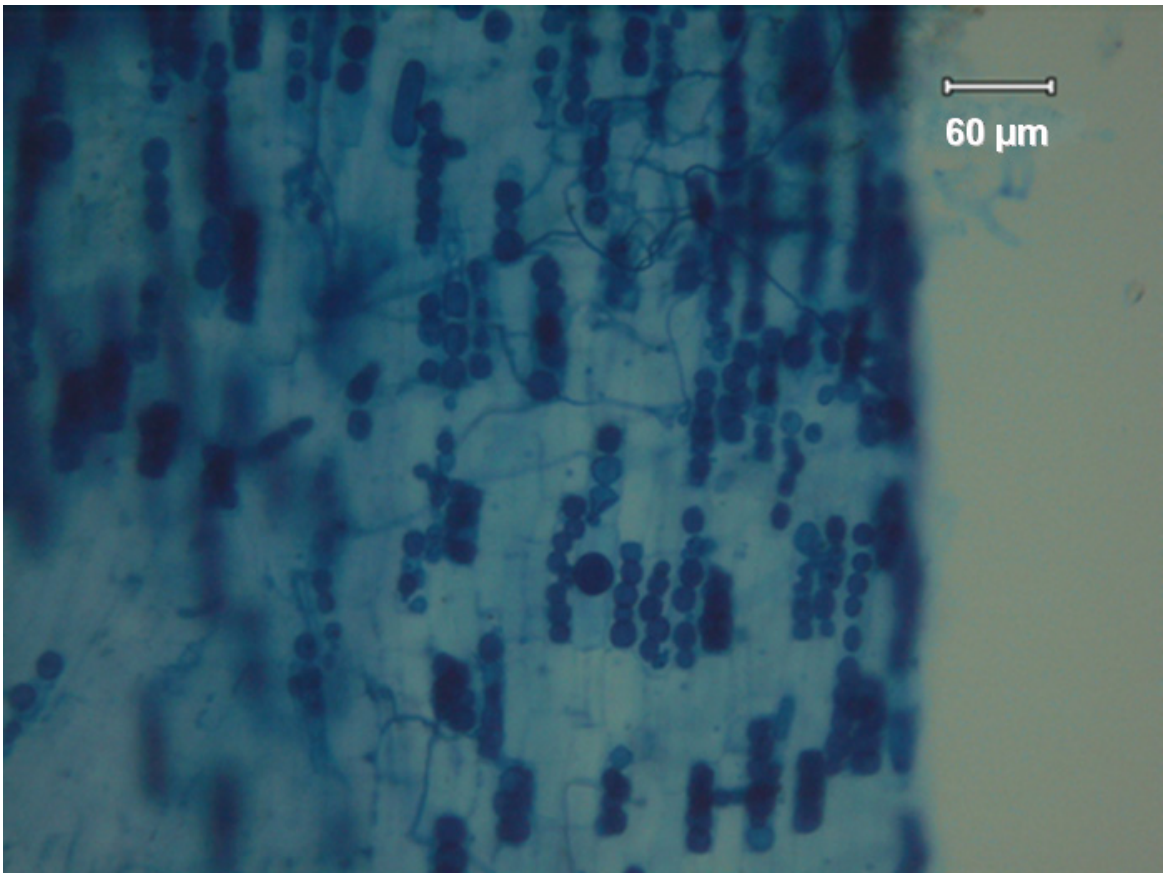

Figure 4-16.Maize roots with colonization of a chytrid *Olpidium* spp. stained with trypan blue. Fungal structures are resting spores and zoospore cysts.

Spores: Enumeration and Identification of Arbuscular Mycorrhizal Fungal Morphotypes

Spore Enumeration of Year Five Trap Culture Soil

Sudangrass cultures were harvested and spores extracted from the root-soil complex for each of the replicate pots that were established. Spores were then counted and a mean derived for each replicate pot to develop an approximation of the number of spores occurring in the rhizosphere of host plants in each treatment. Spore counts were performed in both years of tomato production and in both halves of all plots.

Spores extracted and observed from year five soil were scarce (Figure 4-17). While significant differences existed among treatments, with the bahia pasture and conventional plots having more spores than the other treatments, and organic and disk fallow plots having the fewest, spore numbers in all treatments were very low. Spore numbers may have been slightly to moderately underestimated in both years, however, as a result of adapted harvesting and enumeration procedures that were used to improve spore retrieval for mounting. Also, the final step of spore extraction used vacuum filtration to drain and wash away sucrose solution from spores. When on wet filter paper, spores were remarkably easier to grasp and manipulate for mounting when compared to spores free floating in water within a Petri dish. When observed associated with white filter paper, though, juvenile and hyaline spores are much harder to see and therefore, were undercounted

While year five sudangrass cultures were grown in undiluted field soil, year four cultures were grown in soil diluted with very low P sand in order to stimulate spore generation. Early in the study, it seemed questionable whether sporulation (or colonization of plant roots in the infection potential test) would occur at all at the extreme

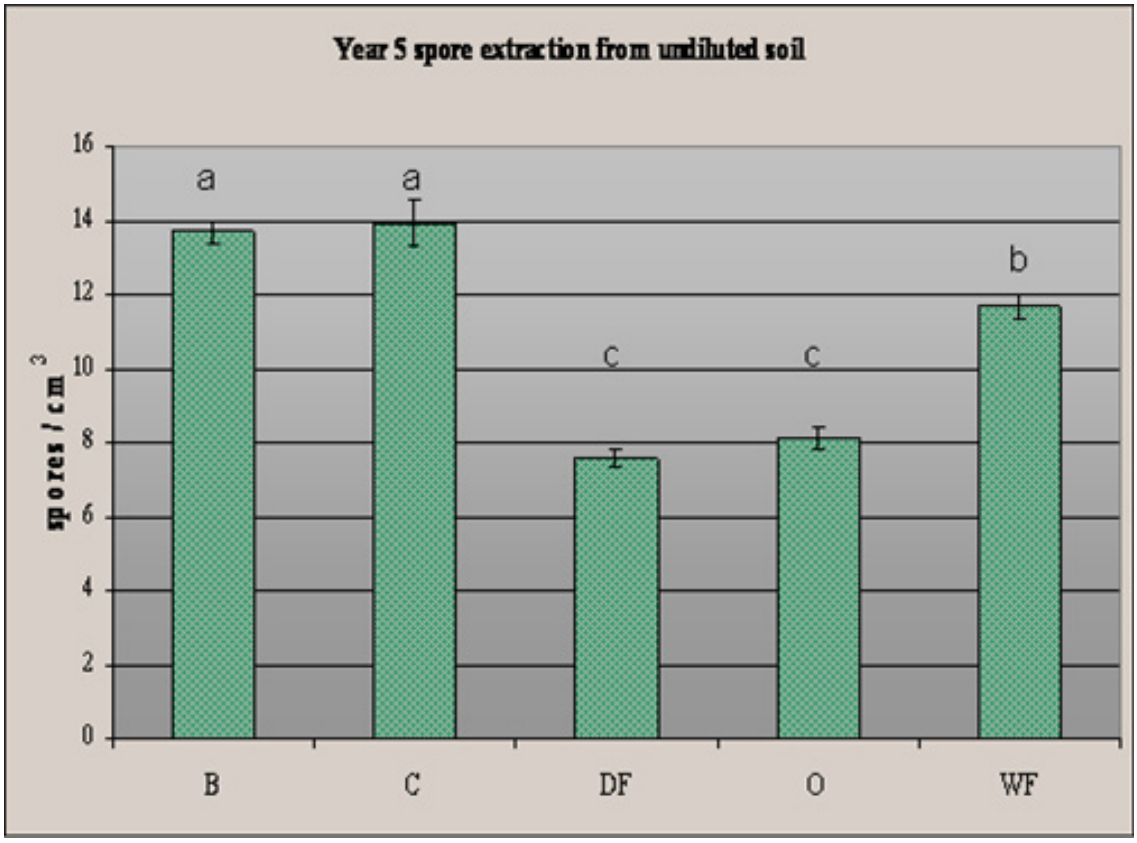

Figure 4-17. Mean arbuscular mycorrhizal fungal spore numbers extracted / cm^3 of undiluted treatment soil from year five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL. Spores were extracted from field soil used to grow a sudangrass trap culture in a greenhouse study. Columns with the same letter above SE bars indicate no significant difference (means separated at $\alpha = 0.05$ using Tukey's HSD). The treatments in the study were bahia pasture (B), conventional (C), disk fallow (DF), organic (O), and weed fallow (WF).

P contents that exist in the Header Canal field soil; therefore, the initial cultures were diluted in half with low-P sand. Since results from trap culture spore enumerations already have diminished value when compared to counts made directly from the field, the spore numbers from the year four extraction using diluted soil, which further modified the edaphic environment, are not included.

Sporulation was approximately three to nine times higher in the diluted year four soil than in undiluted year five soil. It is difficult to ascribe the detected difference in sporulation simply to the reduced P levels derived through soil dilution, however, because the field conditions in year five were significantly altered by climatological conditions. Considerable parasitism existed in spores extracted both years (Figures 4-18 and 4-19) and the extremely wet field conditions in the final year of tomato production may have provided optimal conditions for the many organisms that prey on AM fungal spores. Sporulation also may have been affected by phenological differences in tomato plants that occurred at time of sampling between years, as a result of the later planting date in year five due to the severe weather that was experienced in the end of 2004.

Spore Morphotype Identification

Identification of AM fungal spore morphotypes can be a challenging prospect in the best of circumstances, such as when harvested in abundance from pure trap cultures grown in sterilized soil. Differentiating among the more than 150 morphotypes described in the INVAM collection is a product of the ability of the investigator to capture examples and visualize incremental variability within groups classified as genera. Even inter-familial differences can be hard to ascertain, as crucial diagnostic structures or developmental stages may be absent or otherwise missed from culture.

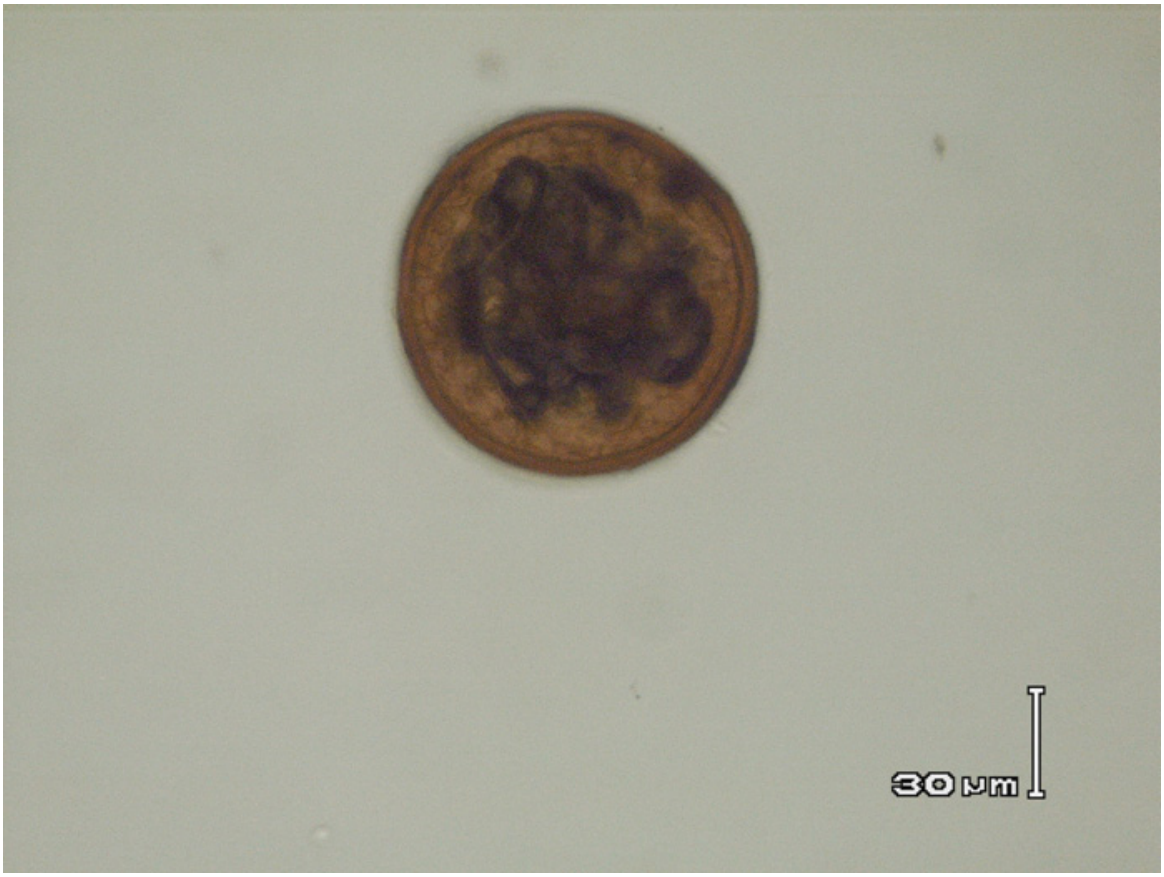

Figure 4-18. Arbuscular mycorrhizal fungal spore with substantial parasitism evident through dark internal swirling; no morphotypic designation could be assigned.

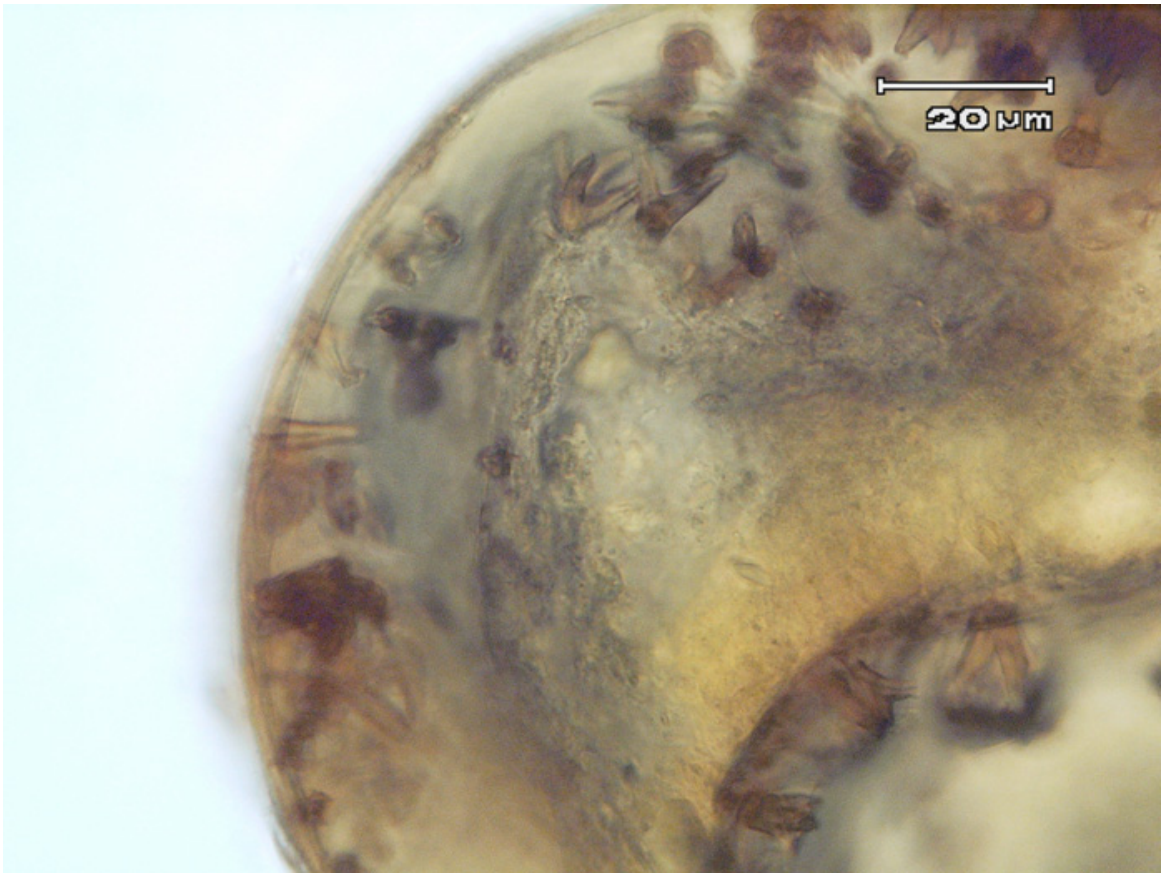

Figure 4-19. Damaged arbuscular mycorrhizal fungal spore with evidence of a defense response initiated by parasitism.

Cultures generated directly from field soil, as in this study, can be even more problematic. In addition to the issues related to parasitism mentioned above, considerable associated debris can obscure field soil cultures and competition among root inhabiting fungi can cause alterations in AM fungal community spore production and representation. Because of this, in addition to the associated sampling technique limitations, the field diversity of AM fungal spore morphotypes detected in this study must be considered as incomplete and may considerably under-represent the richness present at the Header Canal site during the 2 years that sampling and identification took place.

At least 11 individual morphotypes were identified as having sporulated between the two sampling dates; it is likely that more existed, but were not captured or identified. Joe Morton, the curator of the INVAM collection, identified two of the spore types present, *Archaeospora trappei* and *Glomus etunicatum*, and additionally assisted with multiple confirmations of other types. Morphotypes identified from the various treatments are listed in Table 4-4, but the diversity present in the field or even that which was encountered in this study is not definitively revealed. For example, the vast majority of spores fell into one of the last four categories on the table, distinguished as a *Glomus* spp. morphotype. In reality, since substantial variation was observed in each group, these categories are constructs for spores that could represent considerable morphotypic diversity. This variation, however, could not be confirmed to represent different individual morphotypes. Additionally, it is important to understand that many of the observed differences might be attributed to individual spore morphotypes encountered at

Table 4-4. Diversity of arbuscular mycorrhizal fungal morphotypes in year four and five of a 5-year cropping systems study at the Header Canal experimental farm site in Fort Pierce, FL. Morphotypic diversity was determined by extracting spores from sudangrass trap cultures grown in field soil.

Morphotypes extracted	Treatments where morphotype recovered
Acaulospora spp.[a]	Bahia, Conventional, Disk Fallow, Organic
Archaeospora trappei	Bahia, Conventional, Disk Fallow
Gigasporaceae[b]	Bahia, Disk Fallow, Weed Fallow
Glomus ambisporum	Bahia
Glomus clarum	Conventional, Disk Fallow
Glomus etunicatum	Bahia, Weed Fallow
Glomus geosporum	all
Glomus intraradices	Bahia, Conventional, Disk Fallow, Weed Fallow
Glomus mosseae	all
Glomus sinuosum	all
Glomus spp. (orange)[c]	all
Glomus spp. (small hyaline)[c]	all
Glomus spp. (small pale yellow)[c]	all
Glomus spp. (yellow)[c]	all

[a]High quality spores of these morphotypes were not acquired, and sporiferous saccules were not retained after extraction; this grouping may therefore include multiple morphotypes.
[b]Finer taxonomic designations were not possible with the spore types extracted.
[c]These groupings are constructs that could be identified as *Glomus* spp., but could not be classified on a finer taxonomic level; multiple morphotypes may exist within each category.

different developmental stages or affected by the environment to somehow alter morphological characteristics.

The diversity accounted for is not unreasonable for a field with a history of intensive agriculture and constitutes more morphotypes than have been reported as existing in some surveys of natural and disturbed environments (Douds & Millner 1999). Considering this, and magnified by the likely under-representation of spore types in this study, it is possible to speculate that substantial diversity of AM fungal morphotypes has been retained in this field across treatments relative to descriptions from other agricultural environments.

Based on the methods used in this study, the organic plots had the fewest positively identified spore types, while bahia pasture contained the most. This is not unexpected considering the abundance of available P in the organic treatment, a condition previously detailed as being known to contribute to reductions in both colonization and sporulation. *Glomus* spp. spore types were the most common and most frequently identified morphotypes by a large margin in all systems.

Acaulospora spp. spore types were recognized as small to moderately sized spores without a pore connecting to the subtending hyphae. Instead, their spore walls are entire, typically have no associated hyphae and have nothing remaining of their sporiferous saccule after the extraction process. The internal germinal walls diagnostic for this group after staining with Melzer's reagent could only occasionally be visualized, while the ornamentations that are on some of the spore walls of *Acaulospora* spp. provided a more frequent means for identification (Figure 4-20). Spores of *Glomus* spp. are similar in size

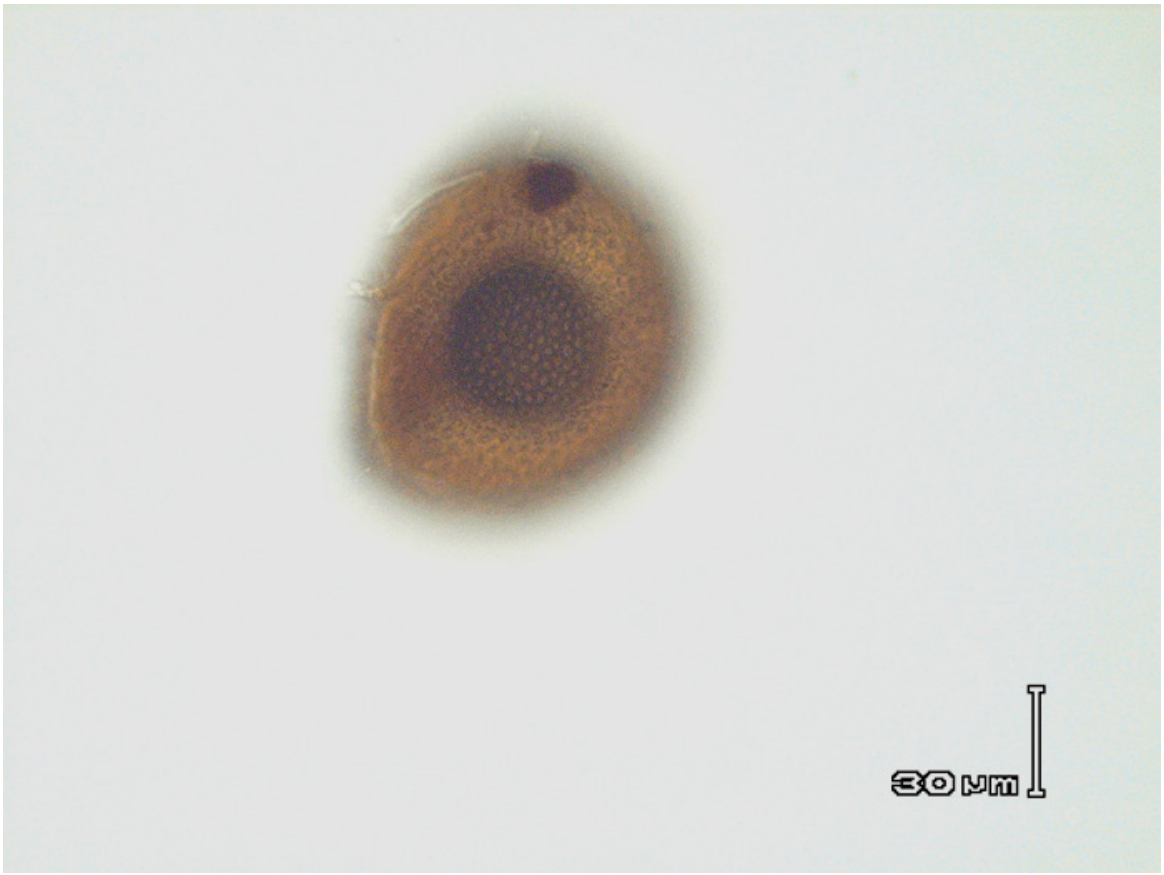

Figure 4-20. Surface ornamentations can be seen on the spore wall of this *Acaulospora* sp.

to the Acaulosporaceae, but often have subtending hyphae, of which some or all of the layers are continuous with the spore walls, which constitutes the pore diagnostic for this spore type (Figure 4-21). No internal germinal walls are formed. Some types of *Glomus* spp. develop aggregates with numerous associated individual spores, producing masses known as sporocarps (Figures 4-22 and 4-23). Spores of members of the family Gigasporaceae are considerably larger than other groups when mature and have an associated, bulbous sporogenous cell that forms during spore development (Figure 4-24). The two groups within this family can be distinguished between each other in a number of ways, including the formation of germination shields in *Scutellospora* spp. that are absent in *Gigaspora* spp., and the number and associated characteristics of the spore walls. Although large spores with sporogenous cells were found, confirming that Gigasporaceae were being observed, none of the other spore-derived features of this group were available for discrimination in this study. Instead, the presence of both groups was confirmed by structures attached to external hyphae in the infection potential study. Both the echinulate auxiliary cells formed by *Gigaspora* spp. (Figure 4-25) and the smooth but knobby auxiliary cells generated by *Scutellospora* spp. were discovered (Figure 4-26).

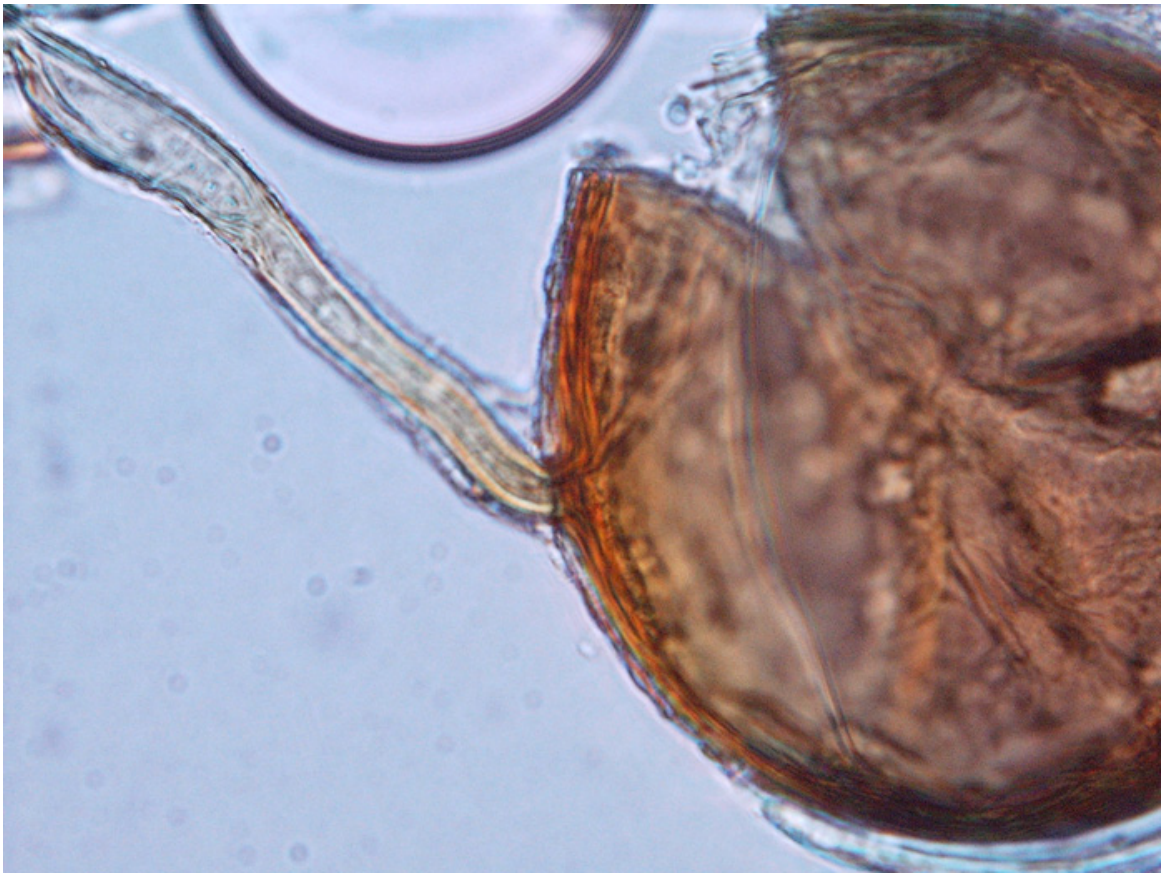

Figure 4-21. Multiple spore walls continuous with the layers of the subtending hypha can be seen in this example of the arbuscular mycorrhizal fungal morphotype *Glomus intraradices*.

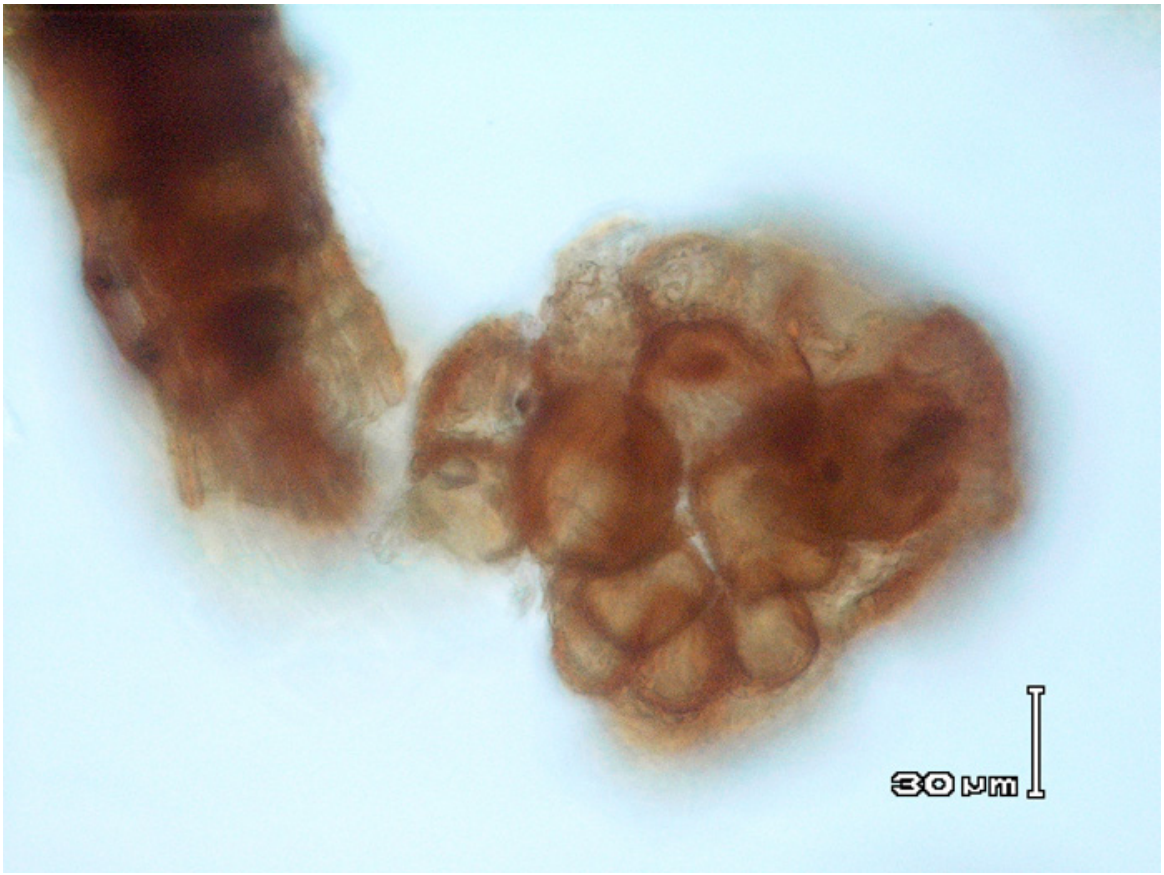

Figure 4-22. Sporocarp of *Glomus sinuosum* covered by peridial hyphae.

Figure 4-23. Portion of a *Glomus ambisporum* sporocarp; this aggregate can be seen associated with a dense mass of mycelium with individual subtending hyphae leading to each spore.

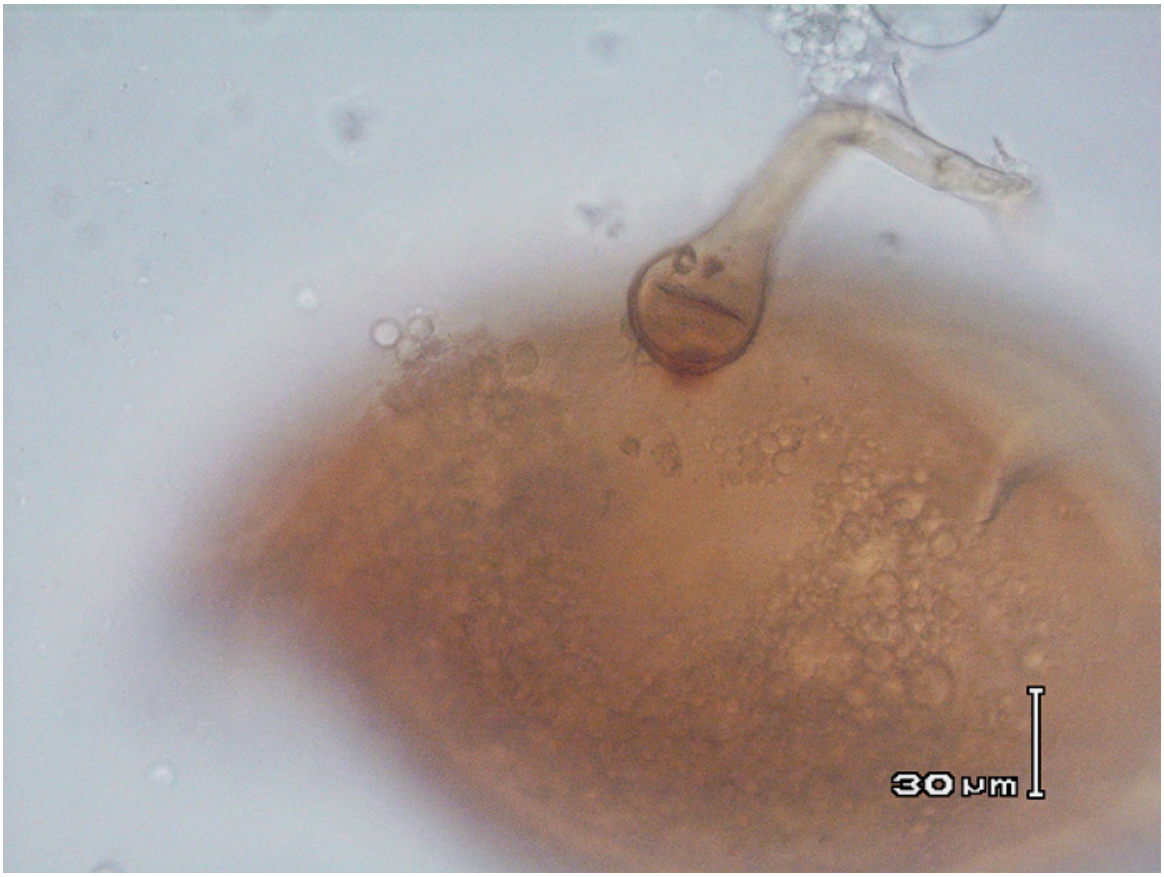

Figure 4-24. *Gigaspora* sp. with its large sporogenous cell still attached.

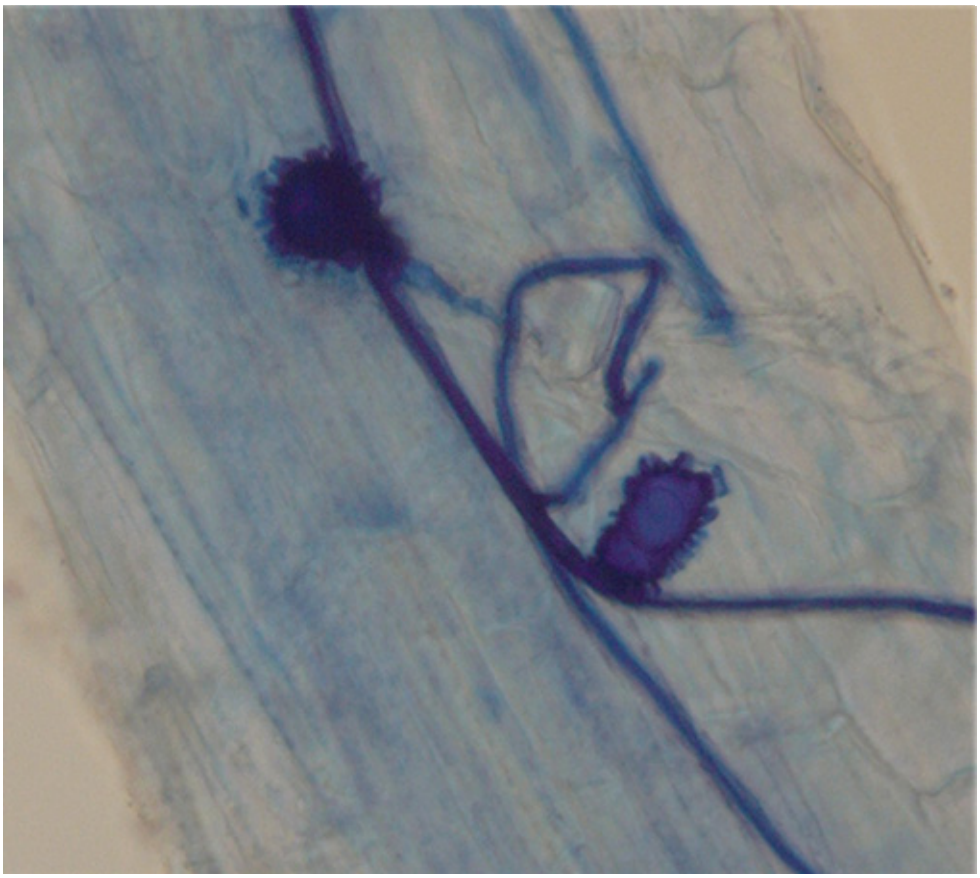

Figure 4-25. Echinulate auxiliary cells diagnostic for a *Gigaspora* spp. These structures were only found produced on external hyphae associated with maize roots in the infection potential study.

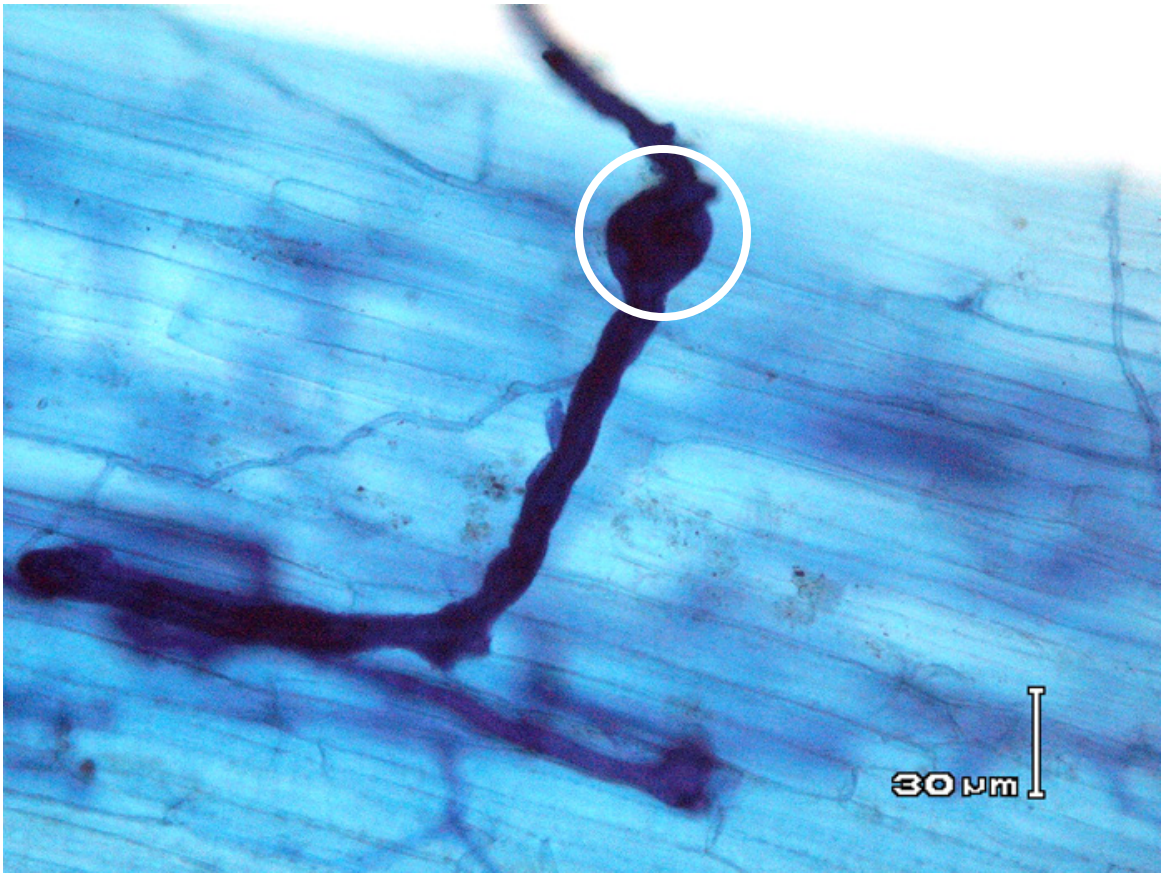

Figure 4-26. Smooth surfaced auxiliary cell diagnostic for a *Scutellospora* spp.; these structures were only found produced on external hyphae associated with maize roots in the infection potential study.

CHAPTER 5
CONCLUSIONS

The experimental farm at the Header Canal site represented a multiplicity of potential adversities to the existence and functioning of AM fungal and other soil microbial communities. The diversity of farming strategies among the treatments occurred across a single soil taxonomic unit, effectively eliminating the substantial influence that soil type and texture would have on microbial communities. Resilient populations of AM and other fungal root endophytes were found to reside within all systems and no permanent impacts related to anthropogenic or environmental alterations could be confirmed. However, field-wide suppression of the mycorrhizal association and short-term changes in AM fungal activity related to management practices and flooding were detected.

Of all investigated agricultural practices, phosphorus fertilization and soil disturbance had the greatest influences on AM fungal infectivity and abundance; however, their impacts were manifested in distinct ways and persisted over different time scales. Historical fertilization practices led to an enormous P bank in all treatments, with the most extreme levels developing in organic plots where P applications were well in excess of annual crop need. The abundant available P supply within the soil in all treatments led to a general repression of AM fungal colonization and probably resulted in a low incidence of sporulation. Tomato roots in the bahia pasture treatment had the highest average levels of mycorrhizal colonization of any roots observed in this study, reaching a maximum of 32 % of root tissue colonized, a degree of association that, in

most environments, would be considered to be at the low end of moderate. This relatively larger amount of colonization in bahia pasture treatments was not due to reduced available P, but to the lack of significant soil disturbance occurring in the plots of that treatment. This P effect represents a long-term obstacle to more extensive AM fungal development in plants and spore production. Mitigation of the soil nutrient content would require long-term crop production and harvest without significant future P additions.

Tillage mediated soil disturbance did not present a long-term suppression of AM fungal infectivity, although primary infection was dramatically decreased in soil actively undergoing intensive tillage. Subsequent to the cessation of disking and following a full season of crop growth, field crop colonization levels and infection potential of soil were indistinguishable from other, less disturbed production systems. The ability of populations to quickly recover infectivity after years of host root absence to levels identical to those in a minimally disturbed system in the course of a single season represents an intriguing finding of this research.

There was no clear evidence that either the history of fumigation at the farm site with MeBr + CP or the continued use of alternative fumigants in the conventional plots had a lasting effect on the ability of AM or other fungal endophyte communities to colonize field roots, or to initiate primary infection on maize roots in a greenhouse study. Analysis of AM fungal signature FAME's to measure biomass within soil did indicate very low habitation in conventional plots in year five; however, this method detected that a similar response occurred in disk fallow plots, suggesting these differences may have been largely a consequence of saturated field conditions prevalent in late 2004.

Repression of these fungal populations would be expected to have taken place directly after fumigation, while results from these studies only included end of season colonization and soil infection potentials, apparently allowing substantial time for recovery.

Widespread waterlogging and a shortened growing season in year five, caused by two consecutive hurricanes, may have reduced the amount and infectivity of all fungal root endophytic propagules as measured by the infection potential study, but colonization levels of full season tomato roots were largely unaffected. This seeming dichotomy can be explained. The low incidence of field root colonization by AM fungi in the systems with regularly mixed soil may represent a minimum level of occurrence, as determined mainly by the P status of the host plants and the periodic disturbance of the soil. The levels of field root colonization were relatively higher in the bahia pasture plots as a result of the spatially dense web of infective hyphae associated with the sod roots. With the exception of AM fungi in the organic treatment, all fungal root endophyte infection potentials within soil were reduced in year five compared to year four. A field-wide alteration of the degree of primary infection that these varied populations are able to generate is most likely due to reduced root growth as a result of a relatively shorter growing season (105 days in year four and 69 days in year five) in combination with highly stressful field conditions, with a concurrent reduction of root-associated fungal inoculum.

Diversity of AM fungal morphotypes at the experimental site was typical of agricultural fields, as determined through spore extraction from greenhouse grown host crops grown in undiluted field soil. Fewer identifiable morphotypes were found in

organic plots than in other treatments. Additional diversity is likely to exist, as a high proportion of extracted spores were parasitized, and pale spores were not readily detected using the methods undertaken in this study. Spore morphotype diversity could also be expected to be enhanced through extractions occurring directly from field soil, especially if assessed at different points within a season and in association with others of the various plants that were sown or that established naturally in plots during this study.

Non-AM fungal root endophytes constitute an extensive group of coexisting organisms that can be difficult and confusing to distinguish among using microscopy to visualize within roots. This creates conditions that discourage investigators from assessing levels of colonization. While some fungal structures stain using non-vital dyes and are therefore mistaken for AM fungal development in some studies, others have no pigmentation and do not stain using conventional dyes; this can lead to overestimation of AM fungal colonization as well as inaccurate evaluations of the extent of other endophytic development. The presence of unstained, hyaline hyphae within roots in this study makes it likely that non-AM fungal root endophyte colonization levels were underestimated; *Microdochium bolleyi*, a saprophytic endophyte that was predominant only in tomato roots harvested from organic plots, undoubtedly suffered from underestimation for this reason. With the exception of the exclusive occurrence of *M. bolleyi* in organic plots, there were no dramatic differences in the types or levels of non-AM root endophyte colonization in end-of-season field plants or infection potential host roots that could be confirmed among treatments. In addition, there was no antagonism detected between AM and other endophytic root fungi with regards to their ability to colonize plant roots, as root co-occupancy was positively correlated.

While many of the management practices associated with intensive agricultural production have long been considered detrimental to the ability of AM fungal populations to form associations with plants and even to persist in the soil, little research has examined the degree to and rate at which recovery is likely to occur upon cessation or alteration of farming strategies. In addition, relatively little work has been accomplished regarding the impact farming strategies have on the diverse group of non-AM fungal root endophytes that are known to be widespread in agricultural fields; nor has the extent that these organisms occur together in the cortex with their mycorrhizal counterparts been thoroughly investigated.

This study has shown that general repression of field root colonization by AM fungi occurs in this field when abundant available P and periodic soil disturbance takes place, although persistent infective propagules remain. Continuous tillage has the clearest negative impact on the ability of AM fungi to initiate primary infection, but this effect is lost after a single season in the presence of host substrate. Field-wide waterlogging in year five seems to have contributed to reducing the production of infective propagules of all root endophytic fungi except for the AM fungi in the organic treatment. The mechanism through which flooding reduced the ability of endophytes to cause primary infection was likely related to reduced root growth and propagule formation in the soil of treatments with higher bulk density, as well as slower rates of water drainage. Morphotypes of *Glomus* spp. were the most common spore type extracted from host crops and no reduction in morphotype diversity could be verified in any treatment. Non-AM fungal root endophytes were more abundant and infective than AM fungi in all treatments; *Phialophora* spp. were among the most commonly identified

fungi within field roots, while *Olpidium* spp. colonized maize roots heavily in the infection potential study. Coexistence between groups of endophytic fungi was general and positively correlated. The fungal endophyte *M. bolleyi* was unique to organic tomato roots, probably due to significant organic matter inputs and the production of a favorable cover crop directly preceding tomato transplantation.

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BIOGRAPHICAL SKETCH

Chris Rasmann was born in Olympia, Washington, but grew up in central and southern Maryland. While attending the University of Maryland at College Park pursuing an undergraduate degree in agronomy, he worked with Dr. Judith Abbott at the USDA Horticultural Crops Quality Lab at the Beltsville Agricultural Research Center (BARC), and later assisted with the research of Dr. Les Vough, the University of Maryland's forage crop specialist. Prior to graduating, Chris took a sabbatical from school to seek a practical application of the theory he had been studying. After visiting farms in the eastern provinces of Canada and the northeastern United States as a WWOOFer (Willing Worker On Organic Farms), Chris managed a small organic farm in Greeneville, Tennessee. Returning to the University of Maryland to finish his undergraduate degree in 2001, he began to work with Dr. Michel Cavigelli on the USDA Farming Systems Project at BARC. Upon graduation in 2003, Chris began pursuing a Master of Science degree in the Plant Pathology Department at the University of Florida.