THE RESPONSE OF THE ROOT AND SOIL FUNGAL COMMUNITIES TO COMPETITION AMONG *AMPHICARPAEA BRACTEATA, ONOCLEA SENSIBILIS,* AND THE INVASIVE *MICROSTEGIUM VIMINEUM* IN NORTHERN VIRGINIA

by

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The Response of the Root and Soil Fungal Communities to Competition among *Amphicarpaea bracteata, Onoclea sensibilis,* and the Invasive *Microstegium vimineum* in Northern Virginia.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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DEDICATION

Soli Deo Gloria.

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LIST OF ABBREVIATIONS AND SYMBOLS

AM.....Arbuscular Mycorrhizal Fungi
ARISA .....Automated Ribosomal Intergenic Spacer Analysis
BL .....Burke Lake Park
BLAST..... Basic Local Alignment Search Tool
BSA .....Bovine Serum Albumin
C ..... Celsius
CLS-VF..... Cell Lysis Solution for plants
CLS-Y..... Cell Lysis Solution for fungi
DEPC ..... Diethylpyrocarbonate
DES..... DNase/Pyrogen Free Water
diH_2O ..... deionized water
DSF.....Dark Septate Fungi
FAM..... Fluorescein Amidite
HM..... Huntley Meadows Park
HCL .....Hydrochloric acid
ILS ..... Internal Lane Standard
KOH..... Potassium Hydroxide
M..... Molar
Mg..... Magnesium
ml..... Milliliters
NaOCL..... Sodium Hypochlorite (Bleach)
NH_4OH.....Ammonia Hydroxide
NRCS..... Natural Resources Conservation Service
OTU ..... Operational Taxonomic Unit
PCA ..... Principal Component Analysis
PCO ..... Principal Coordinate Analysis
PCR.....Polymerase Chain Reaction
PPS.....Protein Precipitation Solution
SEWS-M .....Salt/Ethanol Wash Solution
Taq.....Taq polymerase
TB ..... Trypan Blue
VAM..... Vesicular Arbuscular Mycorrhizae
µm.....Micrometers

ABSTRACT

THE RESPONSE OF THE ROOT AND SOIL FUNGAL COMMUNITIES TO COMPETITION AMONG *AMPHICARPA BRACTEATA, ONOCLEA SENSIBLIS,* AND THE INVASIVE *MICROSTEGIUM VIMINEUM* IN NORTHERN VIRGINIA.

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Of the many exotic plants occurring in the United States, the Asian grass *Microstegium vimineum* is highly invasive. A number of environmental variables seem to play a part in *Microstegium'*s invasiveness. However, the exact mechanisms that allow *M. vimineum* to outcompete native plants are not altogether known. This research used Brightfield microscopy and Automated Ribosomal Intergenic Spacer Analysis (ARISA) to investigate the role of endophytic fungi including mycorrhizae in this grass' invasive tendencies. Fungal communities of two native plants (*Onoclea sensibilis* and *Amphicarpaea bracteata*) were compared with the fungal communities found in *Microstegium.* All three plants occurred as monoculture communities and mixed communities in Northern Virginia.

Brightfield microscopy identified Arbuscular Mycorrhizal fungi (AM) and Dark Septate Fungi (DSF) in all three plants. AM colonization rates were greater than 50% in all three plants in both monoculture and mixed plant communities.

Principal Coordinate Analysis (PCO) of ARISA fingerprint data identified fungal communities unique to each monoculture. When the three plants co-occurred in the mixed setting, *Microstegium* was found to maintain its unique fungal community while the two native plants were found to share their communities. A few small shifts between the communities in the grass and the fern suggest that the invasive grass might be integrating its fungal community into the native plant community. Unlike the root samples, multivariate analysis of soil fingerprints did not identify fungal communities unique to each plant. Instead, soils showed a random distribution. Overall, the sharing of fungal communities between roots of the two natives may represent the long-standing relationship between the two natives in contrast to the recently introduced exotic grass.

INTRODUCTION

Exotic species are invading many ecosystems in the United States. Well known examples of such invasive species include Kudzu, Garlic Mustard, and Honeysuckle. In addition to these well known species are numerous other less commonly known plants showing equally invasive tendencies. One such species, *Microstegium vimineum* (Trin.) A. Camus, is the focus of this research.

Japanese Stilt Grass

Otherwise known as Japanese Stilt Grass or Nepalese Browntop, *Microstegium vimineum* is an annual C4 grass from Poaceae; it originates from Asia and is now found in more than 20 states (Fairbrothers & Gray, 1972; Warren, Wright, & Bradford, 2011). It is listed as "noxious" or "invasive" in several of them, including Alabama, Connecticut, Massachusetts, and Virginia (Department of Conservation and Recreation, 2009; "PLANTS Profile: *Microstegium vimineum*," 2011; Strickland, DeVore, Maerz, & Bradford, 2011). However, *Microstegium* is not listed as a Federal Noxious Weed (Animal and Plant Health Inspection Service, n.d.; USDA NRCS, n.d.). The Noxious Weed Act was enacted to address the problems caused by "undesirable plants" or plants identified as "[…] noxious, harmful, exotic, injurious, or poisonous, pursuant to State or Federal law (Federal Noxious Weed Act, 1975). More specifically, The Noxious Weed Act requires the Secretary of Agriculture and the Secretary of the Interior to take the

necessary action to control and research noxious weeds while educating the public (Federal Noxious Weed Act, 1975). Although this law does not directly support monitoring or control of this grass, several Virginia state regulations do indirectly or directly mention the monitoring and control indicated in the Federal regulation. Subsequent to the 1974 act, the Virginia Species Council created the Virginia Invasive Species Management Plan of 2005 (Virgina Invasive Species Council, 2005). The council did identify *Microstegium vimineum* as an invasive plant receiving management and/or monitoring at Huntley Meadows park. Within the Fairfax Park System in Northern Virginia, parks are seen as a public good that contribute to citizens' quality of life; the Fairfax County Park Authority has been designated to "manage natural resources on park lands and easements in order to provide an integrated network of natural resources, retain representative species and communities, maintain ecological processes and protect rare or unusual resources" (Fairfax County Park Authority, 2008). And, a Natural Resource Management Plan was created to aid the various parks' employees and public visitors in the management of both native and invasive species (Fairfax County Park Authority, 2009). This research seeks to assist the Fairfax Park Authority in accomplishing its goals of managing native plant resources and increasing awareness of invasive plant ecology.

Microstegium vimineum is often identified by the silvery line running down the center of its lanceolate, pale green leaves. Its culms may grow to more than 1 meter in length, rooting at the nodes; and in autumn, it produces a panicle inflorescence ("Japanese Stilt Grass," 2009; Zheng et al., 2004). Unlike many nonnative, invasive plants, it tolerates the shaded understory of temperate forests as long as sun flecks are

present; and its surrounding soils are often slightly acidic (pH 4.8 to 5.8) and moist (Adams & Engelhardt, 2009; Cole & Weltzin, 2004; Warren et al., 2011). Its seed bank persists for at least three years (Barden, 1987). However, seed germination is limited when leaf litter is present; *Microstegium* appears to grow best in areas with minimal litter; and exotic earthworms exacerbate the problem by reducing the ground litter (Kourtev, Ehrenfeld, & Huang, 1998; Schramm & Ehrenfeld, 2010).

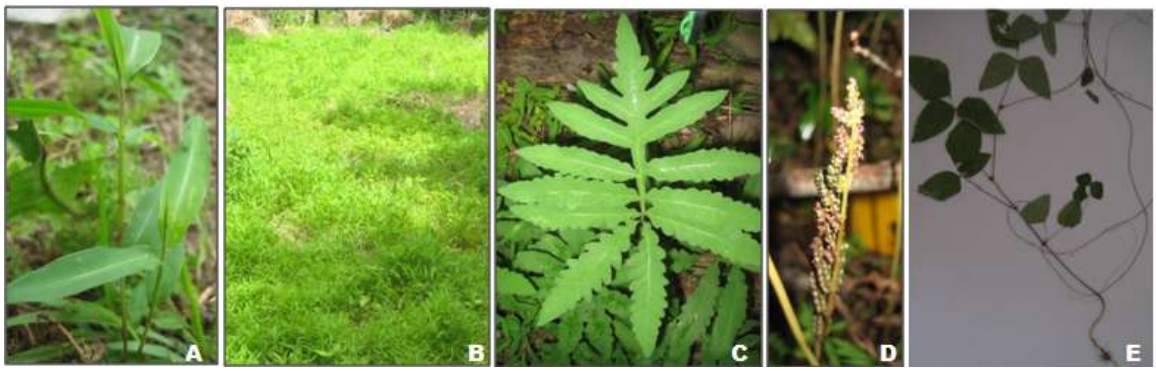

Figure 1. *Microstegium*, *Onoclea*, and *Amphicarpaea* morphology.
Figure1A and B. Microstegium vimineum growing in solitary and community populations in Northern Virginia. Figure 1C and D. Onoclea sensibilis sterile and fertile fronds at Burke Lake Park. Figure 1E. *Amphicarpaea* bracteata collected from Huntley Meadows. Credit: B.N.

As with all invasive species, M. *vimineum* interacts with its new habitat in a different manner than it does with its native habitat. In its Asian habitat, 12 fungi species and 8 arthropod species function as its herbivores and pest pathogens (Zheng et al., 2004). Outside Asia, there are no confirmed natural pathogens or predators (Kleczewski, N. M. & Flory, 2010). However, two recent studies suggest that this grass may have several potential pests and/or pathogens, including several species of Orthoptera insects and a type of leaf blight in the genus *Bipolaris* (Bradford et al., 2009; Kleczewski, N. M.

& Flory, 2010). Further research on these predators/pathogens may provide the foundation for their future use as biocontrols of this invasive grass.

M. vimineum is able to form dense stands in floodplain areas (Barden, 1987; Kourtev, Ehrenfeld, & Häggblom, 2003). Within these floodplain ecosystems, the *Microstegium* stands have been directly linked with habitat changes including losses in native plant species richness, native plant species cover, and seedling regeneration (Adams & Engelhardt, 2009; Oswalt, Clatterbuck, Oswalt, Houston, & Schlarbaum, 2007). The presence of this grass is linked with a reduction in soil particulate organic matter and an increase in soil mineral-associated carbon (Strickland et al., 2011). Likewise, the presence of this plant is correlated with decreases in arthropod populations (Simao, Rudgers, & Flory, 2009).

In addition to the absence of predators and pathogens, it appears that several habitat and phenological traits enable the invasive tendencies of this plant. Warren et al. (2011) recognized M. *vimineum* as possessing great niche breadth as a product of its ability to adapt to various light habitats while Barden (1987) found disturbance to be a prerequisite for habitat invasion. Baiser et al.(2008) provides evidence that deer overabundance creates the necessary disturbance for *Microstegium* invasion by over grazing the native plant understory that would otherwise shade out the stilt grass seeds and prevent their germination and growth. The deer may also provide a mechanism for seed dispersal (Baiser et al., 2008); seeds consumed and later defecated are still viable (Williams, Ward, & Ramakrishnan, 2008) . Cole and Weltzin (2004) indicate that *M. vimineum* alters the soil community; and, Kourtev et al. (2003) supports their findings by

demonstrating that both soil pH and soil nitrogen were changed under *Microstegium* in a New Jersey park. This grass appears to exhibit allelopathic abilities. Pisula and Meiners (2010) found that *Microstegium* has a stronger affect than Garlic Mustard upon seed germination. However, Corbett and Morrison (2012) found that although *Microstegium* is allelopathic, its allelopathic affects are not as strong as the affects of White Snakeroot, a native plant that is able to grow alongside the grass.

Previous research implies that vesicular-arbuscular mycorrhizal (VAM) fungi may be important to *M. vimineum* growth (Kourtev, Ehrenfeld, & Häggblom, 2002; Nord, 2011; Ross, 2008). More specifically, the mycorrhizae function in nutrient acquisition for this plant (Lee, Tu, & Hu, 2009). While mycorrhizae have been identified with this grass, mycorrhizal fungi do not appear to be necessary for the grass' survival (Ross, 2008). Not having an obligate relationship with mycorrhizae may enable the grass to outcompete the native plants that are dependent upon mycorrhizae.

Mycorrhizae consist of several species of filamentous fungi located in the soil; these fungi maintain symbiotic or mutualistic relationships with plant roots (Raven, Evert, & Eichhorn, 2005; Read & Perez-Moreno, 2003). Mycorrhizae are known for their roles in absorbing nutrients (especially phosphorus) from the soil environment and transferring these nutrients to their host plant in exchange for carbon(Chen et al., 2007; Read & Perez-Moreno, 2003). Indirectly and directly, these fungi have numerous impacts on their environment, including soil particle agglomeration (Nasim, 2005; Rillig & Mummey, 2006) and breakdown of minerals and other compounds (Read & Perez-Moreno, 2003). While mycorrhizae and plants can have an impact upon the soil, the

opposite is also true -- soil aggregates and their elemental constituents can have affects on plant and mycorrhizal growth. According to Nadian et al. (2009) large soil particles or aggregates can impede root growth. Nevertheless, mycorrhizae are able counter this problem and assist the plant's growth, resulting in mycorrhizal plants having longer roots than non-mycorrhizal roots (Nadian et al., 2009).

The likelihood that root mycorrhizal fungi provide a key mechanism by which *Microstegium* competes with or manipulates a native plant community is supported by several studies of other exotic plants invading native communities. Andrews (2011) found that the root fungal community of the native plant *Panicum virgatum* shifted toward the root fungal community of the invasive *Lespedeza cuneata* when both species grew in a mixed community. And, similar research by Marler et al. (1999) found that the invasive spotted knapweed (*Centaurea maculosa*) was more competitive when grown with mycorrhizae and the native bunchgrass *Festuca idahoensis*. The invasive, non-mycorrhizal Garlic Mustard (*Alliaria petiolata*) has been directly correlated with a decrease in the mycorrhizae of native plants (Burke, 2008; Roberts & Anderson, 2001; Stinson et al., 2006). And, the invasive, mycorrhizal Cheatgrass (*Bromus tectorum*) of the western US was found to decrease arbuscular mycorrhizal richness in native California grasses and to increase richness in a native dicot (Hawkes, Belnap, D'Antonio, & Firestone, 2006).

In regard to *Microstegium* growing concurrently with other species, previous observation has noted that this grass occurs in conjunction with Japanese Honeysuckle as "monospecific stands which are separated by a strikingly narrow zone of mixing"

(Barden, 1987). The grass has also been found to grow concurrently with the native, allelopathic White Snakeroot in New Jersey forests (Corbett & Morrison, 2012). Another observation provides a third scenario. Monospecific stands of *Microstegium vimineum* are observed to grow in conjunction with monospecific stands of Hog Peanut (*Amphicarpaea bracteata* [L.] Fernald) in the moist soils of a Virginian forest (North, personal oberservations, August 19, 2011). Interestingly, a third species also played a role in this ecosystem. *Onoclea sensibilis* L. (Sensitive Fern) was also identified growing in the "mixed" zone with *Amphicarpaea* and *Microstegium* (North, personal observations, June 16, 2011).

Hog peanut

Amphicarpaea bracteata, one of the native plants observed to interface with *Microstegium*, is a leguminous plant indigenous to eastern North America (Chayka, 2011; "PLANTS Profile: *Amphicarpaea bracteata*," 2011). It is often found in moist woods, and is characterized by alternate, compound leaves made of three ovate, pointed leaflets. It has a vine-like tendency depicted by stems that can wrap or coil around other objects (Chayka, 2011). Flowers, when present, are of two types: cross-pollinated or self-pollinated ("PLANTS Profile: *Amphicarpaea bracteata*," 2011). The cross-pollinated flowers resemble pea flowers and occur as an individual cluster near the end of a stem; resulting fruits are underground pods (Chayka, 2011; Turner & Fearing, 1964). While little is known about its roots, it does appear to support ectomycorrhizal and vesicular-arbuscular fungi (Berliner & Torrey, 1989; Crabtree, Keller, & Ely, 2010; Landis, Gargas, & Givnish, 2004). Its roots support the nodules commonly associated

with leguminous plants; and at least one insect pest (*Rivellia pallida*) is known to feed upon these root nodules (Foote, 1985).

Sensitive Fern

The second species observed to interact with *Microstegium vimineum* and *Amphicarpaea bracteata* – *Onoclea sensibilis* L. – is a fern native to wetland ecosystems of eastern North America ("PLANTS Profile: *Onoclea sensibilis*," 2011). The sensitive fern has both sterile and fertile fronds. Sterile fronds are bright green in color and pinnatifid with approximately 12 opposite or nearly opposite pinnae along a smooth rachis; blade margins are wavy. Fertile fronds are non-leafy, with beadlike pinnules enclosing the sori (Cobb, Farnsworth, & Lowe, 2005). Research by Bell and Klickoff (1979) is inconclusive as to whether *Onoclea sensibilis* is affected by allelopathic chemicals of other plants, specifically ferns. This plant does seem to tolerate the allelopathic chemicals produced by *Juglans nigra* when given extra horticultural assistance (The Morton Arboretum, n.d.). Other research indicates that this fern supports arbuscular mycorrhizal fungi (Shannon M. Berch & Kendrick, 1982; West, Brandt, Holstien, Hill, & Hill, 2009).

Analysis of background information concerning *Microstegium vimineum*, *Amphicarpaea bracteata*, and *Onoclea sensibilis* indicates that abiotic and biotic factors can perform important roles in the ecology of these species and especially in the invasiveness of *Microstegium*. However, the lack of information regarding soil properties and root fungi for *Microstegium vimineum*, *Amphicarpaea bracteata*, and *Onoclea sensibilis* leads to several questions: (1) Do the grass, fern and legume exhibit

root fungal colonization? (2) Are the root fungal colonies of each species (if present) different from each other? (3) If root fungi are present and different among the species, do the legume and fern root fungal communities mimic the grass root fungal community if the three species are growing together in a "zone of mixing"? (4) And, if *Amphicarpaea bracteata* grows in conjunction with the fern, do these native plants share the same root fungi?

Objectives and Hypothesis

The main objectives of this study are: 1) to characterize the root fungal communities of *Microstegium viminium, Amphicarpaea bracteata,* and *Onoclea sensibilis,* and 2) to determine if root fungal communities change (i.e. mirror other nearby fungal communities) when *Microstegium vimineum, Amphicarpaea bracteata,* and *Onoclea sensibilis* grow together.

Hypothesis I: *Microstegium vimineum, Amphicarpaea bracteata,* and *Onoclea sensibilis* plants all support distinct root fungal communities when grown alone.

Hypothesis II: Based on previous research with other invasives, fungal communities from *Amphicarpaea, Onoclea,* and *Microstegium* plants growing in a "mixed" zone will change in a such a manner that the *Microstegium* fungal community is incorporated into *Amphicarpaea* and *Onoclea sensibilis* fungal communities.

METHODS

Study Sites: Huntley Meadows Park

Huntley Meadows (HM), the primary site for this study, is a 1,426 acre park supporting many wetlands with numerous forms of wildlife in Fairfax County, Virginia. In its history, Huntley Meadows has functioned as a plantation, an asphalt testing location, and National Guard and Navy centers. It did not become a park until Gerald Ford's Presidency in the 1970's ("Huntley Meadows Park," 2011). The park supports several trails – one for biking and the others for hiking. A boardwalk with observation platforms and an observation tower border the central wetland and create a popular location to observe wildlife and birds.

Currently, Huntley Meadows is facing a large invasion of *Microstegium vimineun* (K. Munroe, personal communication, October 29, 2011). In 2001, this park participated in an experiment funded by the National Fish and Wildlife Foundation to determine the best method of removing *Microstegium*; test plots from the experiment indicated that chemical control is the most effective method of removing stilt grass (Fairfax County Park Authority, 2009). The park has tried other methods to control the population of this plant. These methods have included hand pulling, seeding with aggressive natives, and burning. The proposed wetland restoration might diminish grass invasion by raising the water table and thereby eliminating the grass's available habitat (K. Munroe, personal

communication, October 29, 2011). In spite of these measures, *Microstegium* continues to maintain a large population in the park.

At the first Y intersection on the main trail at Huntley Meadows (between the Cedar Trail and the Heron Trail), is an area supporting a nearly ideal experimental set-up where monospecific stands (hereafter referred to as monocultures) of both *Microstegium* and *Amphicarpaea* are located next to a large patch of mixed vegetation. The mixed vegetation consists predominantly of *Onoclea sensibilis*, *Amphicarpaea bracteata*, and *Microstegium vimineum*. However, *Onoclea sensibilis* does not occur as a monoculture at Huntley Meadows in the way that *Amphicarpaea* and *Microstegium* occur. Monocultures of this fern were found at a separate site – Burke Lake Park.

Figure 2. An aerial view of Huntley Meadows Park.
Sample site identified by the red box and parking lot identified by the yellow arrow.

Figure 3. Huntley Meadows sample site during the spring. Credit: B. North

Figure 4. Huntley Meadows sample sites during the autumn. Credit: B. North

Study Sites: Burke Lake Park

Burke Lake Park (BL), identified as a second research location, is also located within Fairfax County ("Burke Lake Park, Fairfax County Park Authority," 2011). Its main attractions are fishing at the main lake, hiking on various trails around the lake, camping, and golfing.

Burke Lake hosts a mixed hardwood forest following the description provided by Weakley et al. (2012) of the mesic mixed hardwood forest that occurs in Virginia's Piedmont. The forest canopy consists predominantly of Beeches, Oaks, Maples, and Tulip-poplars; honeysuckle and holly occur in the understory (North, personal observations, June 16, 2011). Along the main trail, several large areas of *Microstegium* are found growing in the herbaceous layer. At mile marker 2.5, more than four populations of *Onoclea sensibilis* are found growing in the herbaceous layer (Figure 2). *Microstegium* is found nearby as is *Woodwardia areolata*, a look-alike to *Onoclea sensibilis* (North, personal observations, June 16, 2011). The populations of *Onoclea* provided the necessary comparison for the monoculture and mixed communities found at Huntley Meadows.

Figure 5. *Onoclea* sensibilis present in the forest understory at Burke Lake Park (spring 2012). Credit: B. North

Figure 6. Burke Lake Park sample site during autumn 2012. Credit: B. North

Field Methods: Collecting Samples

With collection permits from the park authority, research commenced at the two sites of choice during September 2011. The first collections and field assessments were completed during late September and early October 2011 for the first year, and collections were repeated in June the following spring/early summer for verification of data. Precipitation during the autumn was wetter than normal with September rainfall exceeding the average, but the precipitation the following spring/summer was slightly drier than normal with rainfall not meeting the average ("Washington D. C. Precipitation," n.d.).

Individual plant samples consisted of 1 whole fern plant (root and shoot), >10 whole stilt grass plants, and >10 whole hog peanut plants. Representative grass, legume, and fern plants were collected in autumn 2011 to function as herbarium vouchers in the Ted R. Bradley Herbarium. Collection of samples for analysis followed the design used by Batten et al. (2006), modified such that the grass, peanut, and fern formed individual "monoculture" patches with a separate area where they intermingled. Since round quadrats are considered more accurate than square quadrats due to their smaller perimeter (Fidelibus & MacAller, 1993), round quadrats (diameter =71.6 cm) were subjectively placed in each community. Samples were collected from each quadrat. In the monoculture populations, a single sample was collected from each quadrat. Nine quadrats were identified for the monocultures (three for each plant). Where the three plant populations mixed (i.e. the "mixed community"), three plant samples were collected from each quadrat: one sample from each species. There were three quadrats total for the mixed plant community. The five communities analyzed at Huntley Meadows were as

follows: (1) the *Microstegium* monoculture community, (2) the *Amphicarpaea* monoculture community, (3) the *Amphicarpaea* community in the "zone of mixing," (4) the *Microstegium* community in the "zone of mixing," and (5) the *Onoclea* community in the "zone of mixing." The single community analyzed at Burke Lake was (6) the *Onoclea* monoculture community. Several grams of rhizosphere soil were also collected from each quadrat during fall 2011 and spring 2012 for a total of 24 soil samples. These samples were later analyzed for pH to verify that soils between sites were similar. Soil samples were also analyzed for rhizosphere fungal communities to determine if the fungal communities present in roots were also present in the soil.

Sample quadrats were analyzed for aerial plant species richness, aerial percent vegetative cover, and rhizosphere soil color. Following this ecological analysis, plant samples were gathered from *Microstegium, Amphicarpaea,* or *Onoclea* depending on the community type. Each field collection consisted of 18 samples total: 9 from the monocultures and 9 from the mixed community. More simply, each plant was represented by 3 monoculture samples and 3 mixed samples. Individual plant samples consisted of sufficient plant tissue to conduct molecular and microscopic analysis – enough tissue to fill 0.25-0.5 of a 3.78 L freezer bag. Since *Microstegium* is a stoloniferous grass, *Amphicarpaea* is a climbing legume, and *Onoclea* is rhizomatous, individual plants can be difficult to ascertain. Therefore, samples included both aerial leaves and underground root biomass in order to ensure that roots of one species were correctly identified and not confused with roots of another species. Sampled roots and

soil were placed on ice and transported to the laboratory where they were stored at -20°C until processed for microscopy and molecular analysis.

Field Methods: Mapping Plant Communities

Two mapping expeditions occurred between fall 2011 and fall 2012 to document the locations of quadrats and plant communities. The autumn was preferable for mapping sites as reduced forest canopy cover allowed for better satellite connection with the GPS unit. Mapping of quadrats during the late spring or summer when leaf emergence was at its fullest would have interfered with GPS and satellite connections, and thereby produced less reliable coordinates.

Mapping required a data dictionary for the GPS unit. The data dictionary consisted of point features for the 12 quadrats (i.e. 9 monocultures and 3 mixture quadrats), area features for the plant populations (3 monocultures populations and 1 mixed community), and extra features (point, line, and area) for unknown items of interest. The dictionary was transferred to a Trimble GPS (Trimble GeoXT, 2005 Series) and was then used to create a new data file (geographic coordinate system: GCS_WGS_1984; datum: D_WGS_1984). Then, spatial points, lines, and areas were collected at Burke Lake Park and Huntley Meadows Park. For each quadrat or point feature, 25 data points were collected to increase accuracy of latitude and longitude readings. Lines identified trails, and areas depicted whole plant populations. After the second data collection, the data file was transferred to Pathfinder Office and exported as shapefiles for analysis in ArcMap 10.

In ArcMap, analysis included the Pathfinder Office exported shapefiles and the Fairfax County's soil shapefile (obtained from the Natural Resources Conservation Service [NRCS] website: http://soildatamart.nrcs.usda.gov/). The soil shapefile was transformed into the same coordinate system as the other shapefiles to ensure correct placement of features. Layers were placed over ESRI basemap imagery. Later, the "soil names" attribute was added to the soils feature to identify soils found at each respective plant site.

In the field, spider webs and shrubs were avoided when mapping the area features, and this slightly altered the coordinates of the polygon vertices. Therefore, vertices of area features were corrected in ArcMap to more accurately reflect the actual plant areas. The final maps required multiple data frames to give perspective on where the sites were located. Legends, scale bars, North arrows, and titles completed the maps.

Molecular Laboratory Methods

Figure 7. *Onoclea* fine roots after washing.

After field collection, plant samples were washed in deionized water, and roots were separated from stems and leaves using flame sterilized scissors and forceps (Figure 7). Washed roots were stored at -80°C until microscopic and molecular analysis. For molecular methods, 0.5-2.0 grams of thawed wet roots were processed following the protocols employed by Bills and Polishook (1994). Roots were pulverized with autoclaved, deionized water in an autoclaved blender. The resulting products were washed through a series of autoclaved mesh sieves (e.g. 2 mm, 500µ, 210µm, and 106µm). The products on the 106µm sieves were transferred to 50 ml centrifuge tubes where they were washed 10 times with sterile deionized water using the methods followed by Torzilli et al. (2006) and Andrews (2011). Products were stored at -80°C until DNA extraction.

Fungal DNA was extracted from the homogenized root products using the BIO101-FastDNA Spin Kit for Tissues and the modified protocols of Andrews (2011) (Appendix I). The modified protocol indicated by Andrews (2011) used an additional ceramic bead and 800 μL CLS-VF and 200 μL PPS in place of the CLS-Y normally used for fungi (appendix I). Fungal DNA was extracted from the soil using the FastDNA Spin Kit for Soil. The extraction protocol for the soil is included in appendix II.

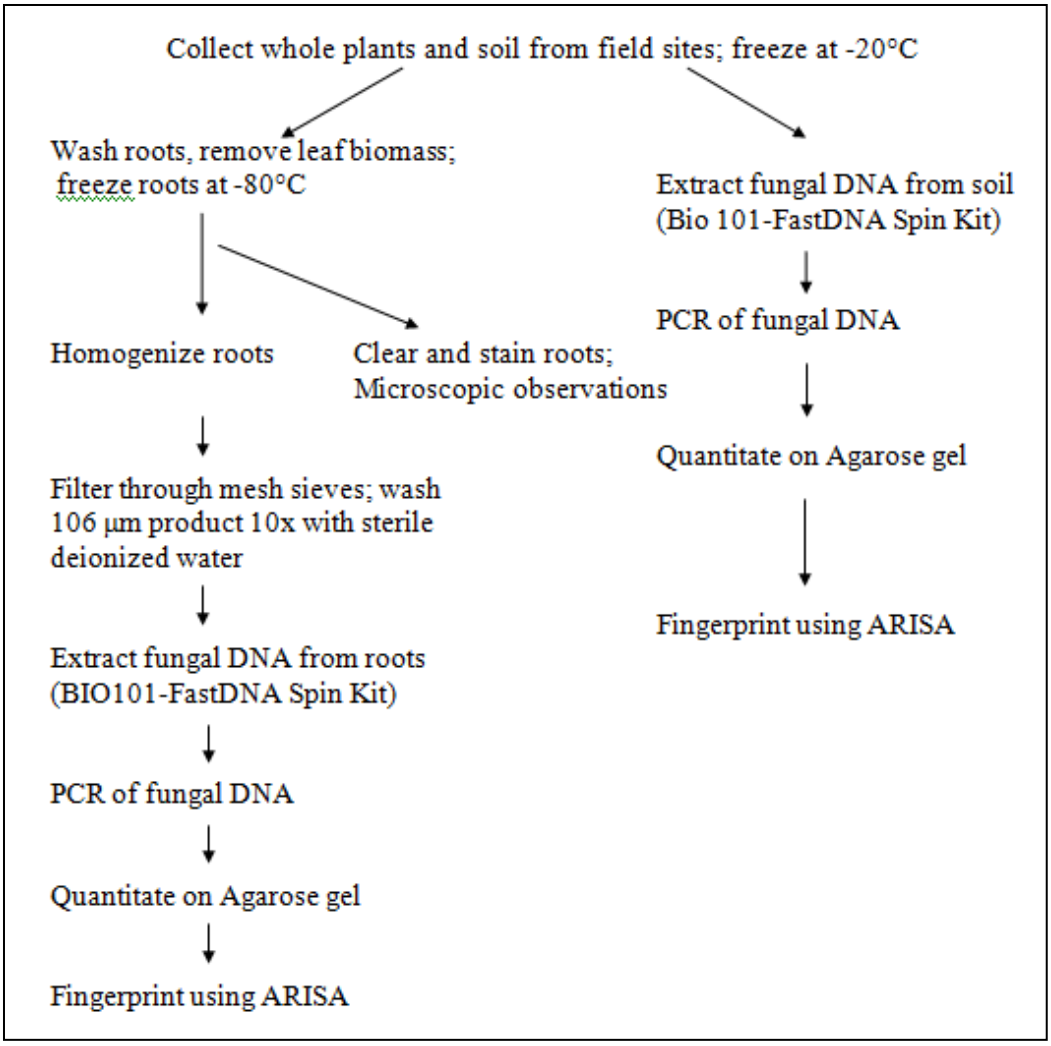

Figure 8. Flowchart showing method for analyzing collected roots and soil.

Extracted DNA from roots and soil was amplified using Polymerase Chain Reactions (PCR) and characterized by subjection to Automated Ribosomal Intergenic Spacer Analysis (ARISA) following the methods used by Torzilli et al. (2006). ARISA characterization distinguishes fungi by differences in DNA amplicon length rather than differences in the nucleotide sequences.

The PCR reaction mixture used DNA from the extraction step mentioned above. Extracted DNA from fall roots samples was diluted 1:5 (1 µL DNA to 4 µL DEPC) for best results. For spring root samples, best results came from undiluted DNA. For soil samples, DNA was diluted 1:50 (1µL DNA to 49 µL DEPC) for best results. Positive control DNA was from lichens and store bought mushrooms. The PCR reaction mixture for the root samples required a master mix of the following components added in sequential order: 7.9 µL DEPC H2O, 2µL 10X Reaction Buffer, 2 µL 25 mM Mg mixture, 2µl dNTPs (2 mM each), 1 µl Forward Primer, 1 µl Reverse Primer, 2 µl 0.1% BSA, 0.1 µl Taq Polymerase (5 units/µl) and 2 µl DNA per reaction. This PCR reaction mixture was also used for the soil fungal DNA. Primers for PCR reactions were the FAM labeled ITS1F forward primer and ITS2 reverse primer (Table 1). These primers amplify fungal DNA fragments from the ITS region of the 18S ribosomal RNA genes (Redecker, 2000).

Table 1. Primers to amplify the ITS region of fungal DNA (White et al., 1990 as cited in Binder and Hibbett (n.d.).

Primer Name	Sequence
ITS1F	CTTGGTCATTTAGAGGAAGTAA
ITS2	GCTGCGTTCTTCATCGATGC

Each PCR reaction began with an initial denaturing step of 11 minutes at 95 °C. Subsequently, the PCR reaction mixture underwent 35 cycles of DNA denaturing (30 seconds at 95.0 °C) , annealing (30 seconds at 52°C), and progressive extension (2

minutes plus 5 seconds per cycle at 72.0°C). Products of the PCR reactions were visualized on 1% agarose gels (TAE buffer) containing ethidium bromide. For each plant and soil sample, one or more PCR replicates verified the repeatability of DNA amplification. The PCR products were then diluted with a mixture of ILS 600 (Internal Lane Standard) and HiDi Formmamide in preparation for fingerprinting using ARISA. More specifically, 1 μL of each PCR product was diluted with 9 μL of the mixture formed by ILS and HiDi Formmamide (22 μL ILS + 978 μL HiDi Formmamide). ARISA fingerprints created electropherograms for each root or soil sample. Each peak in the electropherograms represented a fragment of fungal DNA, otherwise known as an Operational Taxonomic Unit (hereafter referred to as OTU[s]). These electropherogram fingerprints were uploaded to George Mason's Microbiome Analysis Center where the peaks or raw values were transformed into relative abundances for each sample. These relative abundances were downloaded to a personal computer and were imported into Microsoft Excel. Root and soil sample names were then recoded into 8 digit codes in preparation for the multivariate analysis in PC-ORD 6 software. Each OTU was than analyzed for binning errors. Several OTUs had multiple columns assigned to a single fragment length. In these situations, the columns were combined such that only one column was assigned to that OTU.

Microscopy Methods: Root Clearing and Staining

To further characterize the fungal communities of the grass, legume, and fern roots, samples were microscopically analyzed for percent fungal colonization using the methods proposed by McGonigle et al. (1990) and Vierheilig et al. (1998). Roots were

collected, cleared, stained, and measured for fungi colonization using the "field of view magnified intersections" method (McGonigle et al., 1990). The roots of each species differed in morphology and chemical composition. Specifically, *Onoclea* roots were relatively short, quite sturdy, and darkly pigmented. *Microstegium* roots were delicate with little to no pigmentation. And, *Amphicarpaea* fine roots were thick, pigmented, and covered in nodules. Due to this variability, the roots of each species required different clearing, staining, and destaining protocols (Table 2). Since *Microstegium* had the most delicate roots of the three species, optimal results were reached with the least amount of clearing. Two different methods of clearing and staining were used on *Amphicarpaea* roots because the spring samples disintegrated when exposed to the harsh methods used on the fall samples.

Table 2. Clearing and staining methods.

Species	Clearing method.	Stain
Microstegium vimineum	10% KOH for 15 minutes; diH2O rinse; 1 M HCL for 5 minutes	Trypan Blue (TB)
Onoclea sensibilis	10% KOH for 30 minutes; diH2O rinse; acidic bleach; diH2O rinse; alkaline bleach; diH2O rinse;1 M HCL; diH2O rinse	TB
Amphicarpaea bracteata (fall samples)	10% KOH for 30 minutes; diH2O rins; acidic bleach for 1 hr; diH2O rinse; alkaline bleach for 15 min.; diH2O rins; 1 M HCL for 5 min.; diH2O rinse	TB
Amphicarpaea bracteata (spring samples)	10% KOH for 15 minutes; diH2O rinse; acidic bleach for 15 min.; diH2O rinse; 15 min alkaline bleach; diH2O rinse; 1 M HCL for 5 min; diH2O rinse	TB

For all samples, between 0.1 and 0.2 grams of wet roots were weighed out for each sample. Roots were then cut into 0.5 -1.5 mm length fragments, transferred to 100 ml flasks, and covered with 10% KOH. Roots were then autoclaved for 30 minutes (all *Onoclea* samples and 2011 *Amphicarpaea* samples) or 15 minutes (all *Microstegium* samples and 2012 *Amphicarpaea* samples). After autoclaving, the roots were rinsed with deionized water. At this point, the heavily pigmented fern and hog peanut roots went through additional clearing steps using an acidic bleach (1 Molar HCL + 250 ml NaOCL) and an alkaline bleach (2 ml NH_4OH + 15 ml 30% H2O2 +83 ml H_20). The fern roots were placed in the acidic bleach for 1 hour, thoroughly rinsed with deionized water, soaked in the alkaline clearing agent for 15 minutes, and rinsed with deionized water. The fall hog peanut samples followed this protocol and the spring hog peanut samples followed a modified protocol (shortened times) to reduce the trauma to the cortex cells (table 2). After the clearing steps, roots from each species were acidified in 1 M HCL for 5 minutes to prepare the roots to uptake the stain. Then, roots were rinsed with diH_2O, and covered with a Trypan Blue staining solution (1:1:1 ratio of diH_2O, lactic acid, glycerol + 0.033g Trypan Blue for every 1/3 L) for at least 24 hours. If roots needed destaining for better contrast of cellular structures, they were placed in 90% lactic acid for 2-3 days. All cleared and stained roots were stored in 50% glycerol.

Microscopy Methods: Brightfield Analysis

In preparation for Brightfield microscopy, root fragments were placed on microscope slides with a few drops of 50% glycerol. Roots were covered with a glass cover slip and gently squashed to expose the root fungi. Slides were then analyzed using

Brightfield microscopy following the methods employed by McConigle et al. (1990). One hundred and fifteen intersects were observed for each plant community at 10X for the following categories of fungi: no AM hyphae, aseptate hyphae, arbuscules only, vescicles only, and arbuscules *with* vesicles. If coils or Dark Septate Fungi (DSF) were observed in a plant sample, they were noted as a comment separate from the category tallies previously mentioned. Fungal structures were further confirmed at 22X and 40X. Total abundances were calculated as percentages out of the 115 intersects.

Statistical Analyses

Summary statistics were used to analyze plant community diversity, soil pH, and microscopy colonization categories. Previous research of a similar nature to this study has utilized Principle Coordinate Analysis (PCO) and Canonical Correspondence Analysis (CCA) to analyze the multivariate components (Andrews, 2011; Martin, 2012). PCO analysis is preferred over the Principal Component Analysis (PCA) for datasets with many zeros (Peck, 2010). This study used PCOs to better understand the relationships between plant and soil samples and Operational Taxonomic Units (OTUs). More specifically, PCOs analyzed OTU communities from 1) all monoculture root samples, 2) all mixed community root samples, 3) both monoculture and mixed roots samples, 4) all monoculture soil samples, 5) all soil samples (both monocultures and mixed), and finally 6) all root and soil samples. This study also used a PCO ordination of microscopy data to analyze the similarities and differences of fungal communities based on fungal morphology.

PCO ordinations used primary matrices for the multivariate analysis and secondary matrices for labeling and graph symbols. The primary matrices listed plant samples (entities) by OTUs (attributes). The total number of OTU attributes varied among PCOs depending upon the number of OTUs present. Secondary matrices listed samples (entities) by community number (attributes); community numbers were assigned based on whether the sample came from a monoculture population or a mixed population. Odd numbers referred to monoculture communities and even numbers referred to mixed communities. The primary matrix for the microscopy PCO listed plant samples (entities) by fungal morphologies (attribute). The morphological attributes came directly from the five categories calculated during microscopy: 1) no VAM fungi, 2) vesicles only, 3) arbuscules only, 4) hyphae, and 5) vesicles with arbuscules. The secondary matrix for this PCO listed samples (entities) by plant community number (attribute). Again, odd numbers refer to monocultures and even numbers refer to mixtures.

All PCOs used the Sorensen (Bray Curtis) Distance metric with weighted averaging. Randomization tests verified the authenticity of calculated eigenvalues and suggested the number of interpretable axes. The number of randomizations was subjectively chosen as some number less than the number of samples for each respective PCO. Most often, the number of runs was the number of samples minus one (n-1). PCOs of the molecular fingerprint data were further interpreted using overlays that showed the relative abundance of OTUs in each root or soil sample. In the overlays, the size of the symbols represents the relative abundance of each OTU over a backdrop of the original

PCO. Finally, overlays of presence-absence transformed matrices verified the number of OTUs shared among samples or specific to individual samples (data not shown).

RESULTS

Site Maps

Two mapping expeditions identified the locations of quadrats and specific plant community boundaries at Huntley Meadows Park and Burke Lake Park. The fall 2011 expedition occurred after the plants in the herbaceous layer had senesced. As such, mapped plant populations were only estimates of the actual populations. The second expedition, occurring a year later, provided a more accurate estimate of the actual populations. The final map output shows the individual locations of quadrats within plant community areas (Figure 9 and Figure 10).

Table 3. Coordinates of sample quadrats.

Site	Quadrat	Coordinates
Burke Lake	fern 1	77°17'43.688"W 38°46'0.761"N
	fern 2	77°17'43.372"W 38°46'0.475"N
	fern 3	77°17'43.222"W 38°45'59.244"N
Huntley Meadows	grass 1	77°6'10.04"W 38°45'18.362"N
	grass 2	77°6'9.76"W 38°45'18.455"N
	grass 3	77°6'9.687"W 38°45'18.439"N
	peanut 1	77°6'10.05"W 38°45'17.801"N
	peanut 2	77°6'9.837"W 38°45'17.906"N
	peanut 3	77°6'9.9"W 38°45'18.058"N
	mixed 1	77°6'9.783"W 38°45'18.17"N
	mixed 2	77°6'9.874"W 38°45'18.172"N
	mixed 3	77°6'10"W 38°45'18.189"N

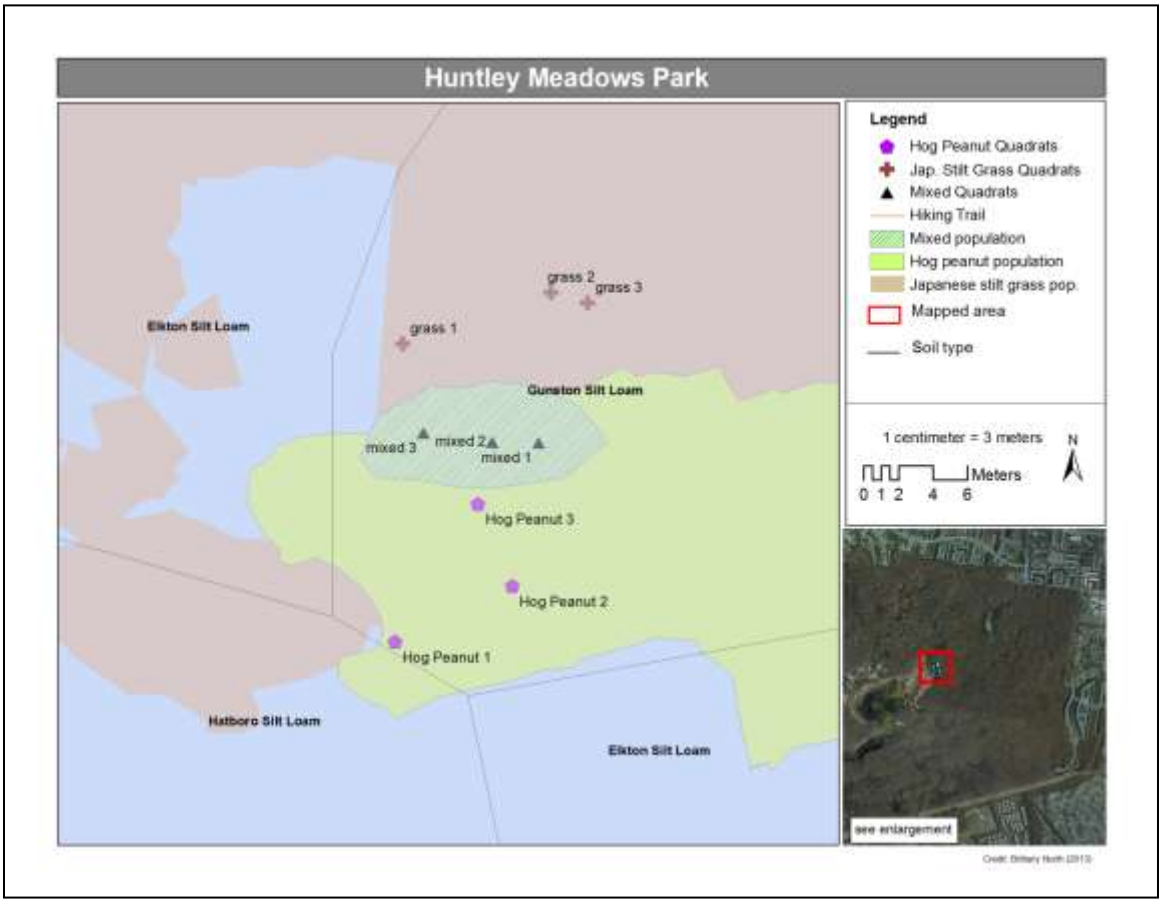

Figure 9. Map of field site at Huntley Meadows Park (software: ArcMap 10)

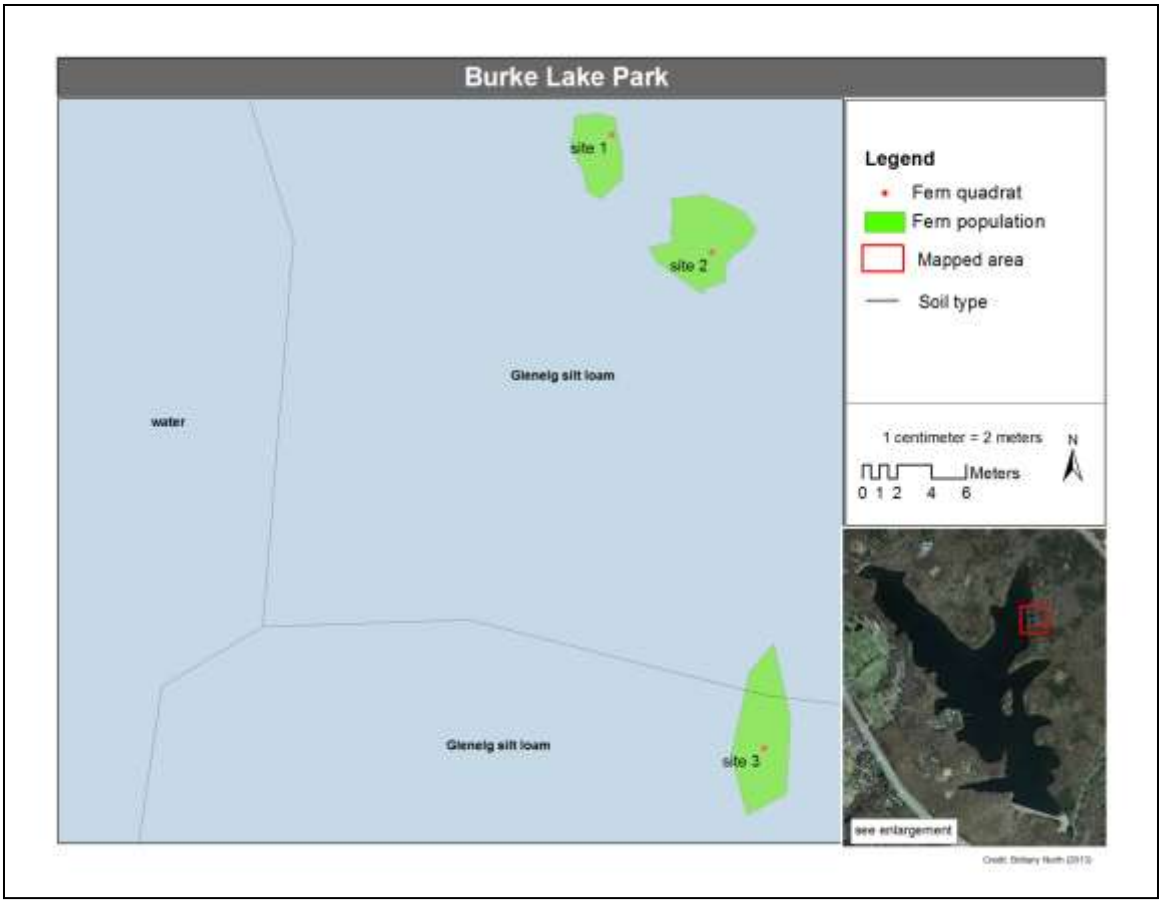

Figure 10. Map of field sites at Burke Lake Park (software: ArcMap 10)

Vegetative cover 2011-2012 of field sites

A general survey of the vegetation within and near the plots identified 24 plant species (Table 4). Twenty-two of these were identified to genus or species. Five of these plants are commonly considered invasive: *Lonicera* sp. (honeysuckle), *Alliaria petiolata* (Garlic Mustard), *Berberis* sp. (Barberry), *Celastrus orbiculatus* (Oriental Bittersweet), and *Toxicodendron radicans* (Poison Ivy).

Assessment of aerial vegetation for Huntley Meadows and Burke Lake sample sites revealed high cover values for all quadrats during both seasons (Table 5). No

quadrats exhibited bare ground, and the total cover for each plant community did not significantly change between seasons (Table 5). The total plant diversity for each community showed an apparent increase in diversity from the fall to the spring (Table 5).

Of all the communities, the stilt grass monoculture plots were nearly pure stands with only two species observed in the fall quadrats and seven observed during the spring (Table 5). While the fern and peanut did not form pure monocultures, they were dominated by the fern or peanut, respectively. The grass monocultures demonstrated the lowest plant richness of all the communities during both seasons (Table 5). In direct contract to the grass, the fern monoculture quadrats had the highest richness with 11 species during the spring sampling (Table 5); the fern dominated the monoculture communities with a mean aerial cover of 65% (fall) and 87% (spring). The peanut monoculture quadrats were also diverse with a mean of 10 species observed during the spring samples (Table 5). The aerial cover of the peanut biomass was lowest during the fall with a mean cover of 67% and highest during the spring with 90% mean cover (Table 5). During the autumn, only three species were observed in the mixed quadrats. However, during the spring, seven species were observed (Table 5). The greater than 100% "total cover" in all quadrats implies stratification as a result of morphological differences among plants. Some plants are taller than others, and some have different shaped leaves. Together, these features can create a dense herbaceous layer to absorb sun flecks. The rest of the cover is accounted for by leaf and twig litter underneath the aerial leaves. The differences between richness values and total cover between seasons for all monocultures and mixed communities implies the importance that temperature,

precipitation, seasonal light changes, and senescence can play in changing plant community structure.

Table 4. Survey of plant species present at Burke Lake (BL) and Huntley Meadow (HM) field sites

Common Name	Scientific Name	Within Quadrat?*	Sites
Avens	*Geum* sp.	yes	BL
Barberry	*Berberis* sp.	no	HM
Bedstraw	*Galium* sp.	yes	BL, HM
Beech	*Fagus* sp.	no	BL
Cinquefoil	*Potentilla* sp.	yes	BL, HM
Clubmoss	*Lycopodium*sp.	no	BL
Dogwood	*Cornus* sp.	no	HM
Garlic Mustard	*Alliaria petiolata*	yes	BL
Greenbrier	*Smilax* sp.	no	BL, HM
Hog Peanut	*Amphicarpaea bracteata*	yes	HM
Honeysuckle	*Lonicera* sp.	yes	BL, HM
Japanese Stilt Grass	*Microstegium vimineum*	yes	BL, HM
Jewelweed	*Impatiens capensis*	yes	HM
Maple	*Acer* sp.	no	BL
Oak	*Quercus* sp.	yes	BL, HM
Oriental Bittersweet	*Celastrus orbiculatus*	yes	HM
Partridge Berry	*Mitchella repens*	yes	BL
Poison Ivy	*Toxicodendron radicans*	no	HM
Sedge	n/a	yes	BL
Sensitive Fern	*Onoclea sensibilis*	yes	BL, HM
Tuliptree	*Liriodendron tulipifera*	no	BL
unknown grass	n/a	yes	BL, HM
Violet	*Viola* sp.	yes	HM
Virginia Creeper	*Parthenocissus quinquefolia*	yes	HM

*"yes" indicates that a species occurred in one or more quadrats; "no" indicates that a species was not identified within any of the quadrats but was observed at the field site.

Table 5. Mean percent cover composition and richness within community quadrats. Richness is the total number of species within community.

	Community							
	MIXED		Grass		Peanut		Fern	
Species	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
Bare Ground	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Litter	33.33	11.33	0.00	20.00	50.00	10.67	35.00	10.00
Microstegium vimineum	50.00	30.00	100.00	83.33	1.00	0.83	4.00	11.67
Amphicarpaea bracteata	17.50	43.33	0.00	2.50	66.67	90.00	0.00	0.00
Onoclea sensibilis	41.67	50.00	0.00	0.00	0.00	0.00	65.00	86.67
***Lonicera* sp.**	0.00	5.00	0.67	8.50	2.67	4.00	1.33	4.00
Parthenocissus quinquefolia	0.00	0.00	0.00	0.00	1.67	0.00	0.00	0.67
***Smilax* sp.**	0.00	0.00	0.00	7.00	0.00	5.00	1.33	2.33
Alliaria petiolata	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00
***Galium* sp.**	0.00	0.00	0.00	0.00	0.00	5.00	1.67	1.67
***Potentilla* sp.**	0.00	0.00	0.00	2.00	0.00	0.00	0.00	2.33
Mitchella repens	0.00	0.00	0.00	0.00	0.00	0.00	3.33	20.00
***Geum* sp.**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67
Impatiens capensis	0.00	5.00	0.00	0.00	0.00	5.00	0.00	0.00
***Quercus* sp.**	0.00	0.00	0.00	0.00	0.00	2.00	0.00	0.00
***Viola* sp.**	0.00	0.00	0.00	2.00	0.00	0.00	0.00	0.00
sedge - unknown	0.00	0.00	0.00	0.00	0.00	8.50	0.00	0.00
unknown grass	0.00	5.00	0.00	0.00	2.00	10.00	0.33	0.33
other	0.00	5.00	0.00	2.00	1.00	14.50	0.00	6.00
Total cover	142.50	154.667	100.667	127.33	125	155.5	112.3	146.33
Total plant cover*	109.17	143.33	100.67	107.33	75.00	144.83	77.33	136.33
Richness	3	7	2	7	6	10	8	11

* excludes bare ground and litter.

Soils

Coordinates from the mapping expeditions were uploaded to the "NRCS web soil survey" to identify soil classifications and characteristics at respective field sites (Figure 11). Further verification of soil classifications involved overlaying the NRCS soil shapefiles on quadrat coordinates in ArcMap. According to the NRCS soil survey and ArcMap overlays, Huntley Meadows Park and Burke Lake Park both support "silt loam" soils in each plant community. These classifications are further supported by the Munsell color notations for soil samples (Table 6). Soils were identified as brown to very dark grayish brown, suggesting the presence of organic matter (Brady & Weil, 2008). Some mottling of colors was also observed and implies the oxidation-reduction reactions indicative of periodically waterlogged soils. Calculated soil pH ranged from 4.7 to 6.0. These values are similar to the literature values of 4.6 and 5.0 (Fairfax County Public Works and Environmental Services & Northern Virginia Soil and Water Conservation District, 2011).

The Elkton silt loam, Hatboro silt loam, and the Gunston silt loam present in the Huntley Meadows sample site are consistent with the floodplain characteristics observed at the site - the plant communities are located in a low lying area that collects water during heavy rainfall (Fairfax County Public Works and Environmental Services & Northern Virginia Soil and Water Conservation District, 2011). The Glenelg soil found at Burke Lake is more often found on slopes and hillsides, has better permeability than other soils, and is considered "undisturbed" (Fairfax County Public Works and Environmental Services & Northern Virginia Soil and Water Conservation District,

2011). This is important to note since *Onoclea* is often associated with native or undisturbed wetland ecosystems (Cobb et al., 2005).

Fairfax County, Virginia (VA059)

Map Unit Symbol	Map Unit Name	Acres in AOI	Percent of AOI
30A	Codorus and Hatboro soils, 0 to 2 percent slopes, occasionally flooded	3.8	7.1%
39B	Glenelg silt loam, 2 to 7 percent slopes	18.3	34.7%
39C	Glenelg silt loam, 7 to 15 percent slopes	22.7	43.1%
78B	Meadowville loam, 2 to 7 percent slopes	5.1	9.6%
93B	Sumerduck loam, 2 to 7 percent slopes	2.9	5.5%
Totals for Area of Interest		52.7	100.0%

Figure 11. Screenshot of Burke Lake Park soils surrounding fern sites according to the "NRCS web soil survey." Soil identical to the soils identified in ArcMAP are highlighted in the red box.

Table 6. Soil pH, classification according to NRCS, and Munsell notation.

Park	Sample site	Calculated pH (mean)	NRCS Expected pH	NRCS Soil classification	Munsell Notation	Munsell Color
Huntley Meadows	Mixed #1	5.93	4.6	Gunston Silt Loam	10 YR3/2	very dark grayish brown
	Mixed #2	5.365	4.6	Gunston Silt Loam	10 YR3/2	very dark grayish brown
	Mixed #3	5.33	4.6	Gunston Silt Loam	10 YR2/2	very dark brown
	Grass #1	5.715	4.6	Gunston Silt Loam	10 YR3/2	very dark grayish brown
	Grass #2	5.84	4.6	Gunston Silt Loam	10 YR3/2	very dark grayish brown
	Grass #3	5.96	4.6	Gunston Silt Loam	10 YR2/2	very dark brown
	Hog Peanut #1	4.975	4.6	Gunston Silt Loam	10 YR3/2	very dark grayish brown
	Hog Peanut #2	4.705	4.6	Gunston Silt Loam	10 YR3/2	very dark grayish brown
	Hog Peanut #3	5.265	4.6	Gunston Silt Loam	10 YR2/2	very dark brown
Burke Lake	site #1	6.04	5	Glenelg Silt Loam	10 YR3/2	very dark grayish brown
	site #2	5.735	5	Glenelg Silt Loam	10 YR 4/3	brown
	site #3	5.87	5	Glenelg Silt Loam	10 YR 4/3	brown

ARISA fingerprinting

ARISA fingerprinting identified 85 OTUs among all plant and soil sample PCR replicates. Of these OTUs, 68 occurred in the monoculture root samples, 59 occurred in the mixed root samples, 44 occurred in the monoculture soil samples, and 9 occurred in the mixed soil samples. Each plant appeared to have a different OTU profile (Figure 12 through Figure 14, different colors indicate different OTUs with a different coding for each figure). A few outlier samples from the peanut and the grass had only one OTU; otherwise, samples had multiple OTUs. Among the three species, the fern replicates had the most OTUs overall with 58 OTUs (Table 7). However, the peanut had the highest mean richness per individual replicate with 7.25 OTUs per sample (Table 7). The monoculture roots appeared to have a higher OTU richness than the mixed roots, but the difference was not statistically significant (Table 7). Both monoculture and mixed root communities were significantly different from the monoculture and mixed soil communities (Table 7).

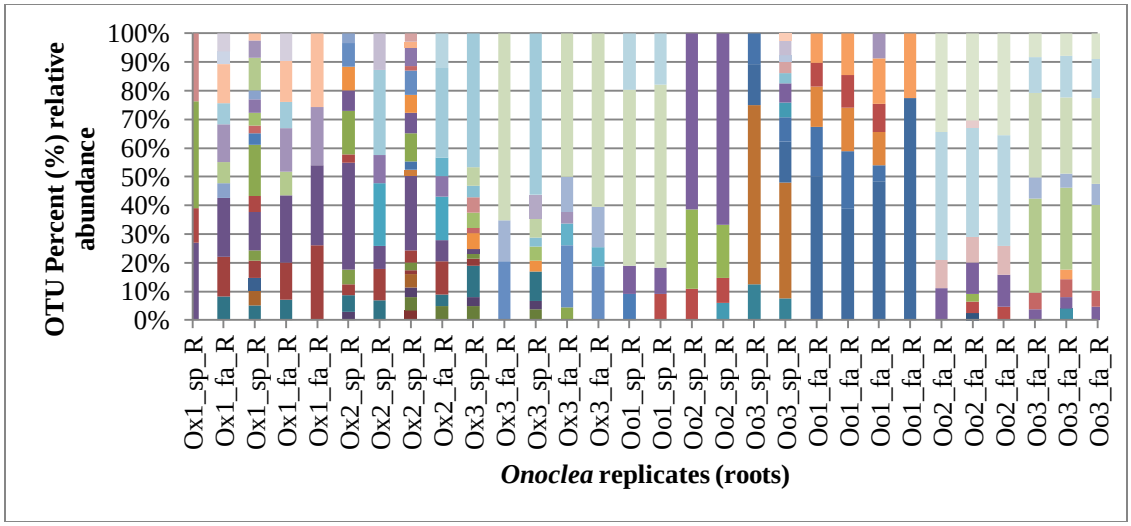

Figure 12. Relative abundances of OTUs in *Onoclea* sample replicates.
Different colors on bars indicate different OTUs; codes for root samples: O = *Onoclea*, o = monoculture community vs. x=mixed; 1,2,3= quadrat #, season of sample: fa=fall or sp=spring, R = root samples.

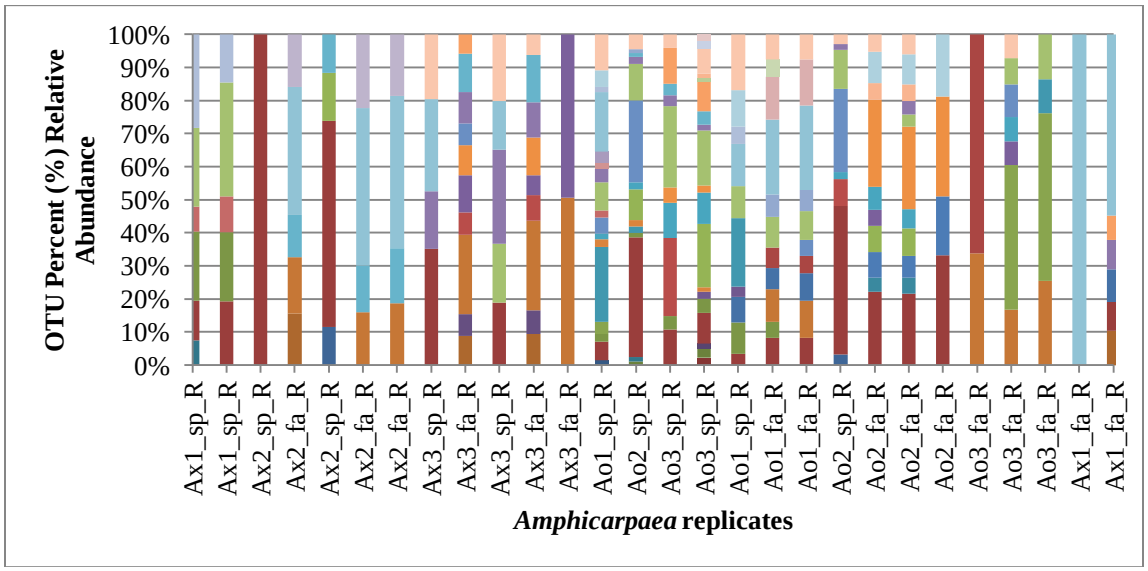

Figure 13. Relative abundances of OTUs for *Amphicarpaea* sample replicates .
Different colors on bars indicate different OTUs; codes for root samples: A= *Amphicarpaea*, o = monoculture community vs. x=mixed; 1,2,3= quadrat #, season of sample: fa=fall or sp=spring, R = root samples.

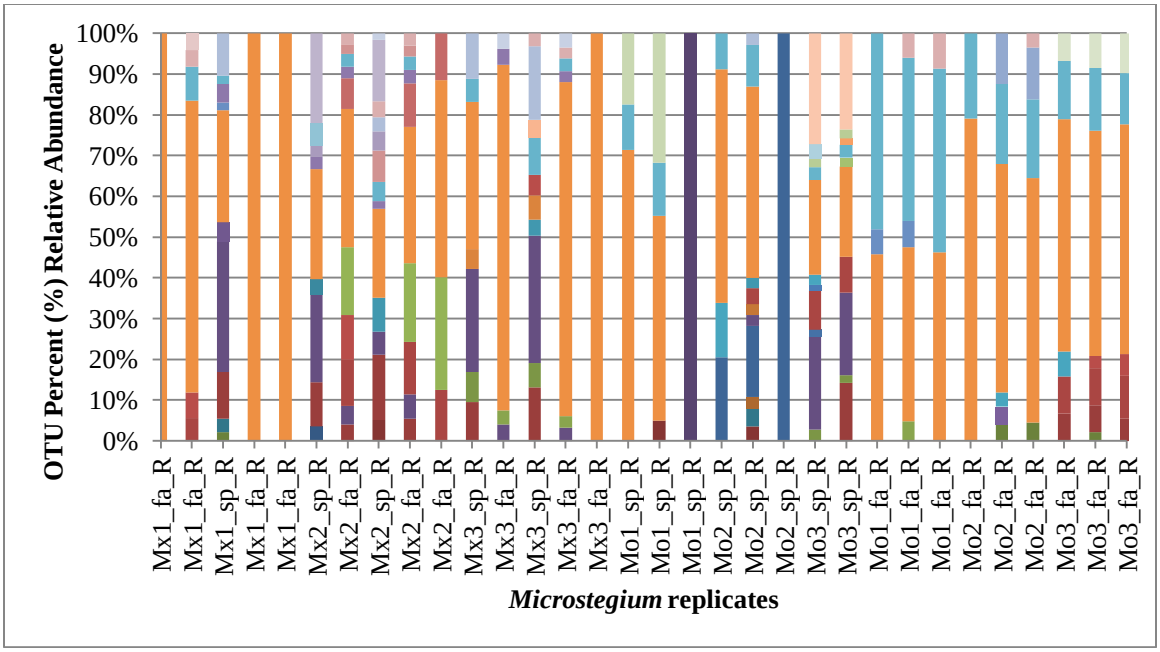

Figure 14. Relative abundances of OTUs within each *Microstegium* replicate.
Different colors on bars indicate different OTUs; codes for root samples: M=*Microstegium*, , o = monoculture community vs. x=mixed; 1,2,3= quadrat #, season of sample: fa=fall or sp=spring, R = root samples.

Table 7. OTU diversity by species or community, standard deviations shown in parentheses.

		N (PCR replicates)	Total OTUs represented	Mean richness per sample
Species*	*Amphicarpaea*	28	44	7.25 (4.57)
	Microstegium	32	45	5.75 (3.61)
	Onoclea	30	58	7.00 (3.88)
Community**	monoculture roots	47	68	6.74 (4.00)^{A}
	mixture roots	43	59	6.60 (4.11)^{A}
	monoculture soils	25	44	4.76 (2.70)^{B}
	mixture soils	8	9	2.63 (2.45)^{C}

*Species are not significantly different from each other.
**Different letters indicate statistically significant differences as determined by the T-test ($p<0.1$)

PCO: Monoculture Roots

The Principal Coordinate Analysis (PCO) for all monoculture roots (excluding all soils) showed three distinct clusters – one for each plant (Figure 15). *Microstegium* clustered to the right on the first axis, away from the fern and peanut. This first axis explained 26.2% of the variance. The second axis separated the fern from the peanut, and explained 13.3% of the variance. Together, the first two axes explained 39.4% of the variance. Addition of the third axis explained a cumulative 51.6% of the variance. The randomization analysis suggested that these first three axes were not random distributions and were useful for interpretation ($p<0.05$; 45 randomization runs).

Of all the OTUs in this PCO, biplot overlays of OTU abundances by plant samples indicated that only three OTUs occurred in all three plants: 253, 272, and 279. Of these three, only OTU 253 is strongly correlated with one of the first three axes (Table 8). Its overlay is shown in Figure 16.

Analysis of overlays of OTU relative abundances on the PCO indicated that there were nine OTUs unique to *Onoclea.* Of these OTUs, four were moderately to strongly correlated with either the second or third axes: OTUs 233, 283, 316 and 362 (Table 8). OTUs 316 and 362 are representative of the other OTUs and are shown in Figure 17. As already mentioned, the second axis is important in separating the fern from the peanut. Biplot overlays of OTU presence-absence transformed data indicate that 17 OTUs are unique to *Amphicarpaea.* Of these OTUs, 280, 304, and 344 were also moderately to strongly correlated with the second axis (Table 8); and overlays of OTUs 280 and 344 are shown in Figure 18. OTUs 276 and 277 were unique to *Microstegium* and were

associated with the first axis which separates the grass from the two native plants (Table 8). Overlays for both OTUs are shown in Figure 19.

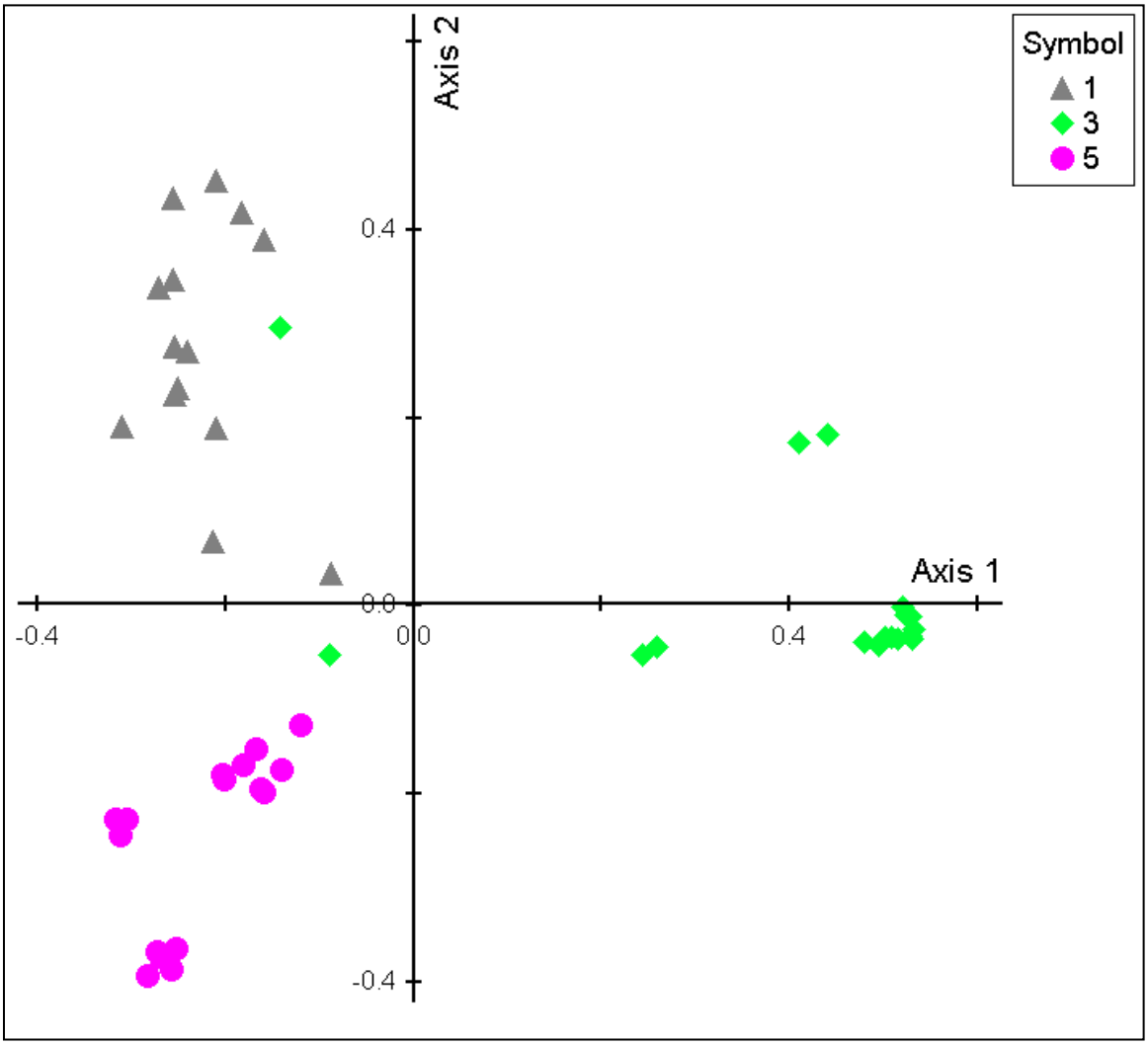

Figure 15. PCO for root fungal communities from "monocultures" labeled by plant.
Ssymbols: [1] gray triangles = *Amphicarpaea*, [3] green diamonds = *Microstegium*, [5] pink circles = *Onoclea*.

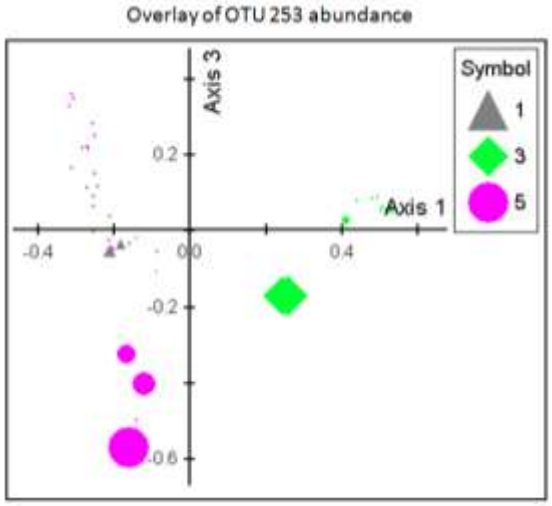

Figure 16. PCO ordination for fungal communities from monoculture root samples showing quantitative overlay of the relative abundance of OTU 253.
Symbols: [1] gray triangle = *Amphicarpaea* roots; [3] green diamond = *Microstegium* roots; [5] pink circle = *Onoclea* roots.

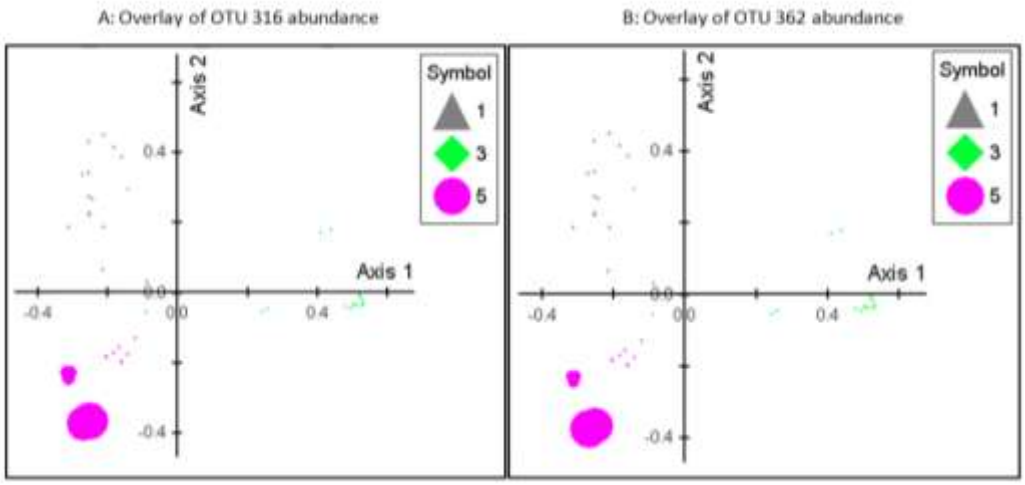

Figure 17 a-b. PCO ordination for fungal communities from monoculture root samples showing quantitative overlays of the relative abundance of two OTUs specific to the fern.
Symbols: [1] gray triangle = *Amphicarpaea* roots; [3] green diamond = Microstegium roots; [5] pink circle = Onoclea roots.

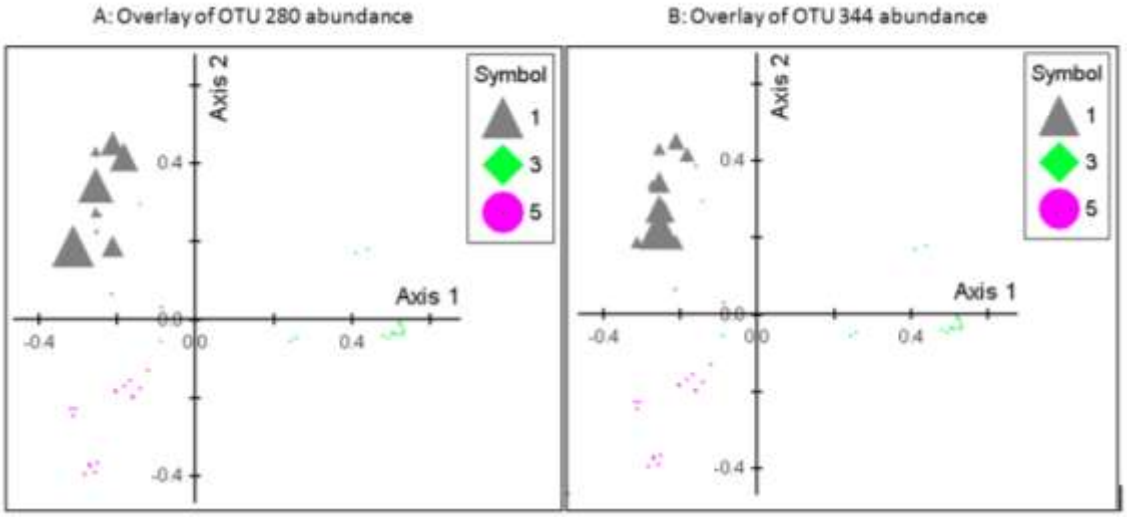

Figure 18 a-b. PCO ordination for fungal communities from monoculture root samples showing quantitative overlays of the relative abundance of two OTUs specific to the peanut.
Symbols: [1] gray triangle = *Amphicarpaea* roots; [3] green diamond = *Microstegium* roots; [5] pink circle = *Onoclea* roots.

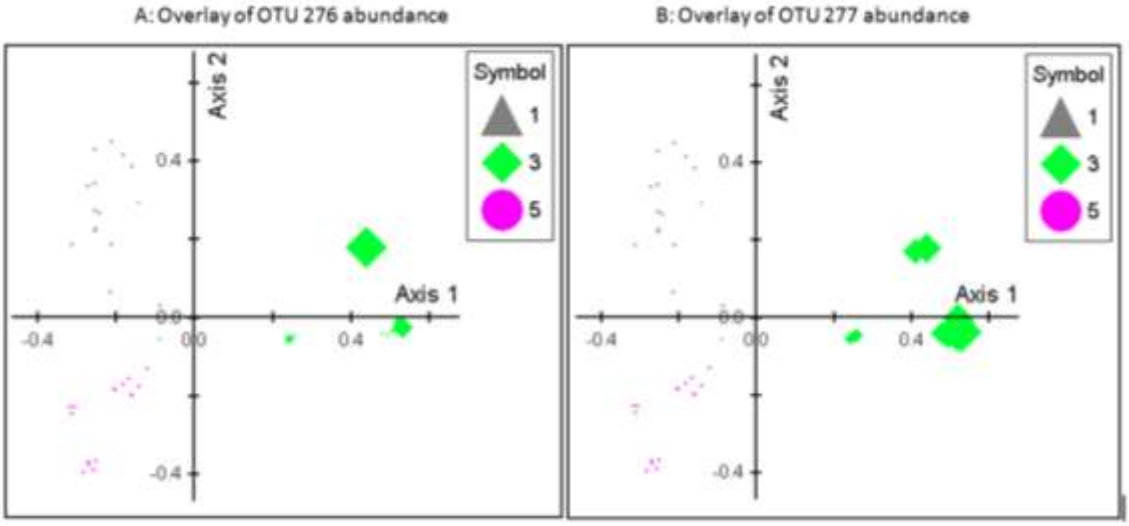

Figure 19a-b. PCO ordination for fungal communities from monoculture root samples showing quantitative overlays of the relative abundance of two OTUs specific to the grass.
Symbols: [1] gray triangle = *Amphicarpaea* roots; [3] green diamond = *Microstegium* roots; [5] pink circle = *Onoclea* roots.

Table 8. Correlation coefficients of root monoculture OTUs with PCO ordination axes.
Only OTUs with correlations greater than an absolute value of 0.3 on at least one axis are shown. Bold numbers indicate strong correlations (>|0.5|).

	correlation coefficients		
variable	axis 1	axis 2	axis 3
OTU 228	0.395	-0.023	0.067
OTU 233	-0.158	-0.254	**-0.773**
OTU 244	-0.172	**0.579**	0.049
OTU 249	0.345	-0.05	-0.121
OTU 253	-0.008	-0.143	**-0.668**
OTU 269	-0.117	-0.203	**-0.659**
OTU 271	-0.332	-0.065	-0.246
OTU 273	-0.035	0.4	-0.029
OTU 275	-0.158	0.466	0.051
OTU 276	0.34	0.088	0.052
OTU 277	**0.963**	-0.007	0.135
OTU 280	-0.288	**0.512**	0.1
OTU 281	-0.16	0.484	-0.039
OTU 282	-0.215	0.409	0.097
OTU 283	-0.155	-0.243	**-0.687**
OTU 285	**0.777**	-0.039	0.139
OTU 287	-0.451	0.107	0.458
OTU 289	0.338	-0.021	0.06
OTU 293	-0.306	-0.038	0.401
OTU 303	-0.224	0.316	0.104
OTU 304	-0.142	0.419	-0.035
OTU 310	0.309	-0.037	0.037
OTU 313	-0.286	-0.441	0.416
OTU 316	-0.333	**-0.59**	0.453
OTU 322	-0.201	0.471	-0.067
OTU 344	-0.354	**0.581**	0.099
OTU 362	-0.268	-0.472	0.359
OTU 405	0.413	-0.014	0.056

Overlays of OTU abundances indicate that twelve OTUs occur in both *Microstegium* and *Amphicarpaea* monocultures. Of these, OTU 244 is strongly associated with the second axis and occurs mostly in the peanut (Table 8; Figure 20). In contrast, OTU 285 is strongly associated with the first axis, and occurs for the most part in the grass (Table 8; Figure 20). None of the 12 shared OTUs are strongly correlated with the third axis.

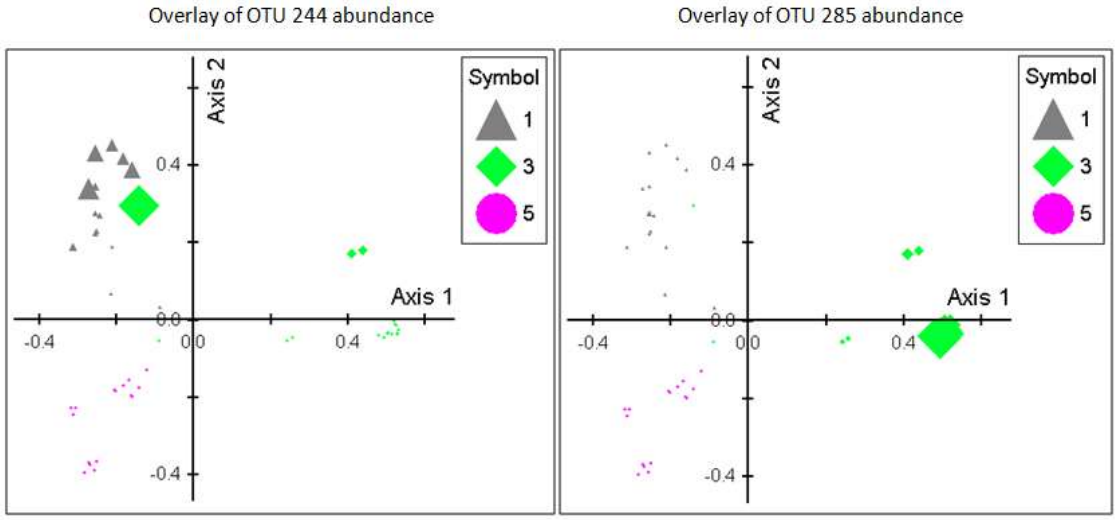

Figure 20 a-b. PCO ordination for fungal communities from monoculture root samples showing quantitative overlays of the relative abundance of two OTUs occurring in grass and peanut samples.
Symbols: [1] gray triangle = *Amphicarpaea* roots; [3] green diamond = *Microstegium* roots; [5] pink circle = *Onoclea* roots.

Overlay analysis showed that only three OTUs co-occur in *Microstegium* and *Onoclea*: 229 (not in table), 269, and 310 (overlays not shown). Although none of these OTUs are strongly associated with the first two axes (OTU 310 is only moderately correlated with the first axis), OTU 269 is strongly correlated with the 3rd axis (Table 8).

Twelve OTUs co-occur in *Amphicarpaea* and *Onoclea* monocultures. Of the twelve co-occurring OTUs, three are strongly correlated with the first, second, or third axes. OTU 287 is strongly correlated with the first and third axes while OTUs 293 and 313 are strongly correlated with the third axis (Table 8; OTU 287 and 293 shown in Figure 21). Because the other eight OTUs are only weakly correlated with the axes, they are not included in Table 8.

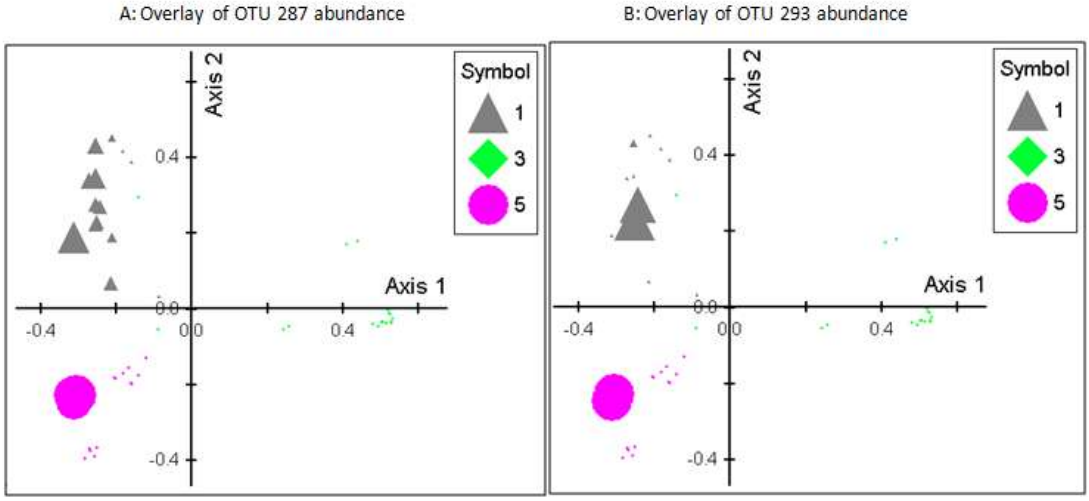

Figure 21a-b. PCO ordination for fungal communities from monoculture root samples showing quantitative overlays of the relative abundance of two OTUs.
Symbols: [1] gray triangle = *Amphicarpaea* roots; [3] green diamond = *Microstegium* roots; [5] pink circle = *Onoclea* roots.

PCO: Mixture Roots

The PCO of all mixed root samples (soils excluded) showed the grass clustering away from the fern and peanut samples on the first axis (Figure 22). The peanut and fern did not cluster separately. The first axis explained 30.7 % of the variance, and the second axis explained an additional 14.3% of the variance. Together the first two axes explained

45% of the variance. The randomization analysis indicated that only the first axis was statistically viable ($p<0.05$; 40 randomization runs). Therefore, the second axis is analyzed with the understanding that it may not be different from a random distribution, and the third axis is included in Table 9 only as it relates to OTU correlation coefficients.

Overlays of OTU abundances indicate that seven OTUs are found in all three plants. This is an increase from the monocultures where only three OTUs co-occurred in all three species. Of the seven, only three are moderately to strongly associated with any of the first three axes: 236, 249, and 303 (Table 9). OTU 236 and 249 are moderately to strongly correlated with the first axis (Table 9), thereby partially explaining the clustering of the grass away from the fern and peanut. OTU 303 is strongly associated with axis 1 and 2 (Table 9), thereby also helping to explain the clustering of the peanut and fern away from the grass. OTUs 236 and 303 are identified in the mixed community mostly with the peanut and fern (Figure 23). Interestingly, OTU 303 was originally identified with only peanut monocultures while 236 was originally identified in peanut and grass monocultures – not the monoculture ferns (data not shown). OTU 249, on the other hand, was originally associated with grass monocultures (data not shown) and is now identified in roots from the mixtures (Figure 23).

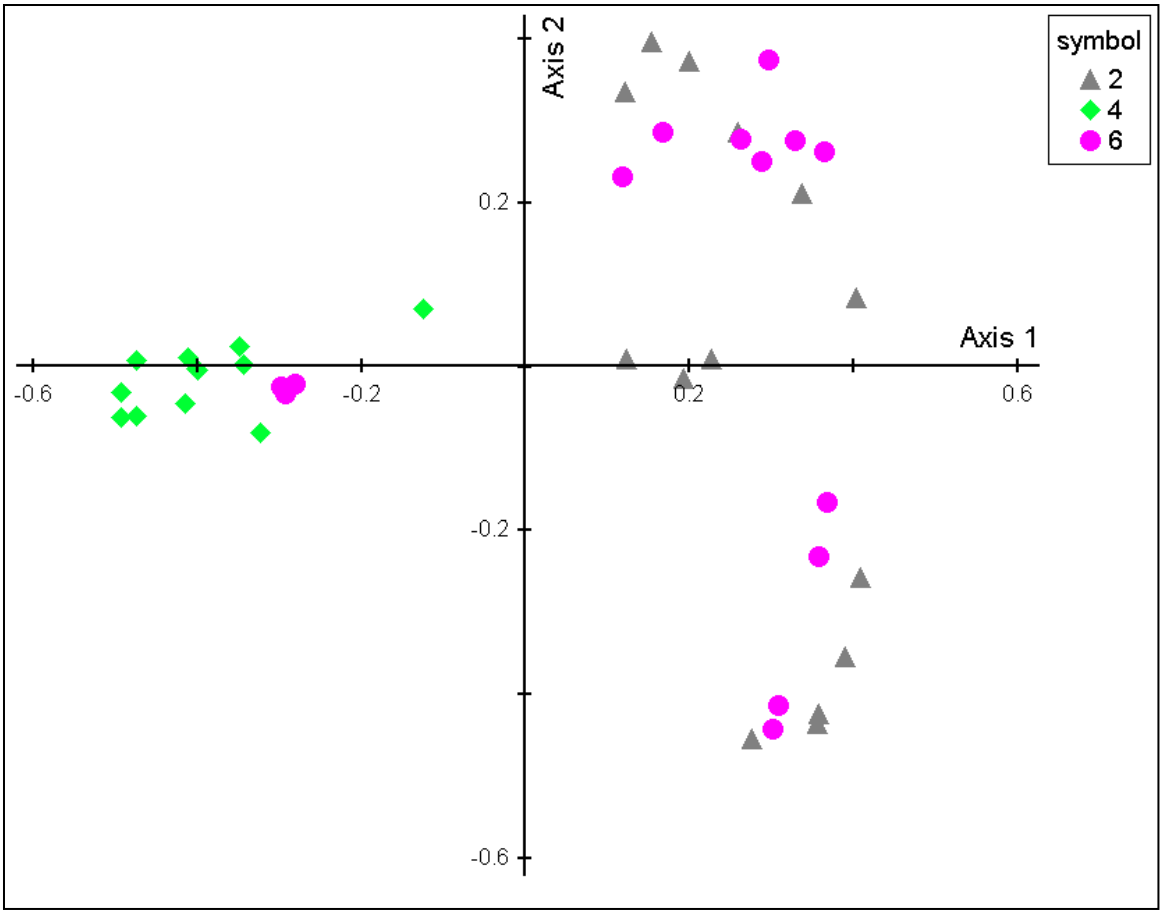

Figure 22. PCO of root fungal communities from "mixtures" labeled by plant species.
Symbols: [2] gray triangles = *Amphicarpaea,* [4] green diamonds = *Microstegium*, [6] pink circles = *Onoclea;* black dots represent OTUs.

Table 9. Correlation coefficients for ordination axes using OTUs from the mixed plant root community. Only OTUs with correlations greater than an absolute value of 0.3 on at least one axis are shown. Bold numbers indicate very strong correlations (>|0.5|).

	correlation coefficients		
variable	axis 1	axis 2	axis 3
OTU 220	0.38	-0.146	0.141
OTU 236	0.482	0.129	0.005
OTU 244	0.399	**0.601**	0.233
OTU 246	0.089	0.345	0.104
OTU 249	-0.312	0.021	**-0.626**
OTU 253	-0.352	0.013	-0.425
OTU 257	0.323	0.004	-0.482
OTU 261	-0.31	0.084	-0.012
OTU 264	-0.318	-0.045	0.241
OTU 277	**-0.834**	-0.099	0.436
OTU 279	0.095	0.006	-0.352
OTU 282	-0.284	-0.119	-0.395
OTU 285	-0.189	0.372	-0.085
OTU 287	0.223	0.462	0.161
OTU 288	0.427	0.328	0.122
OTU 290	0.384	-0.366	-0.257
OTU 293	-0.312	-0.026	-0.413
OTU 295	0.196	-0.404	0.123
OTU 303	**0.53**	**-0.722**	0.21
OTU 307	-0.026	0.322	0.062
OTU 309	0.254	0.365	0.153
OTU 313	-0.282	-0.041	-0.4
OTU 316	0.334	**-0.506**	-0.065

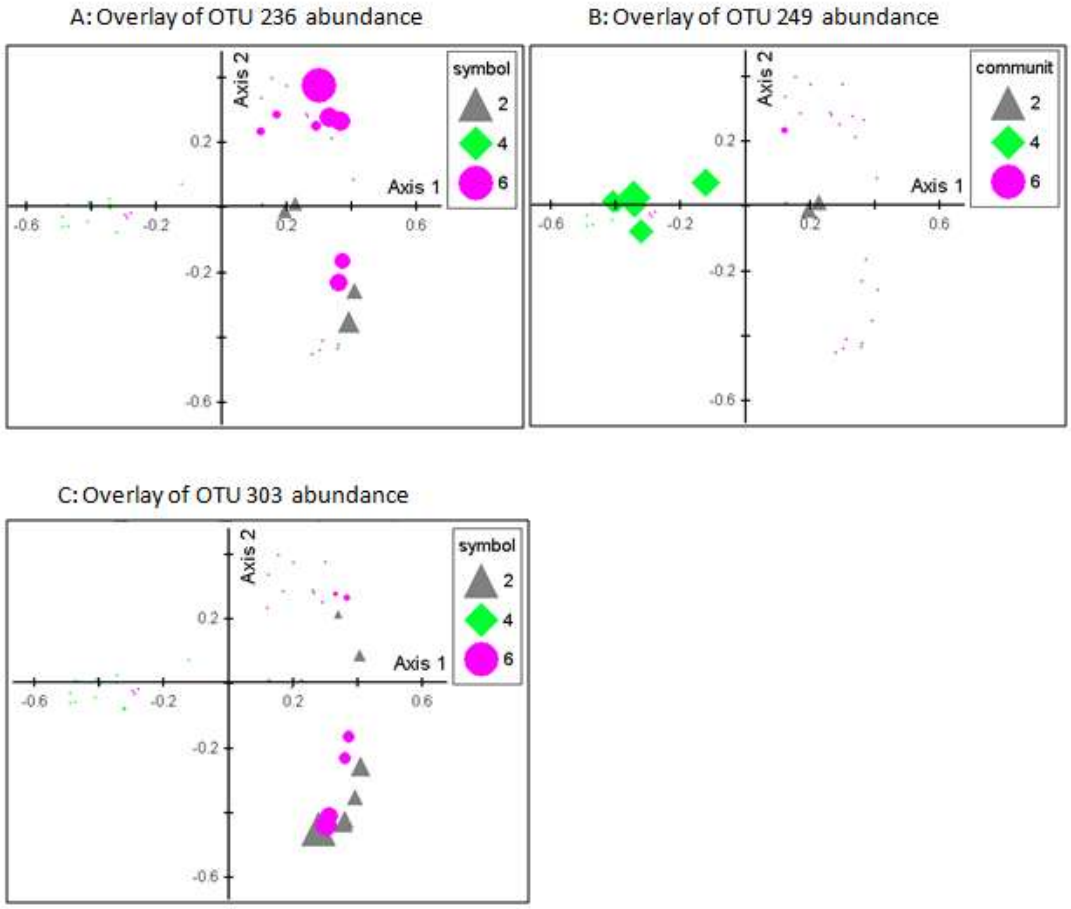

Figure 23. PCO ordination for fungal communities from mixed root samples showing quantitative overlays of the relative abundance of three OTUs occurring in all three plant roots.
Symbols: [2] gray triangle = *Amphicarpaea* roots; [4] green diamond = *Microstegium* roots; [6] pink circle= *Onoclea* roots.

Further comparison of the monoculture samples to the mixed root samples, reveals other notable relationships. While OTU 276 was moderately correlated with axis 1 in the "monoculture roots PCO" (Table 8), it is weakly correlated with the axes in the "mixed roots PCO" ($r < 0.3$; overlay not shown). In the monocultures, the fern alone was associated with OTU 316 (Figure 17). However, in the mixed roots, the peanut is the dominant plant associated with this amplicon and only one fern sample associates with this OTU (Figure 24). OTU 280 does a complete switch. This OTU was strongly

correlated with the second axis in the monocultures (Table 8), and it occurred only in the peanut monoculture (Figure 18). In the mixed, it has a low correlation with all axes ($r<0.2$) and does not occur with the peanut. Instead, it occurs in both the fern and the grass (Figure 24). OTU 277 continues to strongly correlate with the first axis (Table 9). In the monoculture roots, it only occurred in the grass roots (Figure 19), but in the mixed roots, it occurs in both grass and fern roots (Figure 24).

In the monocultures, *Amphicarpaea* has 17 OTUs unique to itself. This number drops to 3 in the mixed samples (overlays not shown). Of these three, only OTU 246 and 307 are moderately correlated with axis 2 (Table 9); all the others are weakly correlated with the axes. In contrast, *Microstegium* maintains a similar OTU richness with 13 OTUs in the monocultures and 12 in the mixed (overlays not shown). Unlike either *Amphicarpaea* or *Microstegium, Onoclea* increases from 9 OTUs in the monocultures to 17 OTUs in the mixed community (overlays not shown). None of the OTUs specific to mixed *Onoclea* or *Microstegium* are strongly correlated with the first three axes.

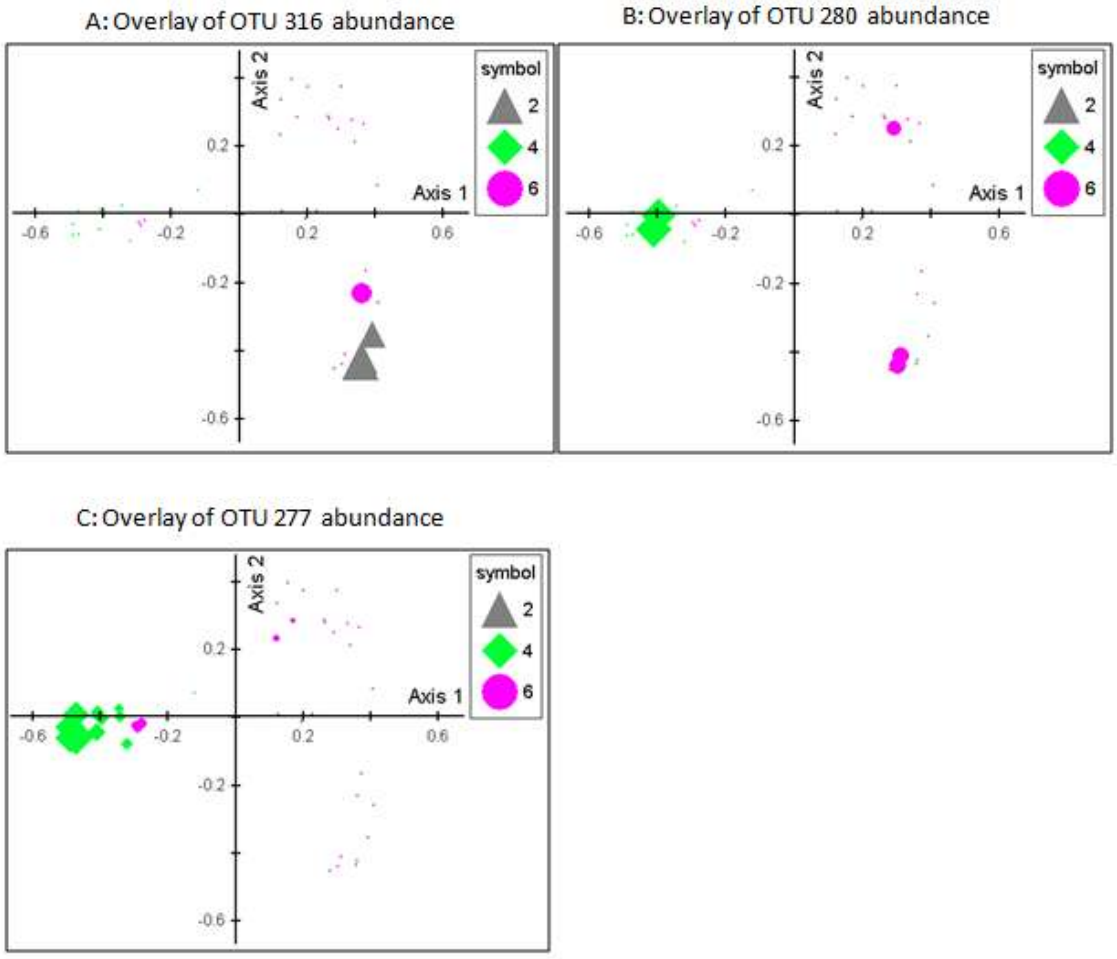

Figure 24 a-c. PCO ordination for fungal communities from mixed root samples showing quantitative overlays of the relative abundance of three OTUs occurring in grass, peanut, and/or fern samples.
Symbols: [2] gray triangle = Amphicarp; [4] green diamond = Microstegium; [6] pink circle.

PCO: All Roots (monocultures and mixed)

A PCO of all root samples was used to better understand the similarities and differences between OTU communities from monocultures and mixed roots. This PCO explains 22.7% and 10.9% of the variance in the first and second axes, respectively (Figure 25). An additional 8.2% and 7.3% are explained in the third and fourth axes.

Together, all four axes explain a cumulative 49.2% of the variance; and all four axes are considered statistically robust ($p<0.05$, 89 randomization runs).

This PCO shows that all *Microstegium* samples cluster away from *Onoclea* and *Amphicarpaea*. The distinct fungal community identified in the grass monocultures appears to remain consistent in the mixed community (Figure 25). As seen in the first two PCO ordinations (Figure 15 and Figure 22), clustering of *Microstegium* away from the fern and peanut occurs along the first axis. In contrast, the monoculture ferns cluster away from all *Amphicarpaea* samples based on the second axis. In the PCO of all root samples, the mixed ferns do not separate from the peanut samples (Figure 25).

OTUs 277 and 285 are strongly correlated with the first axis (Table 10). As previously noted, OTU 277 occurs in both monoculture and mixed *Microstegium* communities (Figure 26). The presence of this OTU in several mixed *Onoclea* roots helps to explain the clustering of these *Onoclea* samples near the *Microstegium* samples in the PCO. OTU 285 is predominantly in the monoculture grass communities but also occurs in small abundances in mixed roots from all three plants (Figure 26).

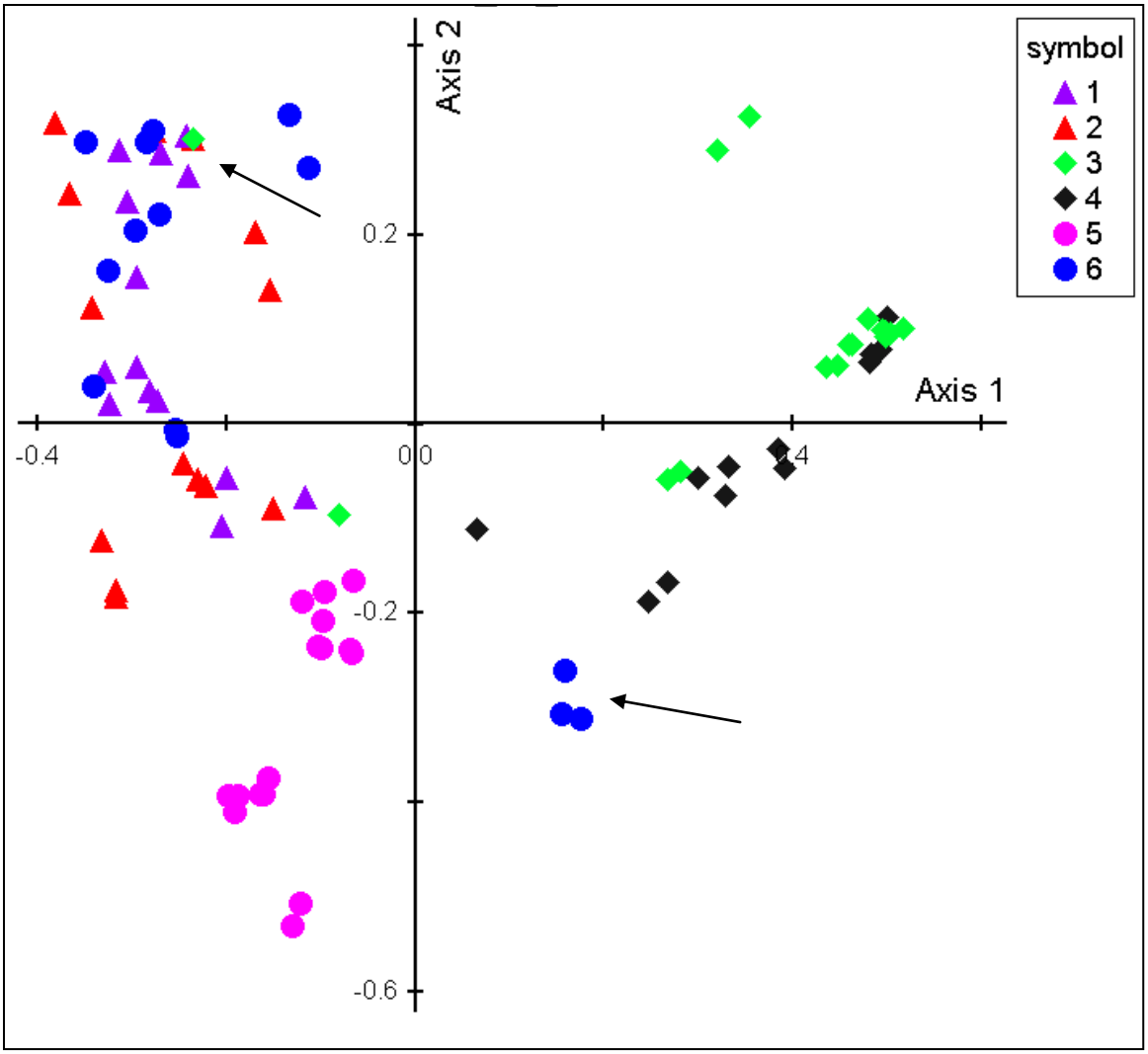

Figure 25. PCO ordination for fungal communities from monoculture and mixed root samples. Symbols: [1] purple triangle = monoculture *Amphicarpaea,* [2] red triangle = mixed *Amphicarpaea;* [3] green diamond = monoculture *Microstegium*; [4] black diamond = mixed *Microstegium*; [5] magenta circle = monoculture *Onoclea;* [6] blue circle = mixed *Onoclea,* black dots indicate OTUs. Black arrows identify outliers.

OTUs 244, 313, and 316 are strongly correlated with the second axis (Table 10). OTU 244 occurs in all samples except for monoculture *Onoclea* (Figure 27). This OTU appears to at least partially explain the clustering of the monoculture *Microstegium* outlier with the monoculture and mixed *Amphicarpaea* and mixed *Onoclea* samples (Figure 25). OTU 313 appears to undergo a shift from native to invasive plant

communities. For the most part, it occurs in the monoculture and mixed *Onoclea* root samples. It occurs to a small extent in the mixed *Microstegium*, but it does not occur in *Amphicarpaea* (Figure 27). OTU 316 occurs for the most part in the monoculture *Onoclea* and mixed *Amphicarpaea*, and to a very small extent in the mixed *Onoclea* samples (Figure 27).

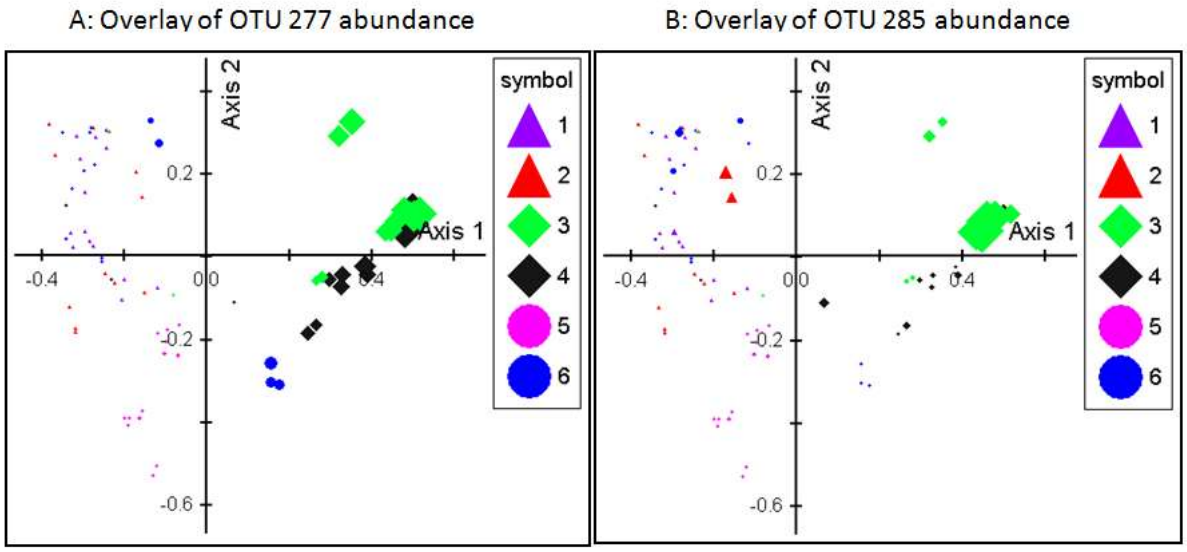

Figure 26 a-b. PCO ordination for fungal communities from monoculture and mixed root samples showing quantitative overlays of the relative abundance of two OTUs that strongly correlate with the first axis. Symbols: [1] purple triangle = monoculture *Amphicarpaea,* [2] red triangle = mixed *Amphicarpaea;* [3] green diamond = monoculture *Microstegium*; [4] black diamond = mixed *Microstegium*; [5] magenta circle = monoculture *Onoclea;* [6] blue circle = mixed *Onoclea.*

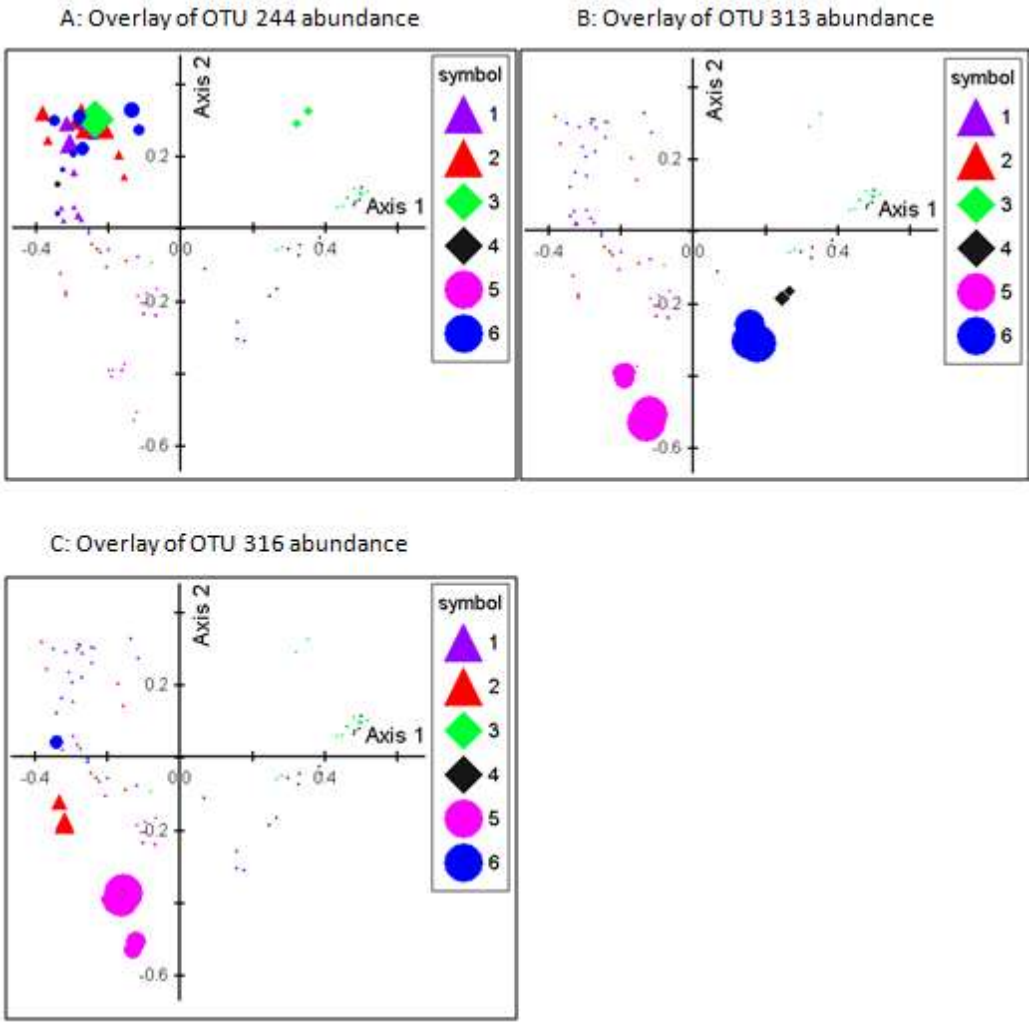

Figure 27 a-c. PCO ordination for fungal communities from monoculture and mixed root samples showing quantitative overlays of the relative abundance of three OTUs that strongly correlate with the second axis. Symbols: [1] purple triangle = monoculture *Amphicarpaea,* [2] red triangle = mixed *Amphicarpaea;* [3] green diamond = monoculture *Microstegium*; [4] black diamond = mixed *Microstegium*; [5] magenta circle = monoculture *Onoclea;* [6] blue circle = mixed *Onoclea.*

Additional analysis of OTU abundance overlays indicates that in the monocultures, the fern and the peanut share twelve OTUs (overlays not shown). In the mixtures, they share ten OTUs (overlays not shown). For the most part, these OTUs are not the same between the monocultures and the mixed; in fact, only two OTUs (288 and

290) are consistent between the monoculture and mixture roots (overlays from "monoculture" and "mixed" PCOs not shown). These OTUs are only moderately correlated with the first three axes in the "all roots" PCO (Table 10). The three OTUs shared between the invasive grass and the fern in the monocultures, increases to eight in the mixed community. Only three of these eight OTUs are shown: OTU 280 is shown in Figure 24, OTU 277 is shown in Figure 24 and Figure 26, and OTU 313 is shown in Figure 27. More importantly, the eight shared OTUs in the mixed are different than the three in the monocultures. Of the eight, only 277 (Figure 26), 313 (Figure 27), and 293 (Figure 28) are moderately to strongly correlated with one of the first 3 axes in the "all roots" PCO (Table 10). While the peanut and the grass share twelve OTUs in the monoculture samples, they only share two in the mixed samples: OTUs 274 and 307 (overlays not shown). These two OTUs are not shared between the invasive and the peanut in the monocultures. And, neither is strongly correlated with the first three axes ($r < 0.3$).

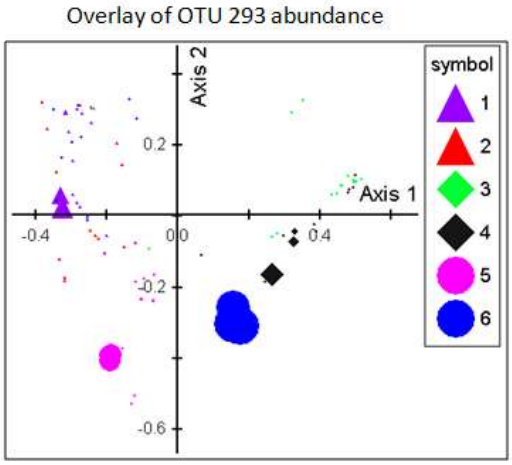

Figure 28. PCO ordination for fungal communities from monoculture and mixed root samples showing the quantitative overlay of the relative abundance of OTU293.
Symbols: [1] purple triangle = monoculture *Amphicarpaea,* [2] red triangle = mixed *Amphicarpaea;* [3] green diamond = monoculture *Microstegium*; [4] black diamond = mixed *Microstegium*; [5] magenta circle = monoculture *Onoclea;* [6] blue circle = mixed *Onoclea.*

Table 10. Correlation coefficients for all roots with the first three PCO ordination axes; only OTUs with correlations greater than an absolute value of 0.3 on at least one axis are shown and bold numbers indicate strong correlations (>|0.5|).

	Correlation Coefficients		
OTU	**axis 1**	**axis 2**	**axis 3**
OTU 217	-0.185	0.092	-0.318
OTU 220	-0.263	0.2	-0.322
OTU 236	-0.305	0.253	-0.217
OTU 244	-0.358	**0.591**	0.347
OTU 249	0.305	-0.11	0.09
OTU 277	**0.898**	0.184	-0.157
OTU 285	**0.54**	0.183	-0.056
OTU 287	-0.344	-0.032	0.293
OTU 288	-0.346	0.304	0.012
OTU 290	-0.289	-0.07	-0.402
OTU 293	0.026	-0.367	0.119
OTU 295	-0.123	-0.008	-0.381
OTU 303	-0.378	0.011	-0.78
OTU 313	0.01	**-0.558**	0.192
OTU 316	0.226	**0.551**	-0.124
OTU 344	-0.356	0.245	-0.018
OTU 362	-0.122	-0.411	0.065

PCO: Monoculture Soils

The PCO of monoculture soil samples created two clusters of *Amphicarpaea* along the first axis; both clusters overlap with *Onoclea* and *Microstegium* (Figure 29). The first axis in this ordination explains 17.5% of the variance and the second explains 14.1% of the variance for a cumulative total of 31.6%. However, neither of these axes are considered viable for analysis ($p>0.05$; 20 randomization runs).

Of the 44 OTUs present in the soil samples collected from the monocultures, the majority are associated with single monoculture communities and only a few occur in multiple monocultures (overlays not shown). More importantly, very few of the soil OTUs are strongly correlated with the axes. As such, few OTUs play a significant role in clustering of the communities. Only seven OTUs show strong correlations, and only these OTUs are included in Table 11. While OTUs 220, 257, and 270 are strongly associated with the first axis, OTUs 283, 286 and 313 are associated with the second axis and 277 is strongly correlated with both axes (Table 11).

Overlays of OTU abundances indicate that five OTUs were shared among soil communities obtained from the three plant monocultures: 220, 260, 274, 277, and 303. Of these five OTUs, only OTU 220 and 277 are strongly correlated with any axis (Figure 30; Table 11).

Of the 39 OTUs not shared among all three monoculture soils, most were specific to an individual monoculture's soil (overlays not shown). OTU richness ranged from 9 OTUs unique to the grass' soil to 11 OTUs unique to the peanut's soil and a different 11 OTUs unique to the fern's soil. The remaining 8 OTUs co-occurred between monocultures. More specifically, two co-occurred in the grass and the fern; of these two,

only OTU 313 was strongly correlated with the second axis (Table 11; Figure 30). Two also co-occurred in the peanut and fern but neither were strongly correlated with either axis. And, four occurred with the grass and peanut. Of these, only OTU 257 was strongly correlated with axis one (Table 11; Figure 30).

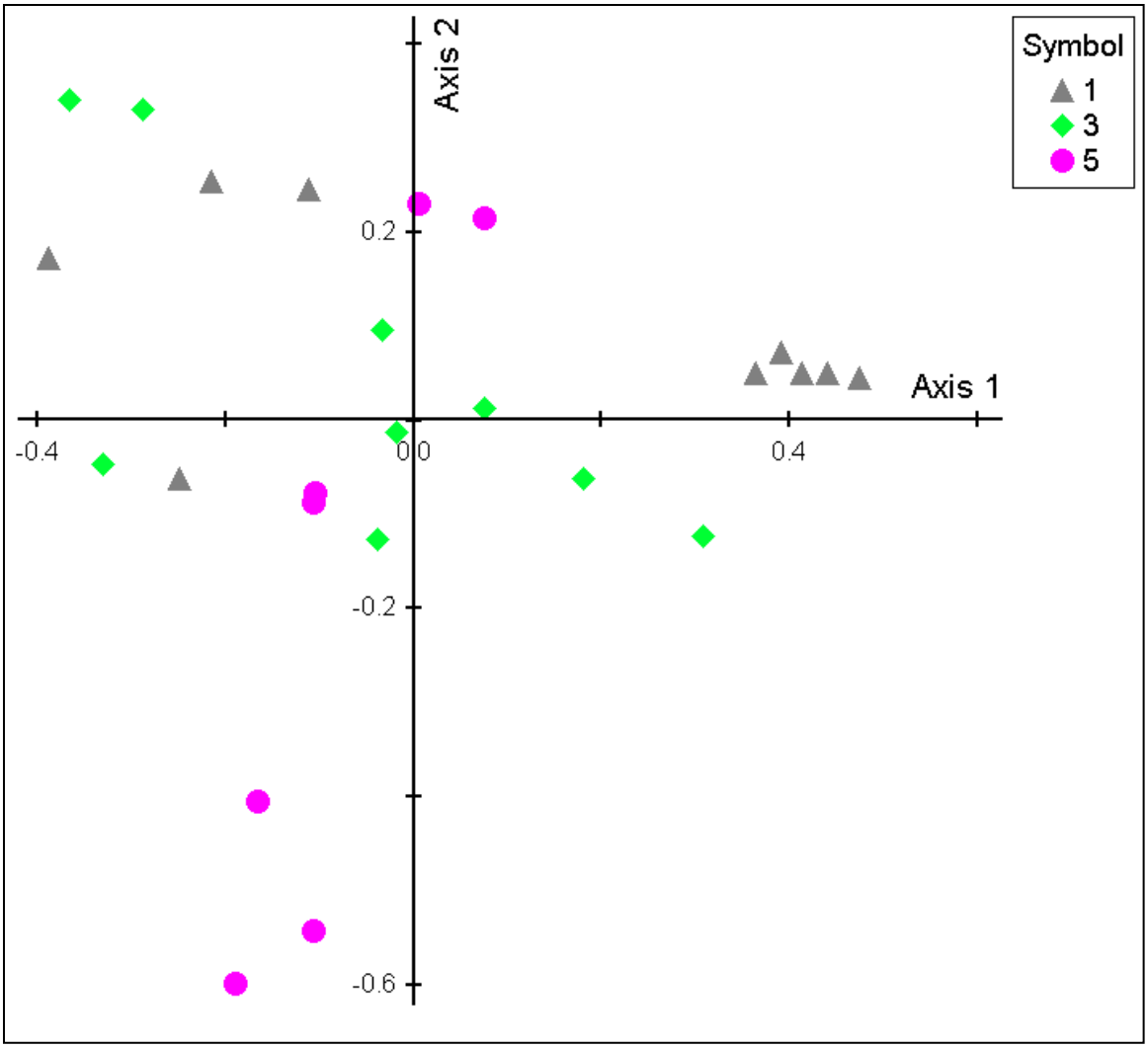

Figure 29. PCO of fungal communities from soil monocultures and labeled according to plant communities. Symbols: [1] gray triangles = *Amphicarpaea* monoculture soil, [3] green diamonds = *Microstegium* monoculture soil, [5] pink circles = *Onoclea* monoculture soil.

The overlapping of OTU communities suggests that the OTUs are randomly distributed among the soils. Nevertheless, the presence of OTUs 277 and 313 in the monoculture soils is informative since 277 only occurred in monoculture grass roots (Figure 19) and 313 occurred in monoculture fern and peanut roots (figure not shown). The clustering of *Amphicarpaea* monoculture soils to the right along axis 1 appears to be

a result of OTU 220 and 257 (Figure 30). OTU 220 is also present to a large extent in the monoculture fern and grass soils.

Table 11. Correlation Coefficients for OTUs from monoculture community soils.
Only OTUs with correlations greater than an absolute value of 0.4 on at least one axis are shown and bold numbers indicate very strong correlations (>|0.4|).

	correlation coefficients	
variable	axis 1	axis 2
OTU 220	**0.658**	0.166
OTU 257	**0.816**	0.08
OTU 270	**0.409**	0.102
OTU 277	**-0.52**	**0.596**
OTU 283	-0.317	**0.485**
OTU 286	-0.208	**-0.816**
OTU 313	-0.174	**-0.753**

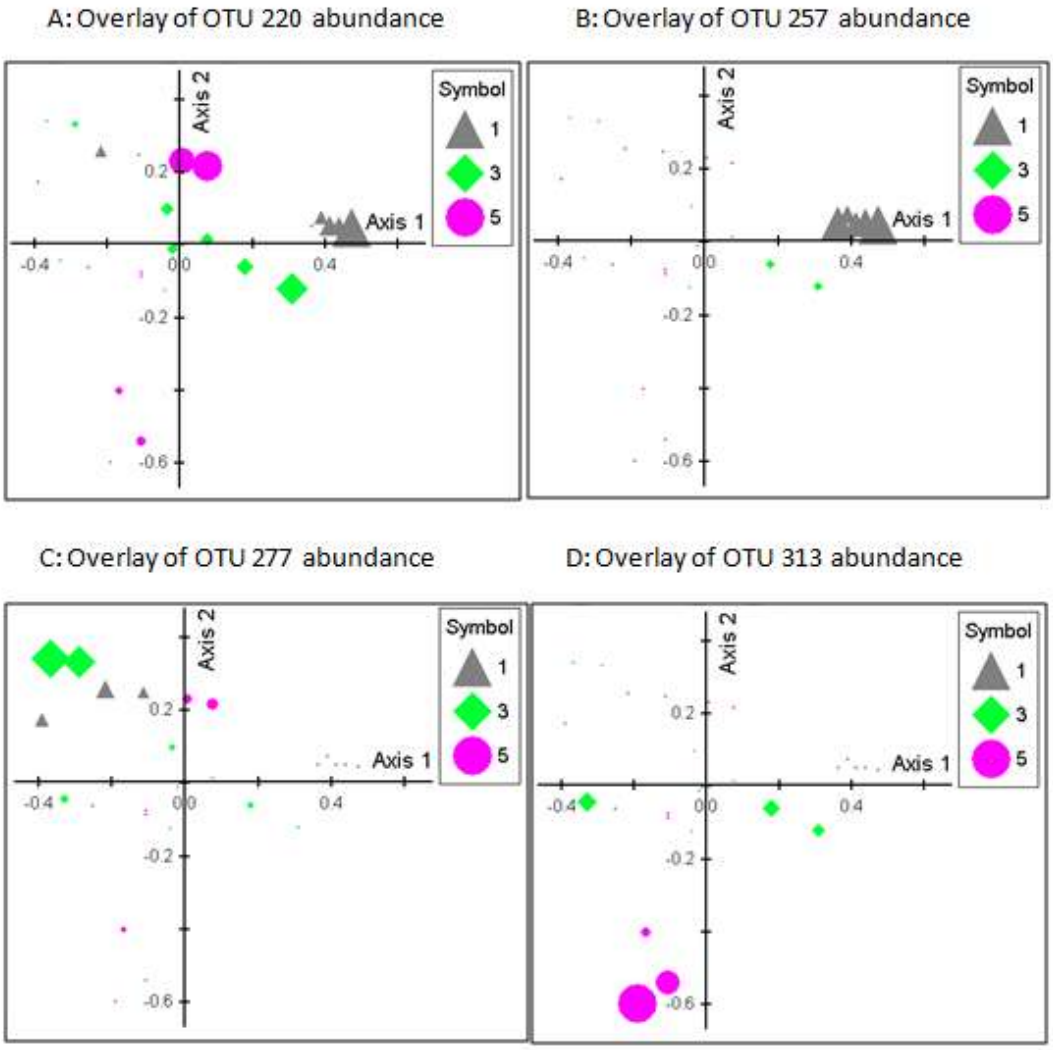

Figure 30a-d. PCO ordination for fungal communities from monoculture soil samples showing quantitative overlays of the relative abundance of two OTUs occurring in grass and peanut soils.
Symbols: [1] gray triangle = *Amphicarpaea* monoculture soil; [3] green diamond = *Microstegium* monoculture soil; [5] pink circle = *Onoclea* monoculture soil.

PCO: All Soils

Combining all monoculture and mixed soil OTU communities into one PCO does not improve the clustering (Figure 31). The first two axes explain 28.2% of the variance, approximately 3% less than the "monoculture soils PCO." None of the axes for this combined PCO are viable for analysis ($p>0.5$, 20 randomization runs). Nevertheless, the

clustering observed in the monoculture *Amphicarpaea* soils is still apparent on the upper right side of the new ordination (Figure 31). The ferns also seem to form a loose cluster along axis one (Figure 31). Overall, the monoculture and mixed soil samples overlap.

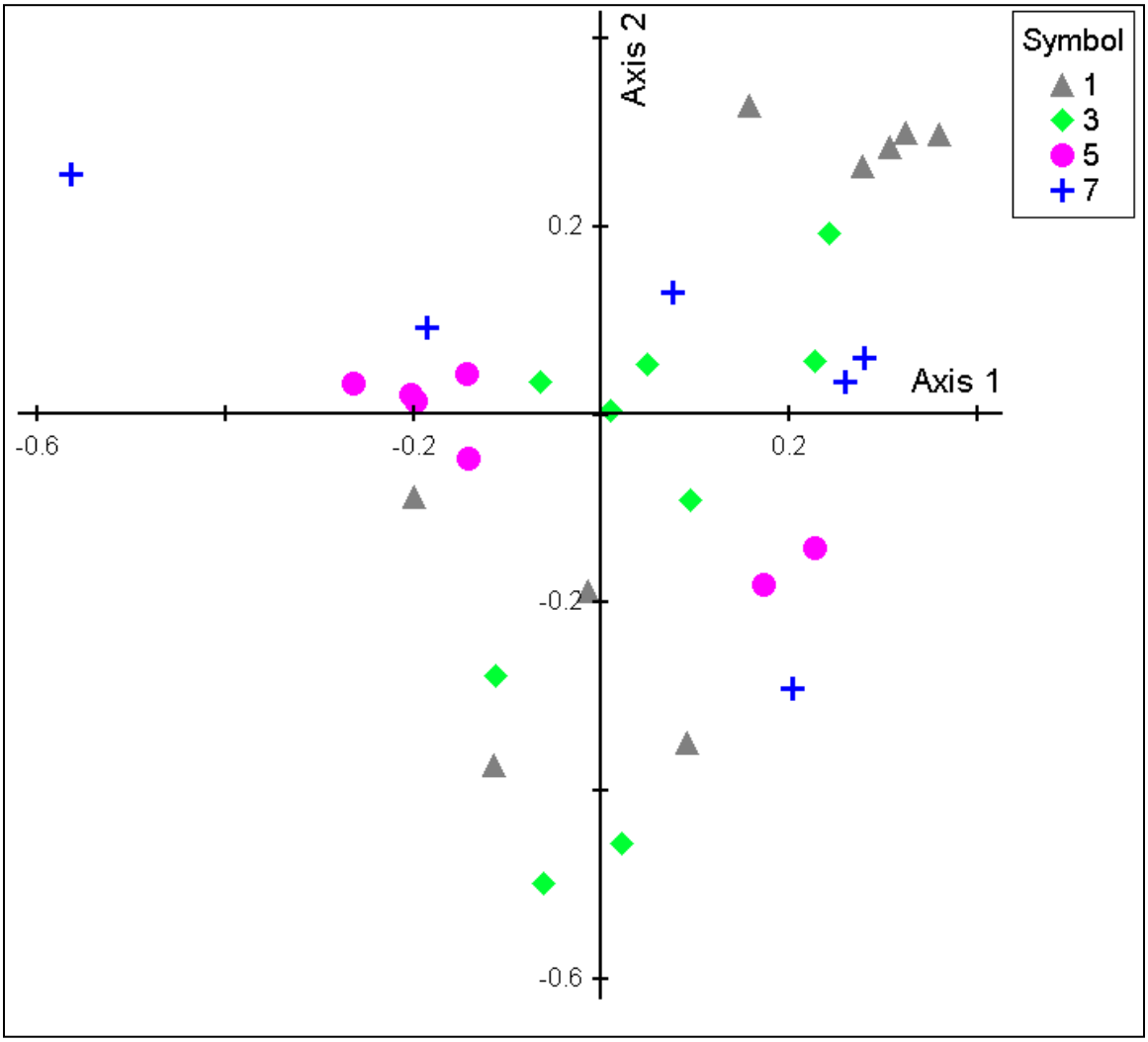

Figure 31. The PCO showing all soil fungal communities labeled according to plant communities. Symbols: [1] gray triangles = *Amphicarpaea* monoculture soil, [3] green diamonds = *Microstegium* monoculture soil, [5] pink circles = *Onoclea* monoculture soil; [7] blue cross = mixed soil.

In this ordination, five OTUs are strongly associated with at least one of the first two axes. OTU 220, 257, and 302 are all strongly associated with the first axis (Table 12). OTUs 257, 277, and 283 are strongly associated with the second axis (Table 12). Of these five OTUs, OTU 220 and 277 occur in both monoculture soils and mixed soils (Figure 32). OTU 257 co-occurs in *Amphicarpaea* and *Microstegium* soil monocultures as well as the mixed soil (Figure 32). OTU 283 occurs in *Microstegium* and *Onoclea* soil monocultures only (Figure 32). And lastly, OTU 302 only occurs in mixed soil communities (Figure 33).

Four OTUs are unique to the mixed soils, but only OTU 302 mentioned above is strongly associated with either of the first two axes (Table 12). The soil from the mixed community where this OTU is found appears to be outliers in relation to the rest of the mixed samples (Figure 33).

Table 12. Correlation Coefficients for OTUs from mixed community soils.
Only OTUs with correlations greater than an absolute value of 0.4 on at least one axis are shown and bold numbers indicate very strong correlations (>|0.4|).

	correlation coefficients	
variable	axis 1	axis 2
OTU 220	**0.676**	0.081
OTU 257	**0.591**	**0.551**
OTU 277	0.115	**-0.807**
OTU 283	0.078	**-0.568**
OTU 302	**-0.709**	0.355

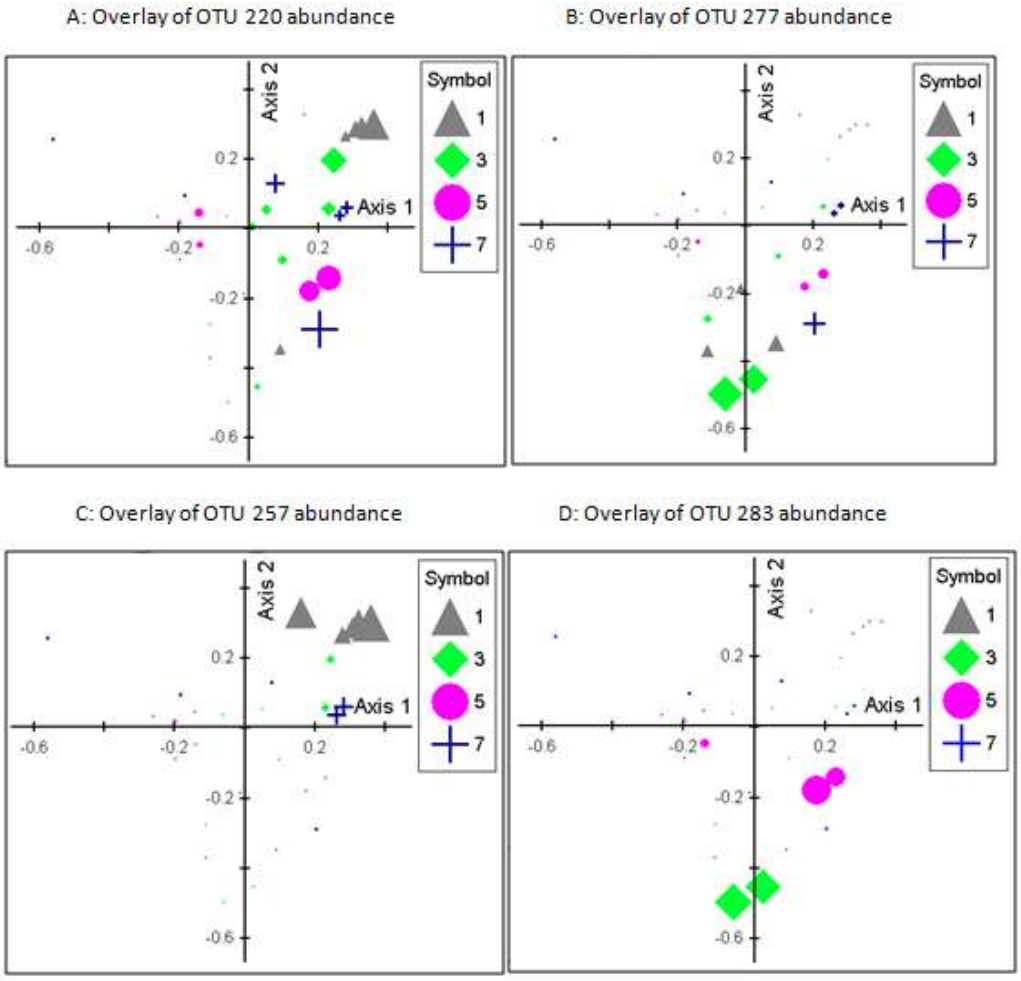

Figure 32 a-d. PCO ordination for fungal communities from all soil samples showing quantitative overlays of the relative abundance of four OTUs occurring in soil samples.
Symbols: [1] gray triangle = *Amphicarpaea* monoculture soil; [3] green diamond = *Microstegium* monoculture soil; [5] pink circle = *Onoclea* monoculture soil; [7] blue cross = soil from mixed plant community.

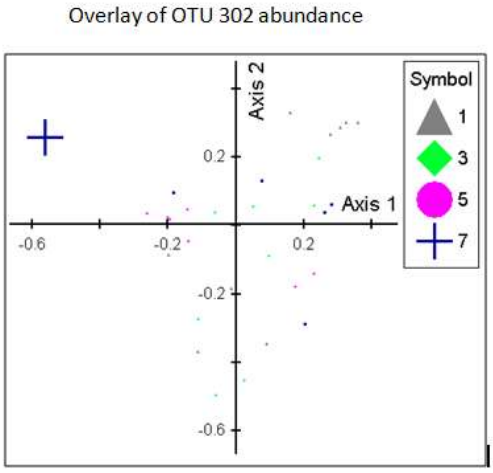

Figure 33. PCO ordination for fungal communities from all soil samples showing the quantitative overlay of the relative abundance of OTU 302 in soil samples.
Symbols: [1] gray triangle = *Amphicarpaea* monoculture soil; [3] green diamond = *Microstegium* monoculture soil; [5] pink circle = *Onoclea* monoculture soil; [7] blue cross = soil from mixed plant community.

PCO: All Roots & Soils

To better understand how soil OTU communities are related to root OTU communities, all root and soil samples were combined into one PCO (Figure 34). The root samples maintain the same clustering pattern seen in the "all roots" PCO (Figure 25), and the monoculture soil samples continue to display a random distribution across all the root samples (Figure 31). However, the mixed soil samples cluster together at the center of the ordination (Figure 34). These mixed soil samples do not share the majority of the OTUs occurring in the roots and monoculture soils (overlays not shown). As such, they are not able to cluster with the other samples.

The first five axes are considered robust for analysis ($p<0.5$, 100 randomization runs). The first two axes explain 17.9% and 8.5% of the variance, for a cumulative total of 26.4%. The third, fourth, and fifth axes explain an additional 6.9, 5.8, and 5.3 %. Together all five axes explain a cumulative 44.4% of the variance. This is clearly a

reduction in explanatory power from the "all roots PCO," where the first four axes explained nearly 50% of the variance. This reduction in explanatory power is informative in that it demonstrates the masking effect that random soil distributions have on the combined PCO.

Table 13. Correlation Coefficients of OTUs from "all samples" PCO.
Only OTUs with correlations greater than an absolute value of 0.3 on at least one axis are shown and bold numbers indicate very strong correlations (>|0.4|).

	correlation coefficients	
variable	axis 1	axis 2
OTU 244	-0.333	**0.65**
OTU 257	-0.337	0.043
OTU 277	**0.902**	0.194
OTU 285	0.356	0.112
OTU 293	0.03	-0.304
OTU 303	-0.337	0.046
OTU 313	-0.003	**-0.534**
OTU 316	-0.183	**-0.424**

Axes 1 and 2 are important in the clustering of communities (Figure 34). And, as expected, the following OTUs are strongly associated with axis 1 or axis 2: OTU 244, 277, 313, and 316 (Table 13). OTU 244 occurs in the monoculture roots and soil of *Microstegium* and in the monoculture roots and soil of *Amphicarpaea*; however, the symbols in Figure 35 do not distinguish between the soils. In the mixture community, this OTU switches to *Microstegium* and *Onoclea* roots alone (Figure 35). As previously

mentioned, OTU 277 is identified in all *Microstegium* roots and soils; and this OTU's strong correlation with the first axis indicates its important role in the clustering of *Microstegium* and several *Onoclea* (mixed roots) samples away from the rest of the samples (Figure 35). OTU 313 is found in the monoculture soil of *Microstegium* and *Onoclea* (shown previously in Figure 30), the monoculture roots of *Onoclea* (Figure 35), and rarely in monoculture roots of *Amphicarpaea* (*Amphicarpaea* is not discernible in Figure 35c due to very low abundance). In regard to the mixed community, this OTU is found in *Microstegium* and *Onoclea* roots but not the mixed soil (Figure 35c). Lastly, OTU 316 is found in *Onoclea* monoculture and mixed roots, *Amphicarpaea* monoculture soil (not labeled separately), and *Amphicarpaea* mixed roots (Figure 35).

Altogether, Figure 35 shows that some of the dominant OTUs occurring in roots are distributed throughout the soils. At the same time, some of the OTUs are not consistent, occurring in roots of one plant and the rhizosphere soil of another (for example, OTU 277 shown in Figure 32 and Figure 35).

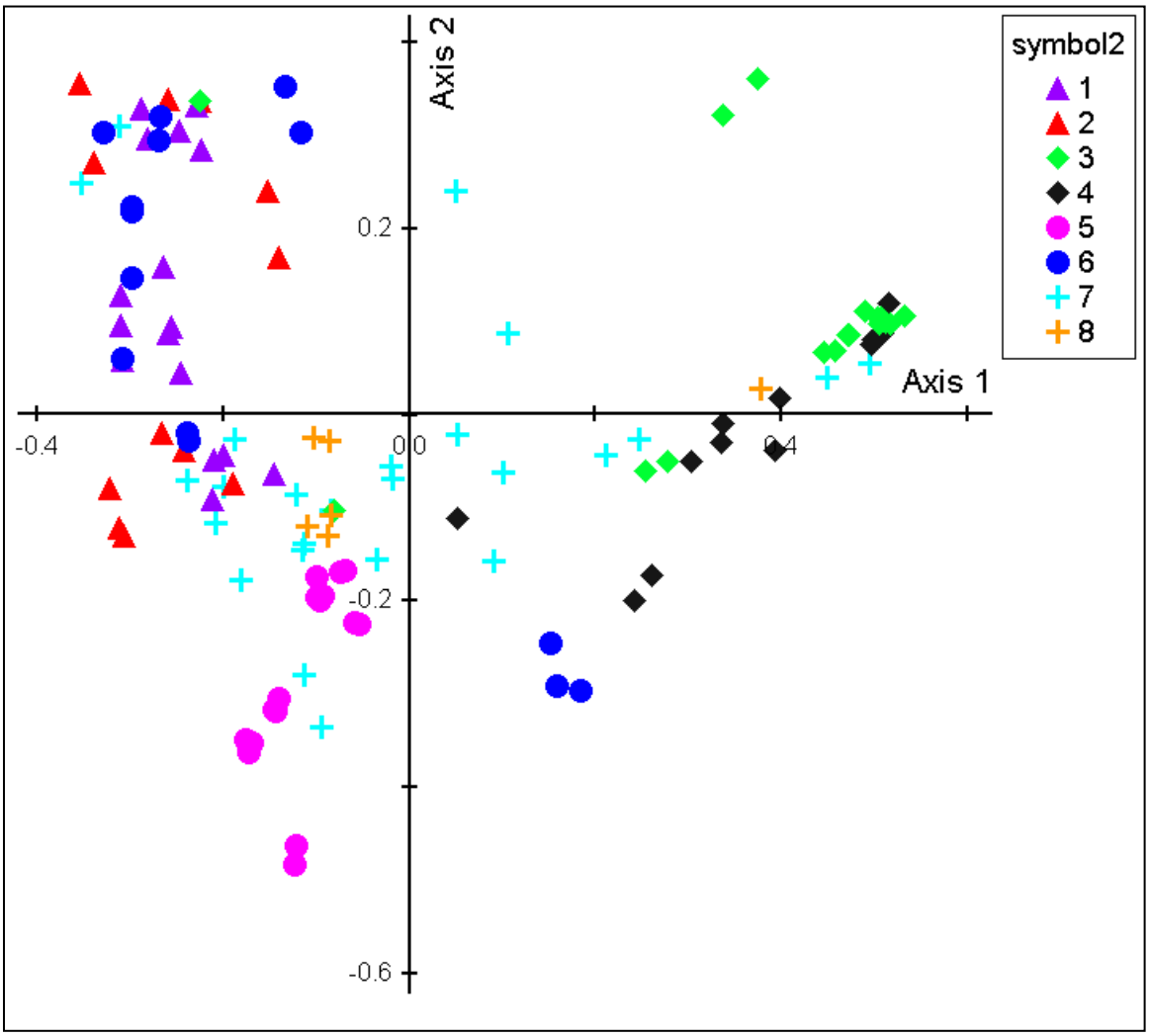

Figure 34. PCO of all root and soil sample replicates.
Symbols: [1] purple triangle = monoculture *Amphicarpaea* roots; [2] red triangle = mixed *Amphicarpaea* roots; [3] green diamond = monoculture *Microstegium* roots; [4] black diamond = mixed *Microstegium* roots; [5] pink circle = *Onoclea* monoculture roots; [6] blue circle = mixed *Onoclea* roots; [7] aqua cross = soil monoculture; [8] orange cross = soil mixture.

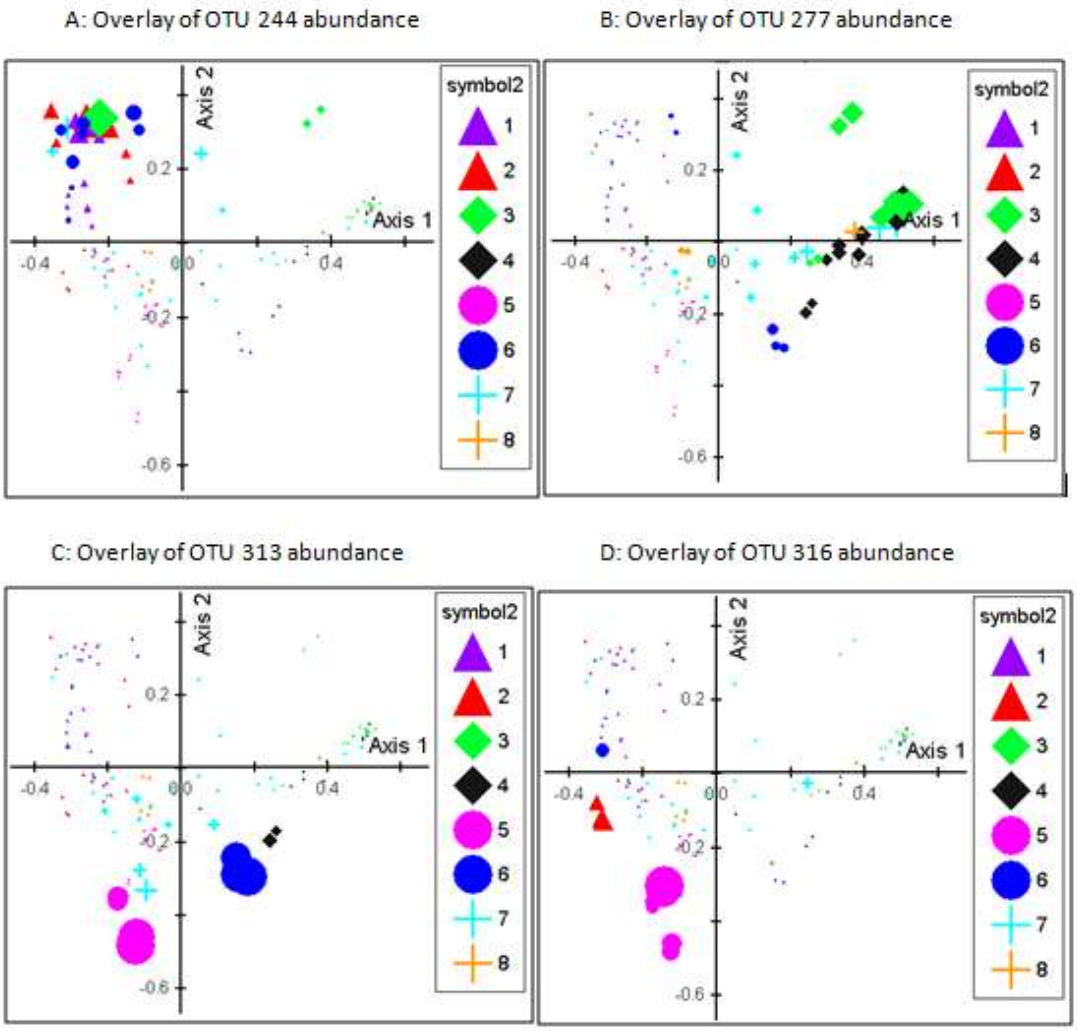

Figure 35 a-d. PCO of all root and soil sample replicates showing quantitative overlays of the relative abundance of select OTUs.
Symbols: [1] purple triangle = monoculture *Amphicarpaea* roots; [2] red triangle = mixed *Amphicarpaea* roots; [3] green diamond = monoculture *Microstegium* roots; [4] black diamond = mixed *Microstegium* roots; [5] pink circle = *Onoclea* monoculture roots; [6] blue circle = mixed *Onoclea* roots; [7] aqua cross = soil monoculture; [8] orange cross = soil mixture.

Brightfield microscopy

Brightfield microscopy revealed high (>50%) colonization rates by arbuscular mycorrhizae. Of the monocultures, *Microstegium* roots were the least colonized of the three species with an average of 63.9% total colonization – similar to colonization

percentages recorded by Nord (2011). *Amphicarpaea* roots had statistically significantly higher colonization with an average of 75.8% colonization ($t=2.46$, $p < 0.05$), and *Onoclea* roots had just slightly higher colonization than *Amphicarpaea* with an average of 80.4% in the monocultures (Table 14). A similar pattern is seen in the mixed samples. *Microstegium* had the least colonization (61.4%), *Amphicarpaea* had higher colonization (71.6%), and *Onoclea* had the highest with 77.1% colonization (Table 14).

All three species appear to have a slightly lower total colonization in the mixed roots as compared to the monoculture roots (Table 14). However, this apparent decrease in fungal colonization is not statistically supported (Table 14).

Table 14. Mean percent of roots colonized by various fungal structures: vesicles only, arbuscules only, vesicles with arbuscules, and hyphae. Values in parentheses are standard errors.

			Colonization frequency by				
	Monoculture vs. Mixed	No aseptate fungi	Vesicles	Arbuscules	Aseptate hyphae	Arbuscule & Vesicles together	Total root colonization (%)*
Amhicarpaea bracteata	Monoculture	0.24 (3.28)	0.06 (0.91)	0.14 (2.44)	0.55 (5.05)	0.00 (0.34)	75.8 (3.28)
	Mixed	0.28 (3.55)	0.06 (1.52)	0.17 (3.08)	0.48 (5.55)	0.01 (0.40)	71.6 (3.55)
Onoclea sensibilis	Monoculture	0.20 (4.67)	0.05 (1.12)	0.41 (6.50)	0.25 (4.49)	0.10 (1.83)	80.4 (4.67)
	Mixed	0.23 (7.31)	0.08 (1.76)	0.34 (6.65)	0.28 (5.66)	0.07 (2.99)	77.1 (7.31)
Microstegium vimineum	Monoculture	0.36 (4.48)	0.06 (2.65)	0.16 (2.53)	0.40 (3.81)	0.02 (1.14)	63.9 (4.48)
	Mixed	0.39 (6.61)	0.06 (1.17)	0.10 (2.46)	0.45 (5.03)	0.01 (0.68)	61.4 (6.61)

* Individual species are not statistically different between monoculture and mixed communities ($p>0.1$).

In spite of variable staining results, hyphae, arbuscules, and vesicles were found in *Onoclea* (Figure 36), *Amphicarpaea* (Figure 37 through Figure 39), and *Microstegium* (Figure 40 through Figure 43). In addition, these structures differed morphologically among the three plants. Aseptate hyphae were found to coil in select samples from all three plants, and coils were noted most frequently in *Microstegium* (representative coils shown in Figure 42 and Figure 43). Hyphae in *Microstegium* were sometimes thicker in diameter than hyphae in *Onoclea.* The large hyphae observed in samples resemble Acaulosporaceae and Archaeosporaceae morphology ("Classification of Glomales," n.d.). The thin hyphae also observed in samples resemble Glomaceae hyphae ("Classification of Glomales," n.d.). Morphological differences carried over to the arbuscules and vesicles, with distinctly lobed arbuscules occurring most frequently in *Onoclea* and "delicate feathery" arbuscules occurring in *Amphicarpaea* (Figure 36 and Figure 37).

Onoclea roots were characterized by high presence of arbuscules in both monoculture and mixed samples (41% and 34%, respectively) while *Microstegium* and *Amphicarpaea* roots were dominated by aseptate hyphae in both monoculture and mixed samples (*Microstegium:* 40 and 45%; *Amphicarpaea*: 55 and 48%, respectively). These observations are supported in the multivariate ordination (Figure 44). In this PCO, *Onoclea* clusters away from *Amphicarpaea* and *Microstegium* based on the first axis (Figure 44). Of the five morphology categories (no VAM, arbuscules only, vesicles only, hyphae, and vesicles *with* arbuscules), the "arbuscules only" category has the highest positive correlation to the first axis (Table 15). In contrast, the "aseptate hyphae"

category has the most negative correlation to this axis (Table 15). The second axis partially separates the peanut from the grass. Of the same five categories, the categories of "no VAM structures" and "aseptate hyphae" are strongly correlated with this axis. More specifically, "no VAM" is negatively correlated while "aseptate hyphae" is positively correlated (Table 15). Altogether, the first axis explains 54% of the variance and the second axis explains an additional 24.9%. Randomization analysis suggests that only the first axis is robust for analysis ($p<0.05$; 35 randomization runs).

Table 15. Correlation coefficients of morphology categories in the "microscopy PCO."

	Correlation coefficients	
Category	**axis 1**	**axis 2**
no VAM	-0.392	-0.911
aseptate hyphae	-0.833	0.519
arbuscules only	0.965	0.064
vescicles only	-0.109	0.485
arbuscules and vesicles	0.725	0.276

During Brightfield microscopy, one peanut root sample stood out from all other microscopy samples. *Amphicarpaea* roots collected from the monoculture quadrat #2 (spring sampling) had a lower hyphae count and higher arbuscule count than the other *Amphicarpaea* samples. And, this sample shows up as an outlier in the PCO (Figure 44).

Microscopic analysis also revealed unique structures that have not been identified. These structures are lobed in appearance, stain blue in the center, but remain brown at the edge. Altogether, they resemble a "morphological hybrid" of arbuscules, microsclerotia, and large hyphopodia (Figure 45). Dark Septate Fungi (DSF) with microsclerotia were identified in all three plants (*Onoclea* and *Microstegium* representatives shown in Figure 45 through Figure 47). In one unique slide from *Onoclea*, a clamp connection indicative of the Basidiomycetes was found among the septate hyphae (Figure 47).

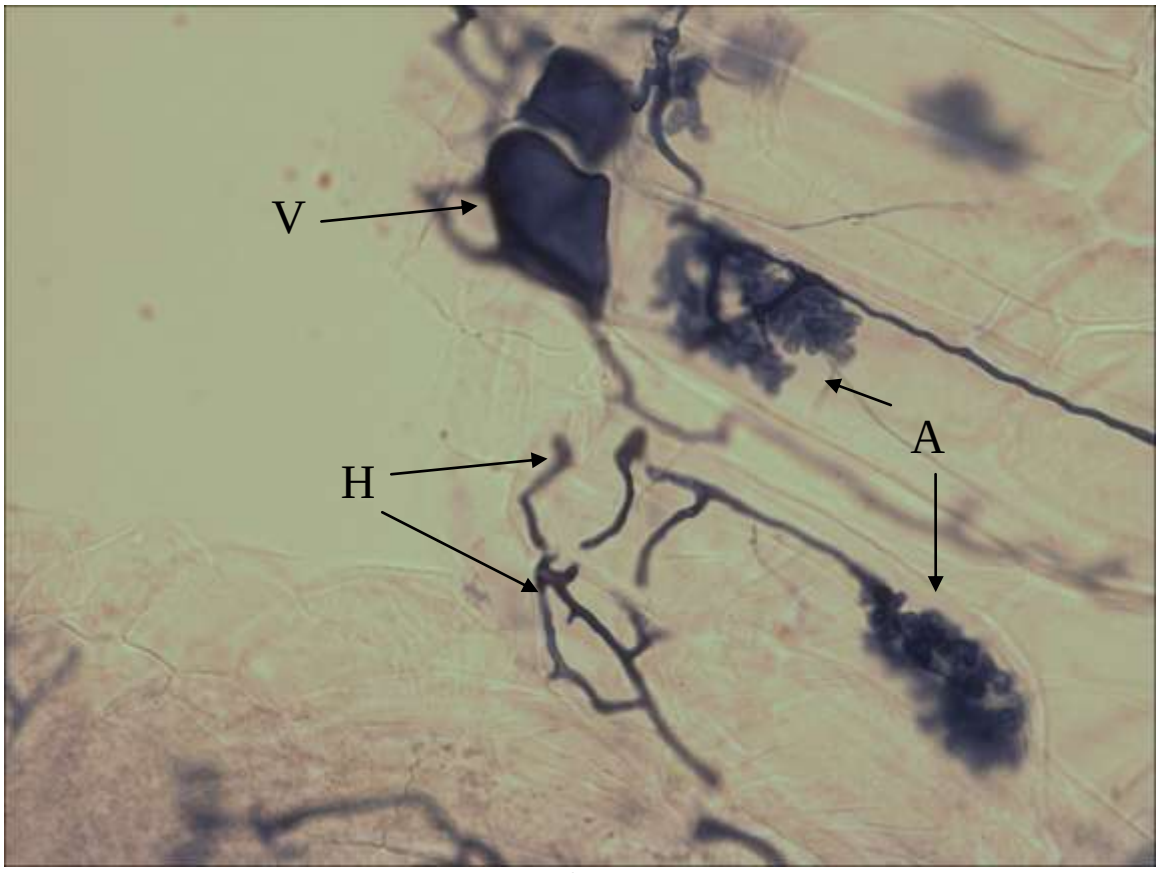

Figure 36. Vesicle (V), hyphae (H), and arbuscules (A) found in *Onoclea sensibilis* (Spring 2012 sample; 40X; Trypan Blue stain). Credit: B. North

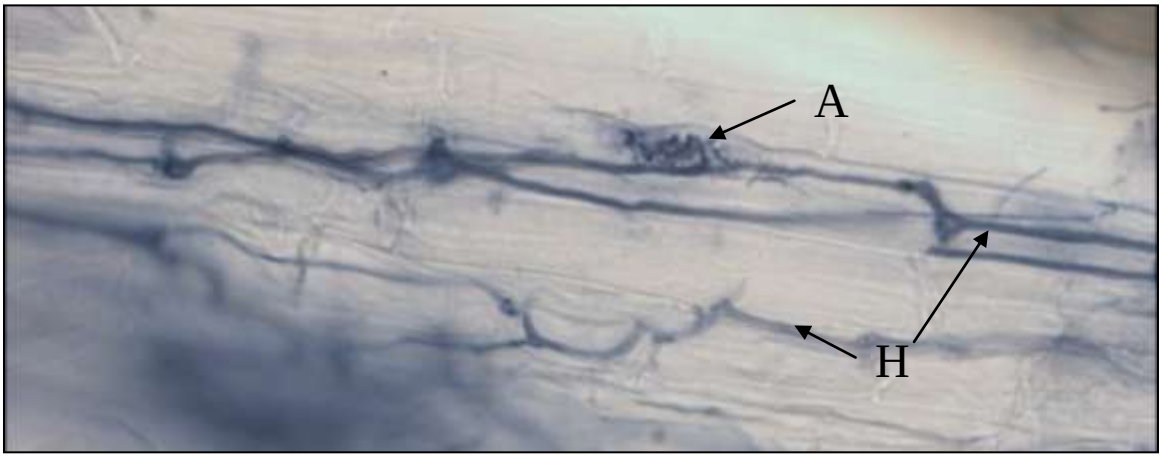

Figure 37. *Amphicarpaea* root and arbuscule (A) with hyphae (H) (fall 2011 sample; 20X; Trypan Blue). Credit: B. North

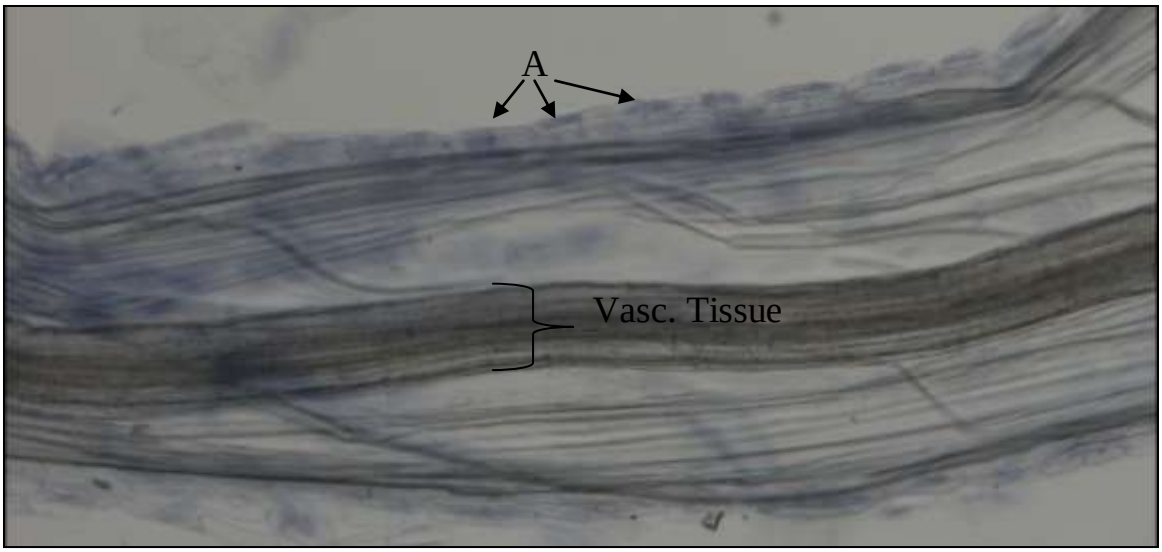

Figure 38. *Amphicarpaea* root showing vascular tissue and arbuscules (A) (spring 2012 sample; 20X; Trypan Blue). Credit: B. North

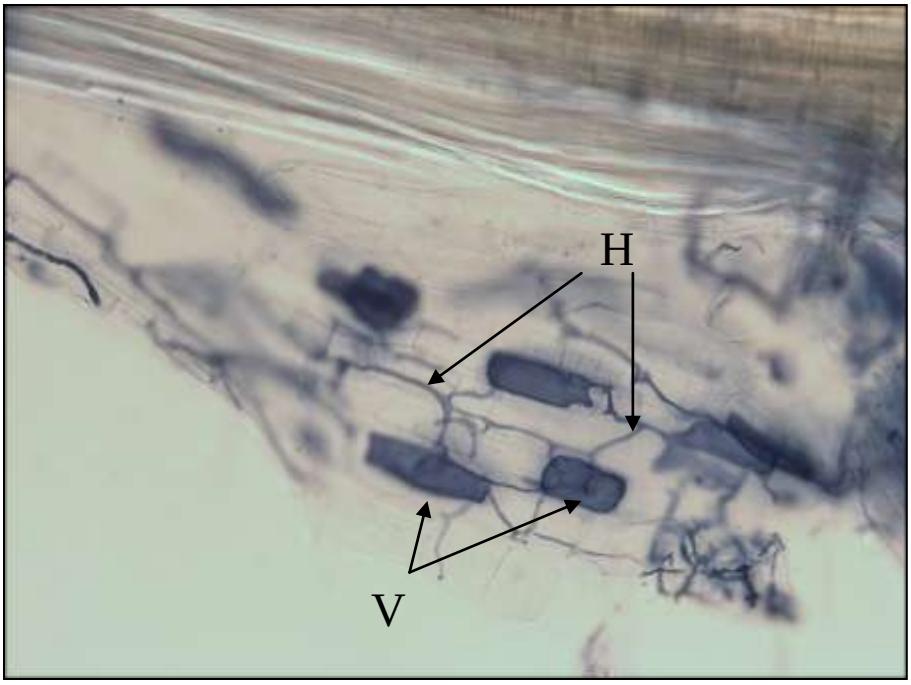

Figure 39. ***Amphicarpaea*** **root and arbuscular mycorrhizal vescicles (V) and hyphae (H) (fall 2011 sample; 20X; Trypan Blue). Credit: B. North**

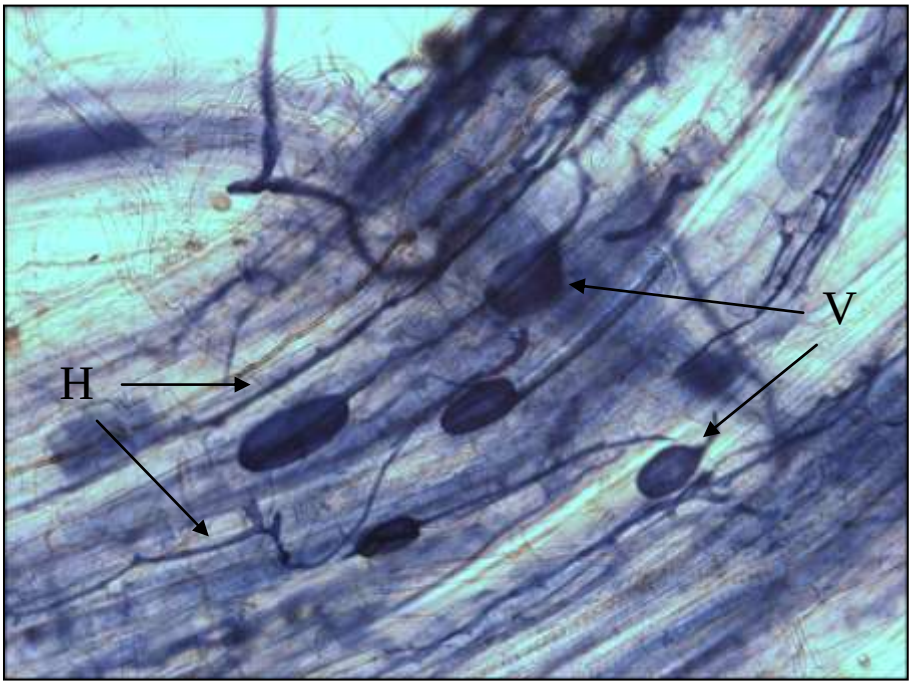

Figure 40. ***Microstegium*** **fine root colonized with mycorrhizal hyphae (H) and vesicles (V) (spring 2012 sample; 20X; Trypan Blue). Credit: B. North.**

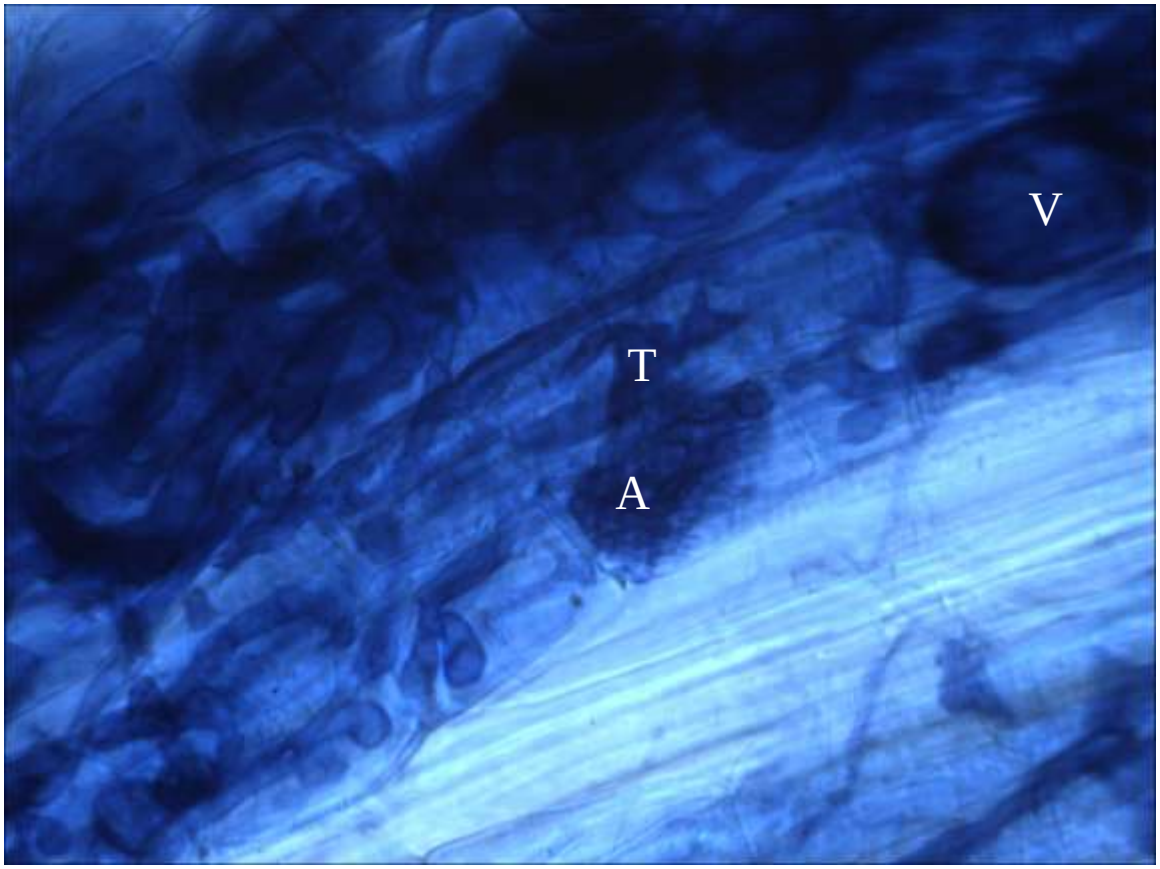

Figure 41. Arbuscule (A), trunk hypha (T), and vescicle (V) in *Microstegium vimineum* (Fall 2011 monoculture site 2; 63X; Trypan Blue). Credit: B. North

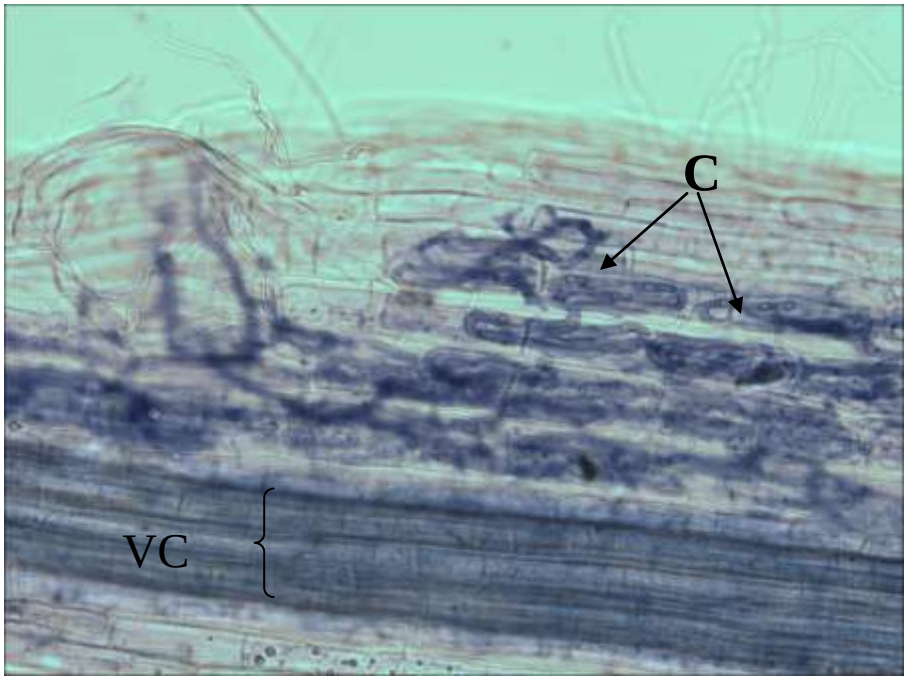

Figure 42. Coiling hyphae (C) near vascular cylinder (VC) in *Microstegium* root (fall 2011 monoculture; 20X; Trypan Blue). Credit: B. North

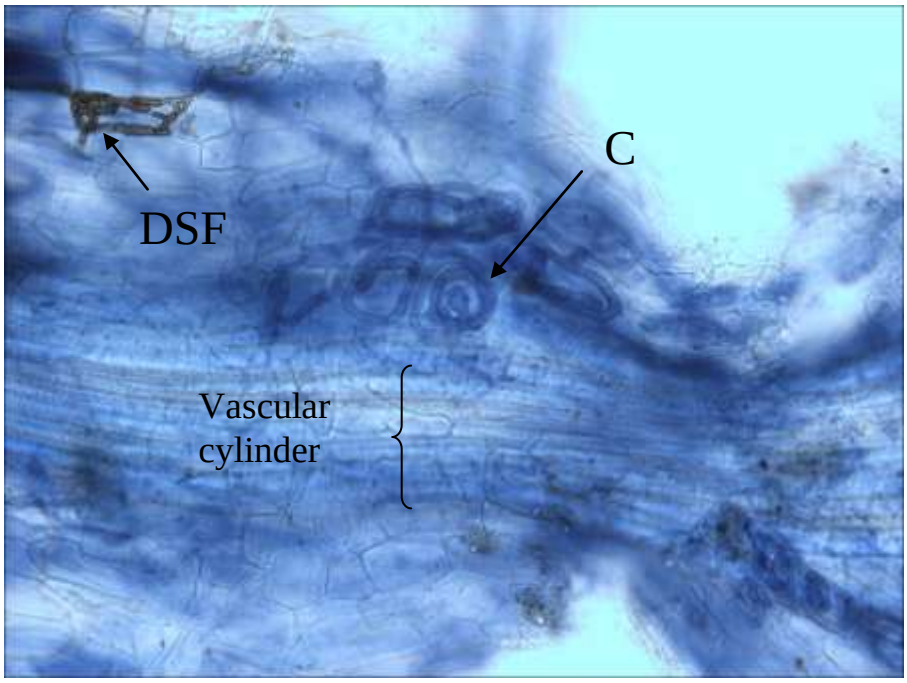

Figure 43. Coils (C) and Dark Septate Fungi (DSF) near vascular cylindar in *Microstegium* fine roots (fall 2011 monoculture; 20X; Trypan Blue) Credit: B. North.

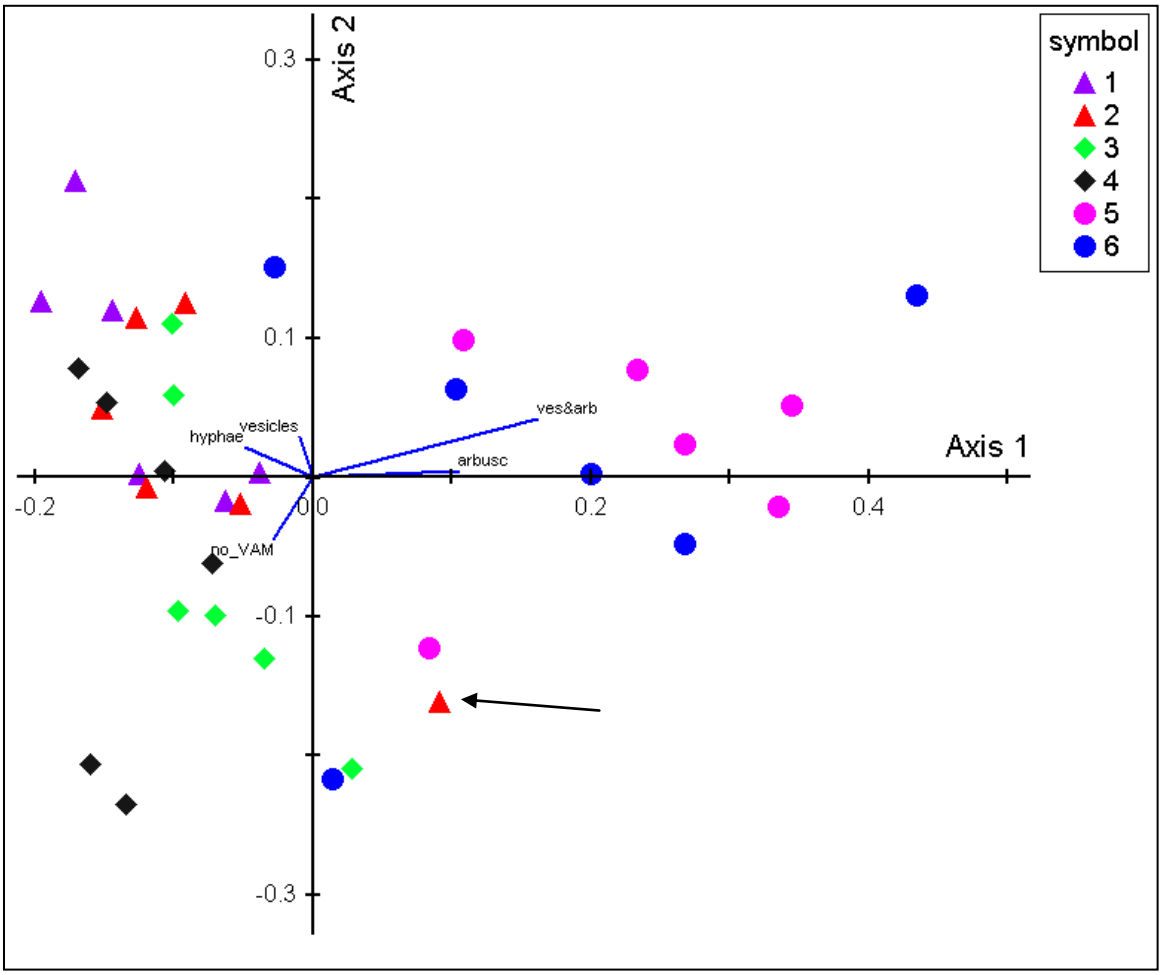

Figure 44. PCO ordination of microscopy samples showing vectors derived from fungal morphology categories. Symbols: [1] purple triangle = monoculture *Amphicarpaea;* [2] red triangle = mixed *Amphicarpaea*; [3] green diamond = monoculture grass; [4] black diamond = mixed grass; [5] magenta circle = monoculture fern; [6] blue circle = mixed fern. The arrow identifies the outlier *Amphicarpaea* sample.

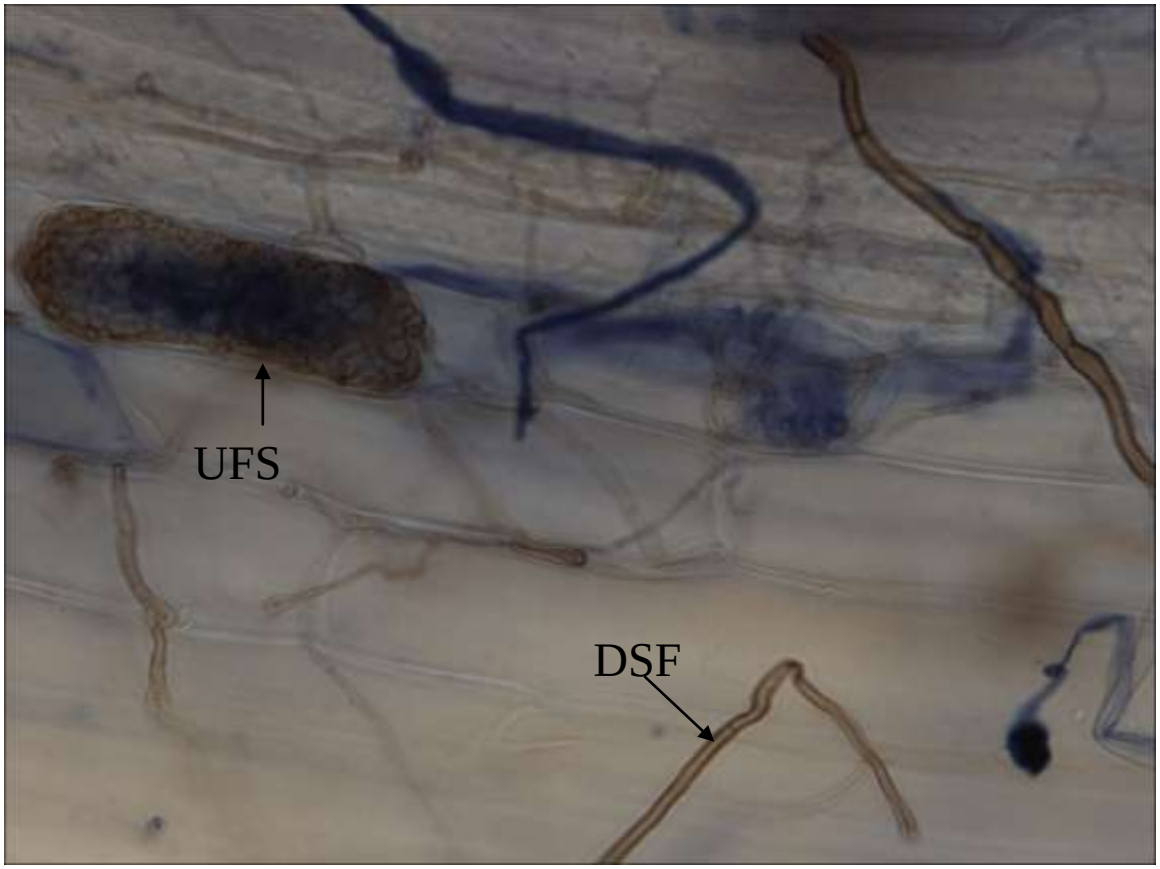

Figure 45. Unidentified fungal structure (UFS) in *Microstegium vimineum* fine root (spring 2012 monoculture sample; 63X; trypan blue). Credit: B. North.

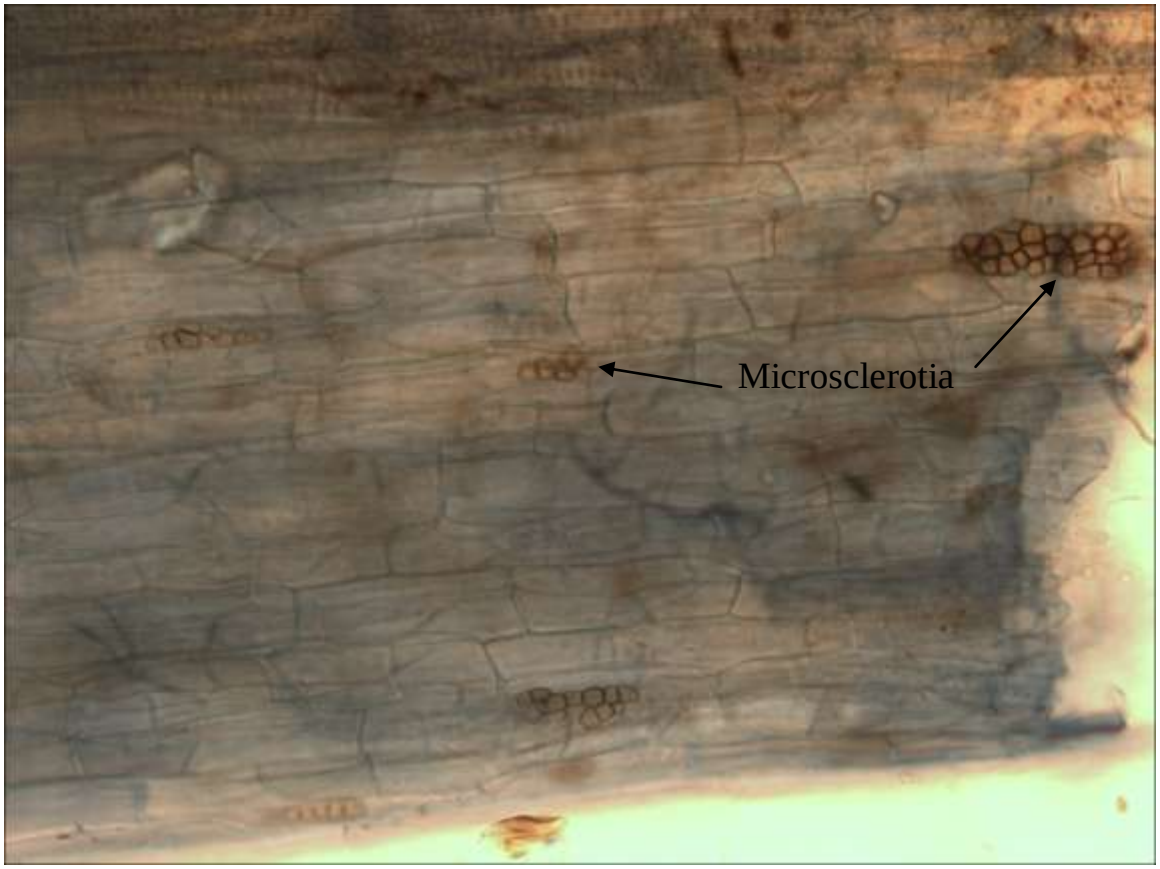

Figure 46. Microsclerotia in *Onoclea*
(fall mixed quadrat #1; 20X; Trypan Blue). Credit: B. North

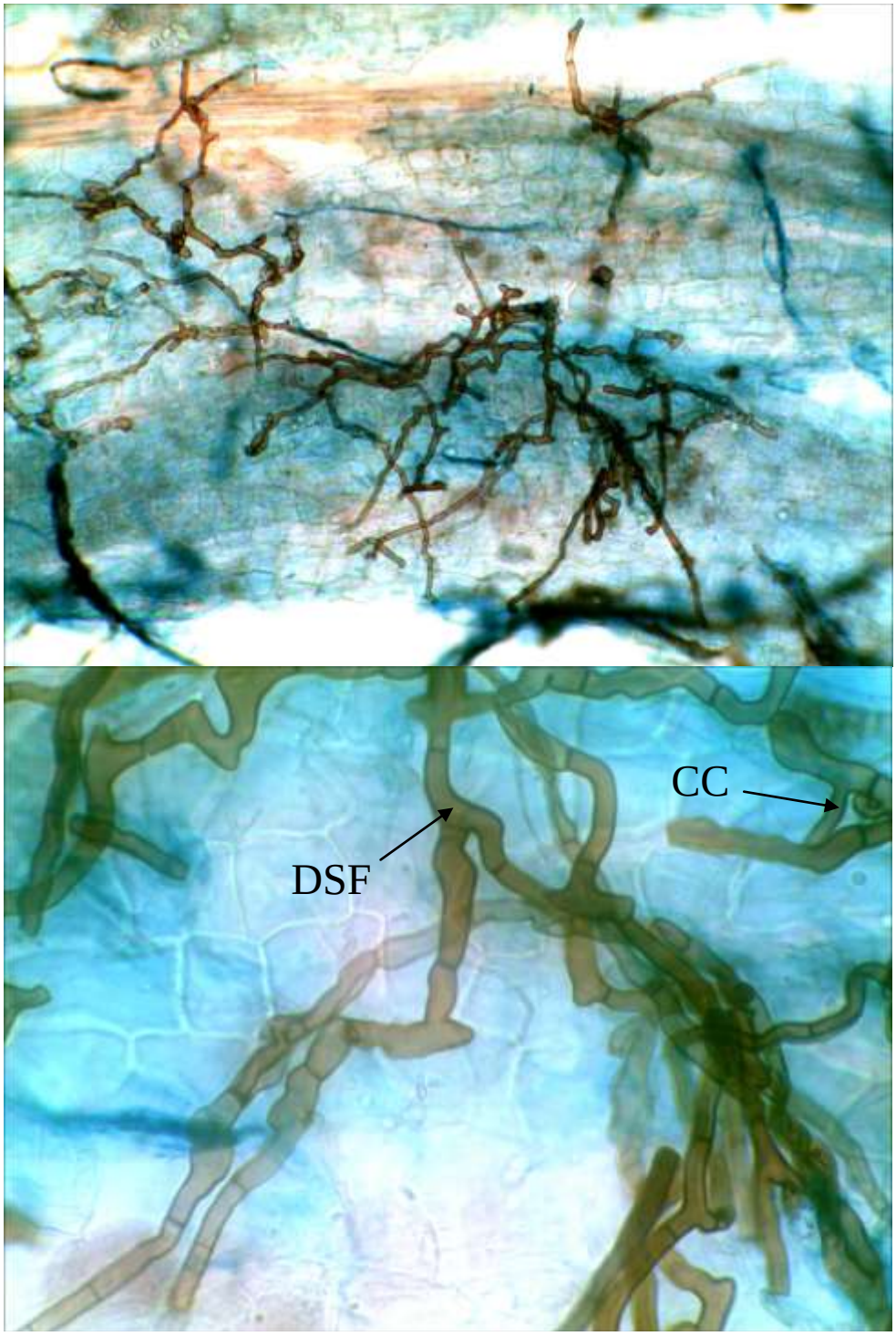

Figure 47. Dark Septate hyphae (DSF) with clamp connection (CC) in *Onoclea* root. (Trypan Blue). Credit: B. North

DISCUSSION

Distinct fungal communities

While molecular analysis did not go as far as sequencing to determine the exact fungal species occurring within each community, it did identify fungal OTU communities. ARISA fingerprints identified fungal communities while PCO ordinations of those communities showed that they were unique by plant species when plants were grown in monocultures found in different locations. While a number of environmental factors (i.e. soil color, pH, species richness) were assessed to verify that the fern monoculture field site at Burke Lake was comparable to the monocultures at Huntley Meadows, there is a possibility that the observed differences in fungal communities were due to differences between field sites rather than differences between plant species.

The finding that fungal OTUs occur in these plants is consistent with previous studies indicating that AM fungi occur in *Microstegium, Onoclea* and *Amphicarpaea* (Landis et al., 2004; Nord, 2011; Ross, 2008; West et al., 2009). These findings are also consistent with Kourtev's (2002) hypothesis that *Microstegium* uses AM fungi to supplement its short roots. Our first hypothesis that *Microstegium vimineum, Amphicarpaea bracteata,* and *Onoclea sensibilis* all support distinct root fungal communities when grown alone is clearly supported.

Plant and fungi competition

Fungal communities from *Amphicarpaea*, *Onoclea*, and *Microstegium* plants were expected to exhibit a shift in fungal communities. More specifically, *Microstegium*'s fungal community was expected to be incorporated into *Amphicarpaea* and *Onoclea* fungal communities. For the most part, this hypothesis was not upheld. *Microstegium* does share the dominant OTU 277 (Figure 35) and several minor OTUs with *Onoclea* in mixed communities (data not shown). Likewise, *Microstegium* shares a couple of minor OTUs with *Amphicarpaea* in the mixed communities (data not shown). These OTU shifts are summarized in Figure 48. This sharing of OTUs does not overtly alter the clustering of the *Microstegium* communities in multivariate ordinations. The molecular evidence predominantly shows that *Microstegium* maintains its own fungal community in both monospecific stands and mixed stands with *Onoclea* and *Amphicarpaea*. Maintaining a distinct community may explain the invasive tendency of the grass. By maintaining a fungal community not found in the native plants, the exotic species may have a competitive advantage over the natives.

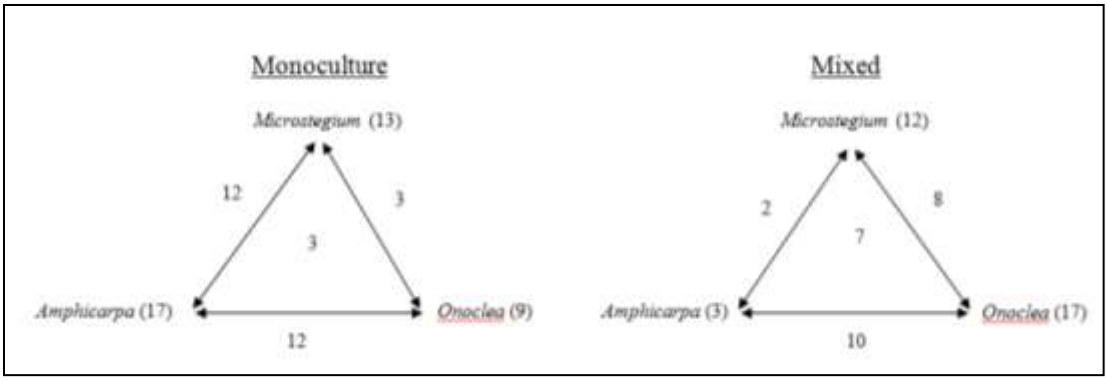

Figure 48. Shared OTUs in the monoculture versus mixture roots.
Numbers in parentheses indicate plant specific OTUs while numbers between species indicate shared OTUs.
Numbers in triangle centers indicate the number of OTUs occurring in all three plants.

While *Microstegium* maintains its own fungal community, *Onoclea* and *Amphicarpaea* do not exhibit distinct fungal communities when grown together in the mixed community. This observed sharing of fungal communities may be the natives' response to invasion by *Microstegium* and may indicate an adverse condition that enhances invasion. On the other hand, the shared communities may be a result of the two natives growing together for many years independent of *Microstegium'*s presence. Verification of either hypothesis requires a separate field experiment where the two natives are grown together without the presence of *Microstegium*.

Both molecular analysis and microscopy showed mixture roots having a slight decrease in total colonization and OTU community richness compared to the monoculture roots. However, the observed decrease in colonization and community richness was not statistically significant. In contrast, the soils *did* show a statistically significant decrease from monoculture soils to mixed soils. A possible explanation for the observed decrease is that the mixed community had additional plant humic

compounds within the soil from the presence of additional plants. These compounds could act as DNA inhibitors during PCR amplification, resulting in a reduced OTU community.

Since the Huntley Meadow site did not have monoculture stands of the fern, fern monoculture data was obtained from the Burke Lake site. Although this might be a confounding factor, care was taken to verify that the same species of fern was sampled at both sites and that the soil type and soil pH were comparable. Multivariate analysis of soil OTU profiles from each monoculture and mixed community showed that some of the dominant OTUs of the roots did occur in the soil. However, a number of the OTUs occurring in the roots did not occur in the soil. This may be due to non-amplifiable "rare" species in the soil that are only amplified when they proliferate in a suitable host root. On the other hand, some of the soil fungal OTUs did not occur in the roots. These OTUs may represent saprotrophic, non-endophytic fungi.

ARISA vs DNA sequencing

There are two caveats regarding ARISA fingerprinting. As mentioned in the methods, ARISA fingerprinting produces amplicons of fungal DNA (i.e. OTUs). These OTUs are assumed to be different species. However, this may not be the case. Different fungal species may have the same OTU fragments during DNA amplification, or a single species may have multiple OTUs of different lengths (Fisher & Triplett, 1999). As a result, it is possible that the fingerprinting in this research has either underestimated or overestimated the total number of fungi in the soil and plant communities. To clarify this

situation, sequencing of PCR products and BLAST (Basic Local Analysis Search Tool) analysis are needed.

Microscopy

The microscopic analysis not only confirmed colonization by arbuscular mycorrhizae but also identified other fungal structures that might be fulfilling important ecological roles both for individual plants and the ecosystem as a whole. Dark Septate Fungi (DSF) were identified in roots from all three plants. These fungi are usually classified in the Ascomycetes (Jumpponen & Trappe, 1998), but the clamp connection identified during microscopy suggests that at least one Basidiomycete species is present. The comprehensive review of DSF by Jumpponen and Trappe (1998) suggests that the ecological role of these fungi is in debate. These fungi may display different morphologies depending on the species of their host; and the exact nature of the relationships between DSF and host plants has been documented to range from parasite to mutualist (Jumpponen & Trappe, 1998; Jumpponen, 2001).

A possible biocontrol for *Microstegium*

Many restoration ecologists are familiar with the importance mycorrhizae can play in restoring an ecosystem to a previous state of plant diversity. This research shows that the native plants have their own fungal community while the invasive grass has a different community. Whether these fungal communities could be manipulated to allow the native species to outcompete the exotic *Microstegium* is worth researching. A short or long term biocontrol experiment has the potential of accomplishing this goal. Individual AM fungi as well as whole communities of AM fungi would need to be added to or

removed from the system to determine the ability of AM fungi to 1) strengthen native plant resistance to invasion or 2) weaken the competitive nature of the invasive grass.

Other research considerations

Do fungi shift between plant hosts as the seasons change? Or, do certain fungal species dominate during different seasons? While this research did not identify any apparent seasonal shifts in the fungal community, it did identify certain OTUs (277, 285, 316, etc.) that were dominant. Future research might include samples over multiple seasons and years to compare shifts between dominant taxa within individual fungal communities. Shifts in taxa abundance might indicate the health of the native plants as a response to the presence of *Microstegium*. For example, an increase in parasitic species could indicate a negative effect of *Microstegium* invasion. Using pyrosequencing, other studies have found that fungal assemblages can be seasonally unique and that certain taxa can dominate within individual fungal assemblages (Dumbrell et al., 2011; Helgason, Fitter, & Young, 1999).

While this research has focused on how changes in fungal community composition can explain the invasion of *Microstegium* into a native plant community, allelopathy also has potential for explaining the effects of these plants. For example, *Microstegium* is known to be allelopathic (Corbett & Morrison, 2012; Pisula & Meiners, 2010). If the White Snakeroot of Corbett and Morrison's (2012) research is able to compete with *Microstegium* because of its allelopathy, might not other native plants such as *Onoclea* and *Amphicarpaea* have the same ability? And, if *Onoclea* and/or

Amphicarpaea are shown to be strongly allelopathic, could they be used as competitors with the grass?

Conclusion

Overall, this research has created a baseline assessment for the fungal communities of *Onoclea sensibilis, Amphicarpaea bracteata,* and *Microstegium vimineum.* While many questions remain regarding the chemical and physiological pathways involved in these plant and fungal interactions, this research indicates that when the three plants grow together in a mixed community, the two native plants share fungi while the exotic grass remains distinct. However, the small shifts in fungal communities among the three species imply that there may be some other functional significance not detected by just observing overall fungal community structure. Further research of these mycorrhizal interactions may show that the fungal community can be manipulated to the overall advantage of the native plant community. Such findings would assist those who are managing and restoring *Microstegium* invaded parks.

APPENDIX I

Modified BIO101-FastDNA Spin Protocol for Fungal Tissues[1]

1. Add extra ceramic bead to each Lysing Mtrix A tube (two beads total).
2. Add up to 200 mg finely ground roots to tubes.
3. Add 800 µl CLS-VF and 200µl PPS to each tube.
4. Put in FastPrep instrument and run 3X at speed 5.0 for 30 seconds; cool tubes on ice between cycles.
5. Centrifuge 10 minutes at max speed (14000 rpm).
6. Transfer 600µl of supernatant to 2 ml centrifuge vial.
7. Resuspend Binding Matrix and add 600µl to vial.
8. Put on rotator for 2 minutes.
9. Briefly centrifuge for <10 seconds to collect pellet.
10. Discard supernatant by pouring directly from vial into waste receptacle.
11. Suspend pellet in 500 µl SEWS-M (ethanol added) and transfer to kit-supplied centrifuge vial with spin filter.
12. Centrifuge for 1 minute at 14000 rpm.
13. Discard contents of catch tube.
14. Centrifuge for 1 minute at 14000 rpm and then place under hood with blower for up to 5 minutes to dry.
15. Move spin filter to new kit-supplied catch tube and gently resuspend the pellet in 100µl of 65°C-warmed DES water and incubate at RT for 2 minutes.
16. Centrifuge for 1 minute at 14000rpm to collect DNA extract in catch tube.

[1] Andrews, 2011

APPENDIX II.

Modified BIO101-FastDNA Spin Protocol for Soil

1. Make sure everything is labeled, put DES in incubator (when pouring DES, don't put a pipette tip into the bottle).
2. Add ~ 500 mg (or ~ 250 µg of liquid) of soil (or equal amount of other tissues) to Lysing Matrix E tube. (The sample and the lysing matrix should not exceed more than 7/8 of the tube volume).
3. Add 978 µl Sodium Phosphate Buffer and 122 µl MT Buffer (this is the detergent).
4. Secure tubes in FastPRep Instrument (never turn off machine; ask for help running machine, if needed) and process for 30 seconds at speed 5.5 (repeat this step if needed).
5. Centrifuge the tubes as 14,000 x g (~14,000rpm on small centrifuges) for 10-15 minutes. Set up 2 sets of clean tubes, label.
6. Transfer supernatant to a clean 1.5 ml microfuge tube (use pipette!).
7. Add 250 µl PPS reagent and mix by inverting the rube by hand 10 times.
8. Centrifuge at 14,000 x g for 5 min. to pellet precipitate. Transfer supernatant to a clean 2 ml microfuge tube.
9. Re-suspend Binding Matrix reagent and add equal volume (~700 µg) to the supernatant.
10. Place on a rotator or invert by hand for 2-3 minute to allow binding of DNA to matrix. Place tube in a rack for 10-15 minutes to allow settling of silica matrix. (this is an ok time to leave for a few minutes)
11. Remove 600 µl of supernatant being careful to avoid settled Binding Matrix.
12. Repeat the last step.

13. Re-suspend the Binding Matrix in the remaining amount of supernatant. Transfer the mixture (approximately 600 µl) to a Spin Filter and centrifuge the spin filter and catch tube at 14,000x g for 1 minute.
14. Discard the flow-through that is now in the catch tube.
15. Add 500 µl SEWS-M (** ETOH added [salt ethanol water]) to the Spin Filter and centrifuge at 14,000xg for 1 minute. Discard the flow-through and place Spin Filter in Catch Tube.
16. Repeat last step and then centrifuge at 14,000xg for 2 minutes to "dry" the matrix of residual SEWS-M wash solution.
17. Remove Spin Filter and place in fresh kit-supplied Catch tube. Air dry the Spin Filter (let it dry with the lid open) for ~ 5 minutes at room temperature (put in the hood with the blower on).
18. Add 150 µl (can be 100-200 µl) DES water (or DNase/Pyrogen Free Water) and gently stir matrix on filter membrane by vortex/finger flip to re-suspend the silica for efficient elution of the DNA. Incubate the tubes at room temperature for 2 minutes.
19. Centrifuge at 14,000 x g for 1 minute to transfer eluted DNA to Catch tube. DNA is now application-ready. Make appropriate dilution of DNA and run original DNA Extractions on 0.7-1% agarose gel to visualize the DNA.
20. Store the original DNA at -20°C (or for long term storage at -80°C).

** Add 100 ml 100% ETOH to original bottle of SEWS-M before using and label bottle "ETOH added".

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BIOGRAPHY

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