INVASIVE CHINESE LESPEDEZA (*LESPEDEZA CUNEATA* [DUM.-COURS.] G. DON) ALTERS THE ROOT AND RHIZOSPHERE FUNGAL COMMUNITIES OF SWITCHGRASS (*PANICUM VIRGATUM* L.) IN NORTHERN VIRGINIA

by

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Dedication

This work is dedicated to my loving wife Holly and son Joseph Scott.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

BLAST	Basic Local Alignment Search Tool
Bp	Basepair
BSA	Bovine Serum Albumin
C	Carbon or Celsius, depending on context
CI	Confidence Interval
CLS-(VF/Y)	Cell Lysis/DNA Solubilizing Solution for Vegetation/Yeast and Fungi
dNTP	Deoxyribonucleotide
DEPC	Diethylpyrocarbonate
DES	DNA Elution Solution (Ultra Pure Water)
FAM	Fluorescein Amidite
M	Molar
MgCl	Magnesium Chloride
N	Nitrogen, Normal, or Number (of Samples), depending on context
NA	Not Applicable
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PPS	Protein Precipitation Solution
PSI	Pounds Per Square Inch
Q	Quadrat
(r)DNA	(ribosomal) Deoxyribonucleic Acid
(r)RNA	(ribosomal) Ribonucleic Acid
Rpm	Rounds Per Minute
RT	Room Temperature
Rx	Reaction
SD	Standard Deviation
SEWS-M	Salt/Ethanol Wash Solution
Sp.	Species
T	Temperature or Transect, depending on context
TAE	Tris-Acetate-EDTA
USDA	United States Department of Agriculture
VA	Virginia
χ	Chi, from statistics
Σ	Summation
$\|a\|$	Absolute Value (of a)

Abstract

INVASIVE CHINESE LESPEDEZA (*LESPEDEZA CUNEATA* [DUM.-COURS.] G. DON) ALTERS THE ROOT AND RHIZOSPHERE FUNGAL COMMUNITIES OF SWITCHGRASS (*PANICUM VIRGATUM* L.) IN NORTHERN VIRGINIA

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Exotic plants can alter the fungal communities of soils outside their native range with possible impacts upon competing plant species. Based on automated ribosomal intergenic spacer analysis (ARISA) of root and rhizosphere-soil fungi across gradients of plant invasion, I demonstrate that the invasive exotic *Lespedeza cuneata* altered the fungal communities of the native prairie grass *Panicum virgatum* at a site along the Washington and Old Dominion Trail in Reston, Virginia. The fungal communities of the roots and rhizospheres of *L. cuneata* significantly differed in structure compared to those of uninvaded *P. virgatum* ($p < 0.02$ and $p < 0.01$, respectively). Additionally, the fungal communities of *P. virgatum* roots and rhizosphere more-closely resembled those of the invader when both plants shared the same quadrat ($p < 0.05$ and $p < 0.01$), especially when the areal cover of *L. cuneata* exceeded 40%. In all, 15 of the 157 fungal operational taxonomic units (OTUs ~ species) detected were significant in differentiating the fungal

communities of *L. cuneata* and *P. virgatum* ($p < 0.05$) and eight of these were responsible for fungal community shifts with invasion, representing a putative crossover to *P. virgatum* of seven OTUs associated with *L. cuneata* and a decline of a single fungus associated with *P. virgatum*. Pyrosequencing of select fungal communities suggested that *L. cuneata* was associated with a variety of fungi, of which members of *Fusarium* were prominent, whereas *P. virgatum* was distinguished by a few uncultured environmental fungi. Additionally, the microscopic analysis of roots for arbuscular-mycorrhizae (AM) and dark-septate endophytic fungi (DSF) revealed that mycorrhizal-colonization exceeded 80% on average in both plants but the presence of DSF was twice as great in *P. virgatum* ($p < 0.005$), presenting the possibility of unique AM-DSF interactions within the invasive and native species. The observed shifts in fungal community structure of *P. virgatum* in the presence of invasive *L. cuneata* could represent a mechanism by which invasive species manipulates the fungal root community of native species in a way that promotes invasion.

1. Introduction

Invasive Plants and Plant-Microbial Ecology

Invasive species cost the United States nearly $137 billion annually through losses and damages to environmental resources and costs toward efforts to control outbreaks (Pimentel et al., 2000). The nation's biodiversity is particularly threatened since roughly 40% of all species listed under the Endangered Species Act are at risk due to predation and competition from alien species. Only human-induced land use changes endanger more US species than biological invasions (Wilcove et al., 1998). Invasive non-indigenous plant species alone account for over $24 billion of annual costs to the US and spread over an estimated 700,000 hectares of the nation's wilderness per year (McNeely, 2001; Pimentel et al., 2000). Since some invasive plants are capable of transforming native habitats into monocultural stands of little wildlife value (Callaway and Ridenour, 2004; D'Antonio et al., 2004; Pimentel et al., 2000) and single plant species have been shown to alter whole ecosystem processes (Vitousek and Walker, 1989), alien invasive flora may cause significant losses of native wildlife habitats and impact sensitive species by altering food webs. Just how a minority of the thousands of exotic plant species that naturalize in the United States become such aggressive invaders is the focus of much speculation (Levine et al., 2003). The discovery of general mechanisms of plant invasion

would both aid natural resource managers in conservation efforts and expand our knowledge of the links between population, community, and ecosystem ecology (Callaway and Maron, 2006; Kourtev et al., 2003; Levine et al., 2003; Smith and Knapp, 2001).

Ecologists have formulated and tested a variety of hypotheses regarding the ability of many exotic plant species to invade native ecosystems, yet empirically supported generalizations remain elusive. The "natural enemies hypothesis," which states that invasive species aggressively spread outside their native range because they have been liberated from specialist herbivores and pathogens (Callaway and Ridenour, 2004; Levine et al., 2003; Reinhart and Callaway, 2004), is the classic explanation for exotic plant invasiveness. A recent expansion of this hypothesis is "the evolution of increased competitive ability" (EICA), which predicts that competitively-liberated exotic plants should lose costly traits that formerly helped defend them against natural enemies and instead devote resources to traits conferring greater competitive advantage in their new habitats (Maron et al., 2004; Muller-Scharer et al., 2004). There is evidence for both hypotheses, but some invasive plants receive similar damage from herbivores and diseases in both native and foreign ranges and evidence of greater sizes of invasive plants outside their native ranges has not been shown to correlate with greater competitive ability or reallocation of resources from defensive traits (Callaway and Ridenour, 2004). An alternative mechanism for EICA, "allelopathic advantages against resident species" (AARS), has been proposed in which "novel biochemicals" exuded by the invasive plants

provide a strong competitive boost via phytotoxic (allelopathic) and antimicrobial means in their new habitats (Callaway and Ridenour, 2004; Hierro and Callaway, 2003). Invasive populations are expected to be more-allelopathic than those in their native ranges, and the competitive superiority need not be directly related to resource uptake (Callaway and Ridenour, 2004). While there is evidence of greater allelochemical exudation by a few invasive species outside their native range, results showing such biogeographical differences are ambiguous due to imperfect experimental design (Bais et al., 2003; Callaway and Ridenour, 2004).

Plant-microbe interactions have recently been proposed as both mediators and mechanisms of plant invasiveness (Callaway and Maron, 2006; Callaway and Ridenour, 2004; Kourtev et al., 2003; Wolfe and Klironomos, 2005). Mutualistic fungi and bacteria of the rhizosphere, or root-soil interface, normally aid individual plants and whole plant communities by providing enhanced nutrient and water uptake, increased disease resistance, improvement of soil structure, and detoxification of pollutants (Bais et al., 2004; Chekol et al., 2004; El-Tarabily and Sivasithamparam, 2006; Gworgwor and Weber, 2003; Joner et al., 2006; Selosse et al., 2004). However, there is growing evidence that plants growing outside their native range can alter soil microbial communities in their new host environments and researchers have proposed that such impacts may aid the spread of invasive exotic plants (Batten et al., 2006; Callaway and Ridenour, 2004; Hawkes et al., 2006; Kourtev et al., 2002ab; Marler et al., 1999; Pritekel et al., 2006; Stinson et al., 2006; Wolfe and Klironomos, 2005). Invasive plants can alter

soil-microbial communities through the root exudation of food-molecules used by soil fauna, the release of anti-microbial compounds through decomposing root and foliage tissues, and the facilitation of symbiotic relationships between roots and soil-microbes (Wolfe and Klironomos, 2005). Additionally, the displacement of native plants through competition can change the microbial landscape because particular plant species tend to harbor unique soil-microbial communities in terms of species richness and abundances (Wolfe and Klironomos, 2005). Invasive plants could benefit from altering soil-microbial communities by interfering with the nutrient-cycling of competitors, harboring pathogens that impact competitors more than the invader, and promoting microbial populations that are beneficial to the invader (Bais et al., 2004; Wolfe and Klironomos, 2005).

Arbuscular-mycorrhizae (AM), ubiquitous soil fungi of Class Glomeromycetes, which form mutualistic relationships with the majority of land plant species through the internal colonization of roots (Van der Heijden, 2002), have recently been posited as competitive allies of some invasive plants (Fumanal et al., 2006; Marler et al., 1999; Nijjer et al., 2008; Sanon et al., 2006; Shah et al., 2008). Normally a given hyphal network of an AM species will connect to one or more nearby plant species and foster plant coexistence through improved water and nutrient, especially phosphorous, exploitation to the plants and possibly nutrient-sharing between individuals (Hart and Klironomos, 2002; Van der Heijden, 2002). In return, the benefited plants reward mycorrhizae with carbohydrates. However, there is evidence that AM forming networks between the roots of multiple plant species “play favorites,” providing more benefits to

those species that are more invested in their services out of necessity, sometimes leading to "parasitic" net nutrient transfers from lesser-to-greater mycorrhizal-dependent[1] plants (Carey et al., 2004; Hart and Klironomos, 2002; Van der Heijden, 2002; Marler et al., 1999). This is contrary to the expected situation where AM networks promote plant coexistence and hence community diversity, which is thought to occur where a large variety of AM species underlie plants with a narrow range of mycorrhizal dependencies (Van der Heijden, 2002). In other words, mycorrhizae are apt to benefit all available host species if none stand out as "favorites." Furthermore, plants with high mycorrhizal dependency tend to form mutualisms with a more-limited variety of AM species (Scheublin et al., 2004; Van der Heijden, 2002), so invasive stands could reduce mycorrhizal species richness by promoting the growth of a select few fungi and thereby reduce the chances of colonizing competitor plants finding a beneficial match to an AM. Newly arrived plants that do manage to establish links with the underlying hyphal network would thus be prone to parasitic interactions if the newcomer were sufficiently less mycorrhizal-dependent than a dominant invasive species. Conversely, an invasive plant colonizing a community dominated by less mycorrhizal-dependent plants may benefit greatly from connecting with a pre-existing hyphal network, essentially freeloading off its established neighbors for nutrition.

[1]The "mycorrhizal dependency" of a plant is measured as the percent dry biomass response of individuals grown with and without AM as determined by pot or greenhouse experiments (Van der Heijden, 2002).

To date there have been at least four studies that have found evidence of exotic plant species benefiting from AM fungal networks over native plants in introduced ranges. Marler *et al.* (1999) observed a strong "parasitic interaction" between the invasive plant *Centaurea maculosa* Lam. and the native bunchgrass *Festuca idahoensis* Elmer. in the presence of AM through a series of greenhouse experiments on interspecies and intraspecies competition that modeled plant community invasion dynamics common in the western North American grasslands. Despite a lack of direct effects of AM on the growth of either plant, *C. maculosa* grown with AM in the presence of large grass individuals were nearly 70% bigger than when the fungi were absent. Similarly, Nijjer *et al.* (2008) observed that the invasive tree *Sapium sebiferum* (L.) Roxb. experienced "unusual positive benefits" to growth and nutrient acquisition in the presence of AM that were not reaped by co-occurring native tree species in pot experiments and a field study conducted in an east Texas temperate forest. Additionally, Shah *et al.* (2008) found evidence of *Anthemis cotula* L. having reliance on AM for its invasiveness in the Indian Himalayas through field studies and pot experiments. Interestingly, *A. cotula*'s AM-mediated invasive traits, such as plant growth and reproductive output, were dependent on the identity of co-occurring plant species, with the two studied AM-host neighbor species aiding *A. cotula* and the single AM non-host significantly reducing the invader's performance. In contrast to studies where AM were found to enhance the performance of invasive exotic plants, Stinson *et al*. (2006) showed that *Alliaria petiolata* (M. Bieb.) Cavara and Grande, a non-mycorrhizal species, secretes compounds that disrupt AM associations with native tree seedlings, likely promoting its invasion of forest habitats. It

should be noted that additional studies of invasive plants and their soil microflora have demonstrated considerable alteration of the AM community with invasion without specifically focusing on plant performance relative to AM community structure or root infection (e.g. Batten et al., 2006; Remigi et al., 2008; Siguenza et al., 2006). Therefore the current body of literature points to there likely being a much larger number of exotic plants that utilize AM as a mechanism of invasion even among those species already studied.

Some plants, including at least a few invasive exotic species, are known to harbor systemic endophytic fungi that are vertically transferred between generations via packaging in seeds alongside the host's embryo (Selosse et al., 2004). As such the fungi are not environmental cohabitants that happen to form mutualisms with a plant but are instead true endosymbionts that wholly require the host's shelter at all stages of their life cycle, in contrast to mycorrhizal fungi that have at least brief periods of their life cycle outside a host and maintain external hyphae. Since both the survival and reproductive success of the systemic endophytes depends on the preservation of the host plant's physical well-being from germination to seed deposition, it is not surprising that a primary benefit of harboring the fungi appears to be host defense against herbivory. For instance, *Neotyphodium coenophialum* is a widespread endophyte of tall fescue (*Schedonorus phoenix* (Scop.) Holub) responsible for cases of poisoning in livestock grazing on infected strains of the grass (Eckart, 2008). Similarly, sleepy-grass (*Stipa robusta* (Vasey) Barkworth) is notorious for causing periods of lost consciousness in

Southwestern US grazing horses due to the presence of hallucinogenic ergot alkaloids produced by an *Acremonium* endophyte in the foliage (Petroski et al., 1992). Furthermore, this defensive role of fungal endophytes extends beyond the grasses since various members of the Ipomoeeae, which includes vines like the morning-glories and bindweeds, are known to have mycotoxins associated with infection by *Claviceps* and other fungi (Eckart, 2008). Nonetheless, tall fescue infected with *N. coenophialum* has been shown to exhibit greater performance in ways similar to that seen in mycorrhizal hosts: enhanced reproduction, vegetative growth, and phosphorous uptake, but with the additional benefit of increased seed germination rates (Eckart, 2008).

In recent years mycologists have begun to study another potential form of mycorrhiza or mutualistic endophyte, dark septate endophytic fungi (DSE or DSF) (Mandyam and Jumpponen, 2005; Newsham, 1999; Peterson et al., 2004; Porras-Alfaro et al., 2008). So-named for their brownish melanized structures and hyphae internally divided by microscopically-visible walls (septa), DSF occur widely in soils and plant roots, frequently alongside AM (De Marins et al., 2008; Porras-Alfaro et al., 2008). In fact, the global distribution and host plant taxonomic diversity of DSF rivals that of AM, having been found in at least 600 plant species in habitats ranging from arctic and alpine regions to temperate grasslands and tropical rainforests (Mandyam and Jumpponen, 2005). Fungal genera with known or suspected DSF member species include *Aspergillus*, *Cryptosporiopsis, Drechslera, Leptodontidium, Periconia, Phialocephala,* and *Phialophora* (Mandyam and Jumpponen, 2005; Newsham et al., 2009, Scervino et al.,

2009), but doubtlessly many more DSF belonging to other genera will be described in the future. DSF have been implicated in the improved performance of host plants via greater phosphorous uptake, increased utilization of C and N from organic nutrient pools, improved drought tolerance, protection from herbivores, and protection from phytopathogens (Mandyam and Jumpponen, 2005). However, unlike the case with arbuscular-mycorrhizae, both the mechanisms by which DSF benefit their hosts and the endophytes' precise ecological roles are poorly understood, in part due to a shortage of experimental studies despite the far-easier cultivation of DSF outside host systems compared to AM (Mandyam and Jumpponen, 2005; Newsham et al., 2009; Peterson et al., 2004).

Current Understanding of Chinese Lespedeza

Chinese lespedeza (*Lespedeza cuneata* (Dum.-Cours.) G. Don.), also known as Chinese bushclover or sericea lespedeza, is an erect, often multi-stemmed, perennial legume with trifoliate, clover-like leaves and a coarse, branching root system (Munger, 2004; Zheng et al., 2004). The plant grows best in full sun but has demonstrated shade-tolerance. Like many legumes (Scheublin and Van der Heijden, 2006), the lespedeza can form nitrogen-fixing root nodules inhabited by *Rhizobia* and associates with arbuscular-mycorrhizae to improve water and nutrient absorption from the soil. Though originally introduced to the United States as a productive forage crop in the late 19th Century from East Asia (Zheng et al., 2004), the plant's agricultural utility is limited because the stems

become woody with maturity and the leaves develop high tannin concentrations (Kalburtji et al., 1999; Munger, 2004). However, Chinese lespedeza is still valued for its resistance to disease and drought, tolerance of poor-quality acidic soils, rapid growth, soil-stabilizing roots, and low-maintenance perennial growth habit, leading to its widespread seeding on disturbed soils in the United States (Mosjidis, 1996; Munger, 2004; Vaughan et al., 1989).

Unfortunately, *L. cuneata*'s hardiness and great production of durable seeds make the removal of established stands difficult and lespedeza populations have demonstrated the ability to both crowd-out competitors and colonize new habitats (Munger, 2004). As a result, Chinese lespedeza is a major invasive plant of grasslands and open woodlands in the Midwestern and Southeastern United States (Munger, 2004). Based on the discriminate analysis of characteristics shared with other invasive plant species, as well as plant distributions and abundances, scientists in the Virginia Department of Conservation and Recreation's Division of Natural Heritage have classified *L. cuneata* as an "A-ranked" invasive plant in the state (Heffernan et al., 2001). While recognized as a major invasive plant, Chinese lespedeza is not regulated as a "noxious weed" or "plant pest" in Virginia (refer to Code of Virginia § 3.2 Chapter 8, amended April 23, 2008).

Although Chinese lespedeza was originally studied by agricultural scientists as a nitrogen-fixing legume and forage crop, its invasiveness in natural tallgrass prairies and resulting classification as a noxious weed in some states has led to research concerning

the plant's role as an invader (Brandon et al., 2004). A variety of competitive traits have since been revealed. First, the plant is known to produce a variety of phenolic, phytotoxic secondary compounds or allelochemicals, in its tissues (Lindroth et al., 1986) and several studies have demonstrated potential allelopathic reductions in growth of cool-season pasture grasses and germination rates, but not ultimate growth, of warm-season prairie grasses exposed to lespedeza shoot extracts and residues (Dudley and Fick, 2003; Kalburtji and Mosjidis, 1993). Stands of lespedeza can shed considerable amounts of leaves on nearby soils (Mosjidis, 1996), which may release their allelochemicals into the soil as leachates during decomposition (Wolfe and Klironomos, 2005). Second, Blair and Fleer (2002) found that *L. cuneata* demonstrated the impressive ability to match a native lespedeza's (*L. capitata* Michx.; roundhead lespedeza) growth of aboveground biomass during the first three weeks after germination while simultaneously exceeding the other species' root biomass by more than double regardless of light level. Similarly, Smith and Knapp (2001) had found that *L. cuneata* grown alongside native *L. capitata* had significantly greater leaf biomass and total leaf number while demonstrating a 50% lower average light compensation point. Third, as expected of the nitrogen-fixing legume, overall Chinese lespedeza growth in terms of biomass has been found to be independent of soil nitrogen[2] levels (Guenther and Roberts, 2004; Sanders et al., 2007) and its shoot biomass is always greater than the root biomass, though a considerably greater proportion

[2]Brandon *et al.* (2004) concluded that high-N levels greatly reduced lespedeza growth and competitiveness based on fertilizer treatments where potash (K), phosphate, and ammonium nitrate were added together. Possible effects of phosphorous and potassium on plant growth and competition were not discussed. Guenther and Roberts (2004) amended soils with only ammonium nitrate, so I feel their conclusions are valid regarding nitrogen's effects (absent other nutrient amendments) on *L. cuneata* growth.

of energy is devoted to belowground than aboveground growth under low-N conditions (Guenther and Roberts, 2004). However, insect herbivory may limit the establishment of *L. cuneata* in low-N soils if the invader provides higher-quality tissues than competitor plants (Sanders et al., 2007). Fourth, field competition experiments have shown that shading effects are paramount to the lespedeza's ability to crowd-out competitors and that mowing treatments aid lespedeza by enhancing its canopy development (Brandon et al., 2004). Fifth, *L. cuneata* has been shown to benefit from partial shading underneath pine forest canopies by managing to persist and spread with little competition from other plants (Pitman, 2006). Sixth, Chinese lespedeza frequently associates with AM and has characteristics typical of high mycorrhizal-dependent plants, such as a low proportion of roots with absorptive ability and the utilization of nitrogen-fixing nodules (Van der Heijden, 2002) and the plant has demonstrated greatly-enhanced growth when experimentally inoculated with AM (Wilson, 1988; Wilson and Hartnett, 1998). Lastly, although *L. cuneata* is a promiscuous host for rhizobial strains, it may associate with the novel species *Bradyrhizobium yuanmingense* and other bacterial strains yet to be described (Yao et al., 2002).

Study Goals and Hypotheses

The primary aim of this study was to use the molecular technique of Automated Ribosomal Intergenic Spacer Analysis (ARISA) to characterize, or "DNA fingerprint," the fungal communities associated with the roots and rhizosphere of *Lespedeza cuneata*

and its competitor prairie grass *Panicum virgatum*, respectively referred to by the common names Chinese lespedeza and switchgrass throughout most of this work, in terms of both the number and relative abundances of fungal species, or the "fungal community structures." The secondary aim was to determine whether the presence of Chinese lespedeza altered the structure of fungal communities in invaded soils and the roots of the adjacent competitor grass, which could help facilitate the "invasiveness" of Chinese lespedeza by influencing the suitability of the soil for other plant species and reflect functional impacts of the lespedeza on switchgrass, respectively. This necessitated the collection of root and soil samples across gradients of plant community structure so that relationships between fungal community structure and different assemblages of *L. cuneata*, *P. virgatum*, and minor, sub-dominant plant species could be evaluated. Additionally, a subset of ARISA-fingerprinted fungal communities was analyzed with the advanced molecular technique of pyrosequencing so that the taxonomic diversity, hence possible ecological roles, of the fungi could be determined. Lastly, the roots of both *L. cuneata* and *P. virgatum* were microscopically evaluated for the extent of colonization by arbuscular-mycorrhizae (AM) and dark-septate endophytic fungi (DSF) as a more direct indicator of functional differences in the plant-fungal associations than the DNA analyses.

Although this study was observational in nature, I tested two hypotheses regarding the effects of invasion by Chinese lespedeza on resident fungal communities. First, it was hypothesized that lespedeza-invaded quadrats (sampling plots) would have

soil fungal communities significantly different from those of lespedeza-free, switchgrass-dominated quadrats. Second, it was hypothesized that soil fungal communities from switchgrass growing in competition with Chinese lespedeza would more-closely resemble the fungal communities of the invader than those of uninvaded switchgrass. While the same shifts expected of the soil fungi were not expected of the root fungi, due to the assumed sheltering of root endophytes from the external environment and supposed interspecies barriers to colonization by host-adapted fungi, these relationships were also evaluated since the methodology allowed for it. In short, invasion was expected to shift the fungal ecology away from a supposedly native state represented by *P. virgatum* toward an alternative, disturbed state represented by *L. cuneata*.

To my knowledge, this study was the first to (1) describe the fungal communities associated with either *L. cuneata* or *P. virgatum* using molecular methods and (2) observe shifts in both the root and soil fungal communities associated with a native grass due to proximity with, or displacement by, an invasive plant.

2. Materials and Methods

Site Selection

This study focused on a site where switchgrass, or tall panic grass (*Panicum virgatum* L.), was in competition with Chinese lespedeza (*Lespedeza cuneata* (Dum.-Cours.) G. Don.). The rationale was that the grass species is a native perennial that is a component of the Midwestern US tallgrass prairies, large tracts of which have been heavily-invaded by *L. cuneata* (Blair and Fleer, 2002; Brandon et al., 2004; Dudley and Fick, 2003). Studies of invasive plants have typically focused on the effects of non-native plants on native plant communities, and the alternative lespedeza-invaded sites in Northern Virginia largely consisted of fields dominated by introduced cool-season perennial grasses like tall fescue (*Schedonorus phoenix* (Scop.) Holub) and Timothy (*Phleum pratense* L.). Additionally, *P. virgatum* and other prairie grasses have recently been proposed as large-scale biofuel sources and researchers have recently found evidence that mixtures of *P. virgatum* and big bluestem (*Andropogon gerardii* Vitman) are sustainable for biomass energy production (Mulkey et al., 2008). Furthermore, *P. virgatum* has been previously shown as more cost-effective for biomass production compared to *L. cuneata* and other forage species and its potential for bioethanol production has been deemed promising (Keshwani and Cheng, 2009; Vaughan et al.,

1989). Therefore, invasion of the tallgrass prairie by *L. cuneata* and the invader's availability as an agricultural crop alongside *P. virgatum* has an additional economic dimension in the context of the developing "green energy" industry.

The study site was located at 38º57.376N, 77º21.689W near where the Washington and Old Dominion (W&OD) Trail crossed the Town Center Parkway near the Reston Town Center, Reston, VA on the east-side of the bridge crossing the parkway and south-side of the asphalt trail. The Northern Virginia Regional Park Authority, with the help of various adopting organizations, maintains the W&OD Trail. A stand of switchgrass grew on the peak and slopes of the hill intermixed with a small amount of Indian grass (*Sorghastrum nutans* (L.) Nash) while a stand of Chinese lespedeza intermixed with other plant species spread to the south and east (see Figures 1 and 2 under Sampling and Sample Processing below). This site was desirable because the distinct stands of invasive lespedeza and switchgrass intermingled over a distinct intermediate zone, allowing for root-and-soil sampling across a "gradient of plant invasion" from the center of one plant community to the other. The grass from 3-5 feet off the path was periodically mowed and bicyclists, hikers, and construction workers frequently used the nearby asphalt and gravel paths, though the field itself seemed to have been left largely untrampled by people or large animals and not subject to a mowing regime (personal observations and communications with park authorities). A low-lying, mowed field or lawn was located south of the study site and was probably maintained by a group other than the park authority; any fertilization or pesticide treatments of this field

were not expected to have impacted the study site due to its downhill location. Over 100 x 25 feet (2500 sq. feet or approx. 232 sq. meters) of field was available for sampling. Significant differences in soil fungal communities by host species were expected over this small scale because adjacent plants of different species can harbor distinct fungal communities (Apphun and Joergensen, 2006; Sykorova et al., 2007). The collection of samples occurred during drought conditions, a fact significant because water-stress is known to promote mycorrhizal-colonization of plant roots. Additionally, saprobic and pathogenic fungi may have been able to grow especially well on distressed and senescent plants.

Sampling and Sample Processing

Figure 1. Westward view of mowed and unmowed field edge adjacent to W&OD Trail, September 2007. A lespedeza stand intermixed with heath aster is at bottom left and a switchgrass stand to far back center-left (inflorescences visible as pale brown vegetation; pale yellow those of Indian grass, *Sorghastrum nutans*). Transect 1 ran parallel about two feet to the left of the white rope.

Figure 2. Westward view of study site taken from gravel path on eastern border, September 2007. The switchgrass bunch surrounded by lespedeza on the bottom right served as the starting point of Transect 3 and Transect 4 ran parallel through the center of the lespedeza stand to the left. Transects 1 and 2 began off-camera to the right of the grass bunch. All transects ended beyond the Autumn olive tree at back-center.

Table 1. Matrix representation of sampling scheme layout.

	T1[2]	**T2**	**T3**	**T4**
Q1	Lesp[1]	Lesp	**Mix (L)**	Lesp
Q2	Lesp	Lesp	**Mix (L)**	Lesp
Q3	Lesp	Lesp	Lesp	Lesp
Q4	Lesp	Lesp	Lesp	Lesp
Q5	**Mix (L)**	Lesp	Lesp	Lesp
Q6	**Mix (L)**	Lesp	Lesp	Lesp
Q7	**Mix (SG)**	**Mix (L)**	**Mix (L)**	**Mix (L)**
Q8	SG	SG	**Mix (SG)**	**Mix (L)**
Q9	SG	SG	SG	**Mix (SG)**
Q10	SG	SG	SG	SG
Q11	SG	SG	SG	SG
Q12	SG	SG	SG	SG

[1]Dominant plant communities by quadrat: Lesp = lespedeza-dominated; SG = switchgrass-dominated; Mix (L) = "mixed" community with more lespedeza than switchgrass; Mix (SG) = "mixed" community with more switchgrass than lespedeza.
[2]Numbered Quadrats (Q) and Transects (T) by rows and columns, respectively.

Soil and root sampling were based on Batten *et al*. (2006), though modified for the harvest of individual plant samples and sampling along full transects that cross both lespedeza and switchgrass patches, versus the arbitrary collection of samples inside, on the perimeter, and beyond invasive plant patches. Four linear transects were established roughly parallel to the W&OD Trail, running from east-to-west for about 20 meters and being spaced 5 ft (1.524 meters) apart north-to-south; the top-most transect being roughly 8 feet south from the trail. Transects were centered over the lespedeza-switchgrass "interface" area, or "mixed plant community," so that ample amounts of samples could be collected from lespedeza-switchgrass "mixed," lespedeza-dominated, and switchgrass-dominated stands. 12 sampling points were set along each transect at 180 cm intervals for a total of 48 sampling points. Plants and their rhizosphere soils were harvested by shovel to approximately 6 cm depth as follows: three to five multi-stemmed[3] lespedezas per sample were harvested from the lespedeza patches and where a sampling point fell on or near lespedeza plants in the interface zone; roughly ½-bunch of switchgrass was harvested per sample from within the grass stand and where a sampling point fell on or near switchgrass bunches in the interface zone. The aerial portions of both collected

[3]Multi-stemmed lespedezas were found to produce nearly an order of magnitude greater weight of fine roots required for microscopic and molecular analyses than single-stemmed individuals (data not shown). Chinese lespedeza produces relatively little fine root material (<1mm width), so the more-efficient collection of larger lespedeza plants was necessary for the execution of the root zone analyses. Hawkes *et al.* (2006) encountered similar issues when harvesting fine roots for both DNA extraction and the measurement of mycorrhizal root colonization, where inadequate root mass remained after DNA extraction for measuring colonization in about half of the invasive grass samples and all native plant samples. Even though the majority of both grass and lespedeza roots were set-aside for DNA extraction, excess fine roots were available for staining and microscopy for all cases in the present study.

switchgrass and lespedeza were cut off an inch above ground level and discarded and the root-soil samples were stored in labeled plastic bags and kept chilled in an ice chest until transported to the lab, where they were promptly frozen in the Mycology Lab freezer at approximately 0ºC for storage prior to further processing within 48 hours of arrival. Samples to be processed at a much later date were stored at –20ºC.

Plant species richness and percent cover by plant species were determined during sample collection by evaluating the aerial portions of all vegetation falling within 60 cm-radius circular quadrats centered over the predetermined sampling-points (Fidelibus and MacAller, 1993; Sorrells and Glenn, 1991; Squiers and Wistendahl, 1976; Zak and Willig, 2004). The purpose was to determine the composition of the plant communities that likely influenced the fungal communities of roots and soils associated with harvested plants (Kernaghan, 2005). Squiers and Wistendahl (1976) successfully used "small," approximately 112 cm-diameter quadrats to sample oldfield vegetation. Additionally, all plants adjacent to transects were surveyed to fully characterize the plant species richness at the site so that the effectiveness of the sampling scheme in capturing the overall plant diversity could be verified. Plants were identified with the aid of field guidebooks and the USDA PLANTS Database (Brown, 1979; Foster and Duke, 1990; Newcomb, 1977; Petrides, 1972; USDA, 2009).

The belowground portions of each field sample were divided into two parts for analysis: rhizosphere soil and fine roots, while lespedeza samples also had root nodules

harvested and kept separate from the fine roots. Rhizosphere, consisting of soils firmly attached to root systems and loose peds held together by fine roots, was separated manually from the root systems of both switchgrass and lespedeza while wearing latex gloves. All rhizosphere soils were sieved to 2 mm, homogenized, and stored at –20ºC (Batten et al., 2006). Soil pH at each quadrat was eventually determined using thawed portions of previously frozen rhizosphere according to the 1:2 soil-to-water method of Gavlak *et al.* (2003). Root samples were collected by removing the fine attached roots with flame-sterilized forceps and gloved hands from the root masses after thorough cleaning with deionized water (Hawkes et al, 2006). All lespedeza root samples with nodules were noted and frozen prior to DNA extraction with nodules plucked off by flame-sterilized forceps. However, some diminutive nodules could not be removed without risking the loss of precious fine roots and may have contributed fungal DNA to samples. As a possible measure of lespedeza's nitrogen fixation or plant maturity, root nodules were later counted, their widths measured, and nodule biomass then determined after drying at 64ºC to constant weight (Hendricks and Boring, 1999). Approximately 0.25 g of wet, cleaned fine roots per sample were set-aside for root staining and microscopic examination and stored at -20ºC until use. The remaining 0.5-2 g of roots per sample was set-aside for DNA extraction and stored at –80ºC (Hawkes et al., 2006). Fine roots were later ground in clean, autoclaved plant blenders (Iberbach Corp.) powered by commercial blender (Waring) and passed through a series of four clean, autoclaved USA Standard Testing Sieves (Fisher Scientific): 2 mm, 500 μm, 212 μm, and 106 μm in descending order (Torzilli et al., 2006). The 106 μm to 212 μm fractions ("106 μm root")

were collected and washed 10 times with sterile deionized water in sterile 50 mL Corning polypropylene centrifuge tubes to remove adhering fungi and spores from the root surfaces (Torzilli et al., 2006), then returned to storage at -80ºC prior to DNA extraction. However, the 212 μm to 500 μm fractions ("212μm root") were collected, processed, and stored just like the 106 μm roots for lespedeza of T1Q2 and T1Q7 because (1) fine root mass was insufficient to yield at least 0.25 g of wet ground root of the 106 μm fraction for DNA extraction in the case of T1Q2 and (2) to allow for the comparison of total fungal communities by root particle size (106 μm vs. 212 μm fractions) in the case of T1Q7.

Root Staining and Microscopy

A modified protocol for the clearing-and-staining of plant roots was used to visualize fungal structures in lespedeza and switchgrass roots for the calculation of percent mycorrhizal colonization by AM fungi and dark-septate endophytes (Appendix 1). This was essentially the same method described by previous authors (Fumanal et al., 2006; Jarstfer and Sylvia, 1997; Phillips and Hayman, 1970). The magnified intersections method (McGonigle et al., 1990) was used to determine the percent mycorrhizal colonization of roots instead of the older, less-accurate gridline intersections method of Giovannetti and Mosse (1979). 100 fields of view were counted per slide at 200X magnification (Hawkes et al., 2006), and fine structures like arbuscules and thin hyphae were also evaluated at 400X magnification to ensure their proper identification. Over 10

cm of stained roots were utilized per sample for 41 evaluated root systems to ensure that 100 intersections with intact root cortex were available; this precaution was necessary to avoid data bias since mycorrhizae primarily colonize the cortex (Brundrett, 1999; Peterson et al., 2004), which may be torn from the tougher root vascular tissue during handling of stained roots (personal observations). Thus, root intersections of "naked vascular tissue" were ignored. Three lespedeza root samples (T1Q1, T1Q7, and T2Q2) and one switchgrass root sample (T3Q2) were only evaluated at 70-75 intersections due to low availability of roots and/or root degradation during clearing and staining; their root colonization tallies were converted to percentages for comparison with the other samples. Separate tallies were kept for the following percent root colonization variables: "AM hyphae," "AM vesicles," "arbuscules," and "DSF." The "DSF" tallies included all melanized fungal structures (hyphae and microsclerotia) for simplicity and since the functions of specific DSF structures are poorly understood (Mandyam and Jumpponen, 2005; Newsham, 1999; Peterson et al., 2004). The visibility of the yellow-brown to brown, naturally-melanized DSF structures was seemingly unaffected by the root staining process based on microscopic comparisons of cleared/unstained and cleared/stained roots of both lespedeza and switchgrass (personal observations) and the contrast between melanin-brown and the Trypan blue stain was adequate for tallying purposes (see Endophytic Fungi: Micrographs of Stained Roots under Results).

DNA Extraction

Two protocols were used to extract DNA from environmental samples. For soils, DNA was extracted from approximately 500 mg of soil per sample using the Q-BIOgene BIO101-FastDNA Spin Kit for Soils (Q·BIOgene, Inc., Carlsbad, CA) and following the GMU Microbiome Center's modifications of the standard protocol. In contrast, approximately 250 mg per sample of 106-212 μm (and several 212-500 μm fractions) of roots were DNA-extracted using a BIO101-FastDNA Spin Kit for Tissues following a modified protocol in which extra ceramic beads were added to Tissue Matrix tubes to maximize lysing of fungi and plant cells during bead-beating (Torzilli et al., 2006; see Appendix 2). Additionally, 800 μl CLS-VF solution and 200 μl PPS were added to Tissue Matrix tubes in place of CLS-Y solution in the root DNA extraction procedure to improve the extraction of DNA from recalcitrant plant tissues (personal communications with Dr. Torzilli). All DNA extracts were stored at –20ºC when not in use (Ritchie et al., 2000).

ARISA Fingerprinting

Automated Ribosomal Intergenic Spacer Analysis (ARISA)[4] was used to characterize mixtures of fungal community amplicons from environmental DNA extracts

[4]Alternatively known as Amplicon Length Heterogeneity PCR (ALH-PCR) or Length-Heterogeneity PCR (LH-PCR)

based on the variation in amplicon length associated with different taxa (Ritchie et al., 2000; Suzuki et al., 1998; Yang et al., 2006). The primer pair ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (TGTGTTCTTCATCGATG**)** was used to amplify the Internal Transcribed Spacer 1 (ITS1) region of 18S ribosomal RNA genes from the total fungal community DNA while not amplifying the co-occurring DNA of bacteria, plants, and other organisms. Both the root and soil DNA extracts were diluted 1:5 for PCR based on the results of preliminary reaction optimization experiments (data not shown). Immediately prior to each PCR run a "master mix" was prepared containing the following components per sample or reaction: 8 µl DEPC water, 2 µl 10X Rx. Buffer, 2 µl of 25 mM MgCl mix, 1.9 µl '2.0mM each' dNTPs, 2 µl BSA 0.1%, 1 µl forward primer 10 µM, 1 µl reverse primer 10 µM, and 0.1 µl Taq polymerase 5 units/µl. After delivering 18 µl aliquots of master mix per sample to separate reaction tubes, 2 µl of diluted DNA extract was added to each tube, with 2 µl of diluted lichen DNA serving as positive controls and master mix minus DNA representing a negative control. The complete reaction conditions were as follows: 35 cycles at 30 seconds denaturation [95ºC], 30 seconds annealing [Tm = 55ºC], 2 mins + 5 sec/cycle extension [72ºC], followed by 45 minutes extra extension step (Torzilli et al., 2006). All PCR reaction mixtures intended for DNA community fingerprinting included ITS1F primers fluorescently labeled by FAM marker to allow analysis by high throughput capillary electrophoresis, whereas preliminary PCR reactions utilized the unlabeled forward primer. Triplicate PCRs were run for each fingerprinted sample to check for reproducibility and aid data processing (Torzilli et al., 2006). All PCR products were

checked for successful amplification by electrophoresis through 1% agarose gel in 1X TAE containing ethidium bromide, using a Phage Lambda molecular weight marker for size comparison, followed by UV-gel photography (working lab protocol of Microbiome Center). The 20 µl PCR products were stored at 4ºC for short -term, and –20ºC for long-term, storage to avoid unnecessary changes in product concentration due to possible evaporation of liquid from PCR reaction tubes during refrigeration. Unused portions of PCR products that were successfully fingerprinted were stored at -80ºC in case of need for future analysis.

PCR products subjected to ARISA generated electropherograms in which amplicons of different sizes on the X-axis and fluorescence intensities on the Y-axis (from the fluorescent-labeled primers integrated during PCR) represented fungal OTUs (operational taxonomic units). Peak heights represented relative OTU abundances. Since each OTU represented a presumptive fungal species, the fingerprint or community profile gave a general snapshot of a particular fungal community's composition (Martin and Rygiewicz, 2005; Suzuki et al, 1998). Peak-calling was performed using SpectruMedix software with peaks below 1% relative abundance threshold not included in the analysis. Data derived from the community DNA fingerprints were exported to Microsoft Excel after processing by a Perl script program written by Dr. Patrick Gillevet (personal communications; Torzilli et al., 2006). Once in Excel, the results for each soil and root sample were verified by comparing the relative abundance values of each fungal OTU with those of the corresponding replicates, abundance values found in two or three

replicates were kept while values found in only a single replicated sample were disregarded as artifacts. Similarly, errors in the automated assignment, or binning, of peaks to basepair numbers were corrected by comparing adjacent columns of relative abundances by OTU for instances where rare or singleton values likely corresponded to unusual gaps in the adjacent column of a more-abundant fungus, the misplaced value being moved to fill the gap where appropriate.

Community Pyrosequencing

Based on the methods of Gillevet *et al.* (2009) and Torzilli *et al.* (2006), fungal community DNA from 17 select samples, representing roots and soils from both plant host species from the three plant community types, was pyrosequenced using the 454 Life Sciences service (Roche Co., Branford, CT). To achieve adequate alignments, the relatively short pyrosequencing fragments were assembled at 90% similarity using Seqman assembler, saved as individual pyro contigs and identified by BLAST analysis.

Approximately 170 sequences of the most frequently detected fungi were selected for a second round of BLAST analysis to verify the data quality and update the original sequence matches, a move necessary due to potentially misleading results from the automated bioinformatic analysis (communications with Dr. Torzilli). Megablasts of the NCBI nucleotide collection without exclusions were performed to optimize the searches for highly similar sequences, including uncultured/environmental sample sequences that

were absent from the initial BLAST search. The identifiers of the old matches were changed whenever a better, higher-similarity match was found and/or the original match could not be found in the top 100 new matches, either of which was the case for most sequences. This greatly improved the quality of the sequence homologies and eliminated all original mismatches between short strands of fungal rDNA and animal sequences.

The sequence lengths of pyrosequencing contigs were recorded from the second round of BLAST analysis and used to find probable matches with fungal OTUs from ARISA based on apparent agreements (within a range of a few basepairs) between the contig lengths and the estimated sequence lengths corresponding to the OTU designations. Matches were confirmed by the visual comparison of the corresponding ARISA and pyrosequencing-based fungal community profiles for congruence in the presence and relative abundances of the fungi. While most contigs and OTUs could not be firmly-matched based on this criteria due to the complexity of the community datasets, both molecular methods produced DNA community fingerprints with overall similar shapes of the fungal species distributions by sample and a selection of fungi were cross-matched with reasonable confidence.

Statistical Analysis

The diversity of both fungal and plant communities was measured by Shannon's log e diversity (H') on Multi-Variate Statistical Package (MVSP) Version 3.13h software

by Kovach Computing Services. Species-saturation was measured by the generation of Mao Tau rarefaction curves without sample replacement at 200 randomizations using EstimateS Version 8.2.0 software (Colwell, 2006). Rarefaction was used to determine the adequacy of the sampling regimen in representing the actual species diversity in the field. Most importantly, diversity values calculated from data sets with asymptotic rarefaction curves can be evaluated with confidence by hypothesis tests as well as compared against literature values based on data sets with asymptotic curves (Gotelli and Colwell, 2001). The rarefaction analyses of the ARISA fingerprints utilized the OTU abundances of each sample averaged across replicates to calculate species accumulation with increasing collection effort, which was necessary to avoid artificial species-saturation based on non-averaged replicates (data not shown). All other data sets analyzed by rarefaction were not replicated and thus evaluated as-is.

Several multivariate ordination techniques were used to compare samples (refer to Cox, 2005; Gotelli and Ellison, 2004; Legendre and Legendre, 1998; and Ludwig and Reynolds, 1988). Principal Component Analysis (PCA) was applied to the fungal root colonization data to evaluate relationships between host plant identity and the extent of colonization by AM and DSF. Principal Coordinate Analysis (PCO) with Bray Curtis distances was used to examine differences between samples in overall fungal and plant community structure. PCOs were carried out for (1) all fungal communities derived from ARISA fingerprinting, the separate evaluation of (2) fungal root and (3) rhizosphere

communities from ARISA, (4) all fungal communities derived from pyrosequencing, and (5) plant communities in terms of areal cover by composing species. Canonical Correspondence Analysis (CCA) with detrending was applied to the four sets of fungal community data above that were subjected to PCO in order to evaluate correlations between fungal community structures by sample and the corresponding environmental variables by quadrat, namely soil pH and areal covers by plant species. PCA and CCA ordinated samples in terms of Euclidian and χ^2 distances, respectively, the latter of which and Bray Curtis distances being suitable for representing species-abundance data (Legendre and Legendre, 1998). In contrast to the rarefaction analyses, ordinations of the ARISA fingerprints used replicates as distinct samples to maximize the sample-to-variable ratios and to avoid changing the original OTU abundance values by averaging. However, nine ARISA replicates were excluded from the ordinations, as well as pertinent group comparisons, as outliers on the basis of weak fluorescent peak heights that led to unusually low OTU richness and relative abundances.

Mean Bray Curtis distance values calculated from all OTUs composing fungal communities were compared to verify conclusions drawn from the PCO scatterplots. These values were also used to test the hypothesis that fungal communities were significantly more dissimilar following invasion. The distance values can be interpreted as the average proportion of dissimilarity between two compared groups of communities (Legendre and Legendre, 1998). As such, they represent differences between groups *in toto* instead of as visualizations limited to only two or three principal axes that cover only

portions of the variance, as with the ordination techniques. Marquez *et al.* (2008) used a similar procedure to compare the average Jaccard's index of similarities of fungal assemblages from different locations.

Both parametric and non-parametric tests were used for group comparisons, the choice of method based on the "normal fit" of the data sets according to Kolmogorov-Smirnov D test with Q-Q plots and/or similarities in overall sample distribution shape based on sample histograms. Group comparisons and tests of normalcy were performed with the Analyze-It for Microsoft Excel Version 2.20 statistics application (Analyze-It Software, 2009). Welch's T-test, a conservative variation of Student's T-test without pooled samples that assumes unequal variances, was performed on mean Shannon diversities and the mean Bray Curtis distances between fungal and plant communities (i.e. ecological resemblance analyses), both types of data having adequately fit the normal distribution. All remaining types of data, which were "raw" and not converted into any metric, were evaluated with Mann-Whitney's U test or Kruskal-Wallis' H ranking tests with Bonferroni pairwise contrasts *post hoc*, the sample distributions having similar shapes despite the lack of normal fit.

Linear regressions were performed on Microsoft Excel between variables to confirm relationships inferred from ordinations and group comparisons. These were straightforward except for the regressions between individual fungal abundances and areal cover densities of lespedeza and switchgrass that were implied by environmental

variable rankings against the CCA biplot vectors. Greater amounts of plant cover seemed to increase the likelihood of associated fungal OTUs being present in a sample and exhibiting linear increases or decreases in abundance. However, zero-value abundances of individual OTUs were numerous in most fungal communities, so the threshold for determining significant linear responses was set at a liberal $R^2 = 0.25$ to balance the effects of putatively-missing values on regressions and each scatterplot was visually inspected for apparent linear responses. Furthermore, strong linear relationships were not expected because neither plant cover nor fungal abundances were precise quantities and both were facades for hidden variables, such as plant cover for plant age or biomass and ARISA-derived OTUs for multiple fungal species with equal-length ITS1 rDNA.

Analyses of fungal community data from PCO, CCA, and group comparisons were performed separately for ARISA and pyrosequencing profiles in order better-describe fungus-plant associations over the gradient of invasion. In the ARISA-based analysis, likely plant affinity was first determined by taking the 24 of the 55 most-abundant OTUs that had CCA variable scores ranking high against the biplot vectors for Lespedeza Cover and Switchgrass Cover (separately for soil and root CCAs) and then determining which of these OTUs also had 1% or greater mean relative abundance for one host plant or the other. The reasoning was that a fungus with an actual, versus chance, association to either dominant plant would have a preference for both the plant's cover density and presence as a host. Second, plant affinity of the selected OTUs was confirmed by Mann-Whitney U tests of the group abundances (all lespedeza vs. all

switchgrass separately by roots and soils) at 95% confidence, and insignificant OTUs were dropped from further analysis. Third, Kruskal-Wallis tests with *post hoc* Bonferroni pairwise contrasts of the mixed plant community vs. unmixed plant community OTU abundances were performed separately for lespedeza and switchgrass samples to detect significant changes in fungi with invasion. Fourth, every ARISA profile (averaged across replicates) from a mixed plant community quadrat was checked for unusually high or low abundances of the OTUs with significant plant affinities to find those responsible for shifting individual fungal community structures. This revealed interesting differences between particular mixed and unmixed-community samples likely deemed insignificant by group comparisons due to their small sample sizes.

The further analysis of the pyrosequencing data paralleled the analysis of fungal OTUs. Fungi occurring in all or most representative samples of a fungal subgroup from the pyrosequencing-based PCO at a relative abundance of 1% or greater were considered to be associated with the corresponding host and plant community. The CCA species variable scores of the 16 sequenced fungi with putative host preferences were evaluated by their rankings against associated environmental biplot vectors to determine which fungi likely occurred in a sample due to host identity and/or plant community structure, as opposed to chance. Finally, a fungus was designated a “core species” of a fungal group if it (1) was associated with the CCA biplot vector corresponding to the dominant plant species of the group and/or (2) was significantly associated with the sample group according to Bonferroni pairwise contrasts following significant Kruskal-Wallis test.

3. Results

Soil Characteristics

Table 2. Mean (SD) soil pH by plant community and transect

Lespedeza Dominated Community	**Mixed Community**	**Switchgrass Dominated Community**	**Whole Site: All Communities**
5.44 (0.47)	5.38 (0.47)	5.49 (0.56)	5.45 (0.50)

Transect 1	Transect 2	Transect 3	Transect 4
5.50 (0.18)^{A}	4.96 (0.30)^{B}	5.27 (0.43)^{AB}	6.06 (0.20)^{C}

Mean pH values with different letter superscripts in the same row significantly differed at $p < 0.001$ according to Bonferroni pairwise contrasts following significant Kruskal-Wallis test results. Values within rows without superscript letters or sharing the same superscript letters lacked significant differences according to Kruskal-Wallis test and Bonferroni pairwise contrasts, respectively.

The soil at the site was rocky, shallow, acidic, brown sandy loam of low clay content (NASA, 2001; personal observations of particulate layering of centrifuged, moist soils). No physical differences could be seen between any collected soils, save for obvious differences in soil moisture based on weather at the time of harvest. Soil pH did not differ appreciably by plant community (i.e. along transects), yet pH differed significantly between transects (Table 2).

Soils from the switchgrass stand had a foul odor that was vaguely "crustacean" and "skunky," a quality also observed in switchgrass roots and foliage (personal observations). The odors of lespedeza-associated soils were either unremarkable or spicy-sweet like the lespedeza roots themselves (which the author likened to carrot root, *Daucus carota* L., or fresh chicory root, *Cichorium intybus* L.). Substances known to possess odors of crab or shrimp include trimethylamine, indole, some alkanes, carbon disulfide, dimethyltrisulfide, 1-pyrroline, some pyrrolidines, geranylacetone, 1-dodecanol, and methyl isopropyl disulfide (Chung and Cadwallader, 2006; Ishizaki et al., 2005). Likewise, "skunkiness" may be due to a variety of sulfurous mercaptans, sulfides, and disulfides (Andersen et al., 1982). On the other hand, aroma compounds associated with carrots and chicory root include a variety of monoterpenes, sesquiterpenes, fatty acids, and alcohols (Alasalvar et al., 1999; Bais et al., 2003; Pazola, 1987).

Root Nodule Yields from Chinese Lespedeza

Nodules were ubiquitous among the root systems of *L. cuneata* but they were consistently small (< 0.4cm diameter) and yields from lespedeza-dominated and mixed-plant quadrats were not significantly different at $p < 0.05$ according to Mann-Whitney U-test. Nodule recovery dry weights from lespedeza-dominated and mixed-plant samples averaged at 20.88mg (SD 10.40) and 20.65mg (SD 11.73) per sample of approximately three plants, respectively, similar to reported September nodule recovery from native, trailing *Lespedeza procumbens* Michx. in Georgia (approx. 8mg dry mass/plant;

Hendricks and Boring, 1999). Root samples tended to yield 10-40 live nodules and anywhere from five to over 100 senescent nodules. Although both live and senescent nodules occurred along the length of fine roots seemingly anywhere in the root system, the highest concentrations of live nodules were observed in the root-crowns immediately below ground level. Live nodules were pale, grayish-brown, plump, ovular bodies and senescent nodules were brown-black, relatively flat, and thin outgrowths of slight mass.

Table 3. Plant species at Washington and Old Dominion field site, Sept. 2007

Common Name/Designation	**Scientific Name**	**Within Quadrat?[1]**
"Weed Y," unidentified trailing herb	Unknown	Yes
Autumn Olive	*Elaeagnus umbellata* Thunb.	Yes
Boneset	*Eupatorium* sp.	Yes
Bradford Pear	*Pyrus calleryana* Decne. "Bradford"	No
Bur Marigold	*Bidens* sp.	Yes
Chinese (Sericea) Lespedeza	*Lespedeza cuneata* (Dum.-Cours.) G. Don	Yes
Common Evening Primrose	*Oenothera biennis* L.	No
Common Ragweed	*Ambrosia artemisiifolia* L.	Yes
Deer-Tongue Grass	*Dichanthelium clandestinum* (L.) Gould	No
Dock	*Rumex* sp.	Yes
Eastern Red Cedar	*Juniperus virginiana* L. *var. virginiana*	Yes
English Plantain	*Plantago lanceolata* L.	(Yes)
Field Thistle	*Cirsium discolor* (Muhl. ex Willd.) Spreng.	Yes
Goldenrod	*Solidago* sp.	Yes
Heath Aster	*Symphyotrichum ericoides* [L.] G.L. Nesom	Yes
Horseweed	*Conyza canadensis* (L.) Cronquist	Yes
Indian Grass	*Sorghastrum nutans* (L.) Nash	Yes
Milkweed	*Asclepias* sp.	No
Mustard	*Brassica* sp.	Yes
Orchard Grass	*Dactylis glomerata* L.	Yes
Path Rush	*Juncus tenuis* Willd.	Yes
Pokeweed	*Phytolacca americana* L.	Yes
Prickly (Wild) Lettuce	*Lactuca serriola* L.	(Yes)
Spreading Dogbane	*Apocynum androsaemifolium* L.	(Yes)
Switchgrass	*Panicum virgatum* L.	Yes
Tall Fescue	*Schedonorus phoenix* (Scop.) Holub	Yes
Teasel	*Dipsacus sylvestris* Huds.	Yes
Virginia Creeper	*Parthenocissus quinquefolia* (L.) Planch.	Yes
Yarrow	*Achillea millefolium* L.	Yes
Yellow Wood-Sorrel	*Oxalis stricta* L.	No

[1]"Yes" without parentheses indicates that the plant species was within at least one sampled quadrat and a "No" indicates that the species was at the field site but not within a sampled quadrat. (Yes) indicates a species that was within sampled quadrat(s) but without corresponding switchgrass and lespedeza root and soil samples that were subject to ARISA for total fungi.

Table 4. Composition of plant communities by mean (SD) percent areal cover

	Plant Community Type			
Areal Cover Type	**Lespedeza Dominated**	**Mixed Community**	**Switchgrass Dominated**	**Whole Site**
Bare Ground/Litter**	30.6 (17.1)^{A}	24.0 (23.1)^{A}	6.39 (7.44)^{B}	20.2 (19.1)
Chinese Lespedeza***	42.5 (16.3)^{A}	31.8 (19.9)^{A}	1.36 (2.73)^{B}	24.8 (23.2)
Switchgrass***	2.25 (3.75)^{A}	21.4 (19.2)^{B}	70.7 (17.6)^{C}	31.9 (34.1)
Autumn Olive	0	0	2.50 (10.6)	0.94 (6.50)
Boneset	0	0.50 (1.58)	4.72 (8.66)	0.31 (2.17)
Bur Marigold	0	0	0.28 (1.18)	0.10 (0.72)
Cool-Season Grasses^{X}	7.50 (12.8)	1.50 (2.42)	0.56 (1.62)	3.65 (8.92)
Common Ragweed	0.25 (1.12)	0.20 (0.63)	0	0.15 (0.77)
Dock	0.05 (0.22)	0	0	0.02 (0.14)
Eastern Red Cedar	0.50 (2.24)	0	0.17 (0.71)	0.27 (1.50)
English Plantain	0	0	0.42 (1.29)	0.16 (0.80)
Field Thistle	2.60 (6.15)	0.20 (0.63)	0.56 (2.36)	1.33 (4.31)
Goldenrod	0.25 (1.12)	0.90 (1.66)	0.28 (1.18)	0.40 (1.27)
Heath Aster*	5.95 (11.4)^{AB}	6.30 (8.45)^{A}	0.28 (1.18)^{B}	3.90 (8.66)
Horseweed**	6.75 (8.62)^{A}	1.80 (3.36)^{AB}	0.28 (1.18)^{B}	3.29 (6.46)
Indian Grass**	0.10 (0.45)^{A}	7.70 (12.6)^{B}	3.61 (4.05)^{B}	3.00 (6.71)
Mustard	0	0	0.83 (1.92)	0.31 (1.22)
Path Rush	0	1.00 (3.16)	0	0.21 (1.44)
Pokeweed	0	0	0.39 (1.24)	0.15 (0.77)
Prickly (Wild) Lettuce	0.25 (1.12)	0	0	0.10 (0.72)
Spreading Dogbane	0	0.50 (1.58)	4.78 (11.3)	1.90 (7.20)
Teasel	0	0.70 (1.64)	1.33 (4.56)	0.65 (2.90)
Unidentified “Weed Y”*	0^{A}	0.50 (1.58)^{AB}	4.72 (8.66)^{B}	1.88 (5.71)
Virginia Creeper	0	0	0.56 (1.62)	0.21 (1.01)
Yarrow	0.50 (2.24)	0	0	0.21 (1.44)

Asterisks denote significance of Kruskal-Wallis test for corresponding Areal Cover Type: *$p < 0.05$; ** $p < 0.005$; *** $p < 0.0001$. Values of different letter superscripts in the same row (save Whole Site listings) significant at $p < 0.05$ confidence by Bonferroni pairwise contrasts. Values within rows without asterisks and values sharing the same superscript letters lacked significant differences according to Kruskal-Wallis test and Bonferroni pairwise contrasts, respectively.

^{X}Mixtures of tall fescue and orchard grass.

Tables 3 and 4 summarize the plant species surveyed and average plant species compositions by community type at the study site. A total of 30 plant species were observed at the site, 29 of which were identified to species and/or genus. Most species happened to fall within the 120 cm-diameter circular quadrats and may therefore have influenced the soil and root fungal communities of samples from the field. Given that some plant species failed to occur within sampling plots but were observed elsewhere in the field (Table 3), it was not surprising that plant species-saturation was not met during sample collection based on the rarefaction curve (*Sobs*; Figure 3).

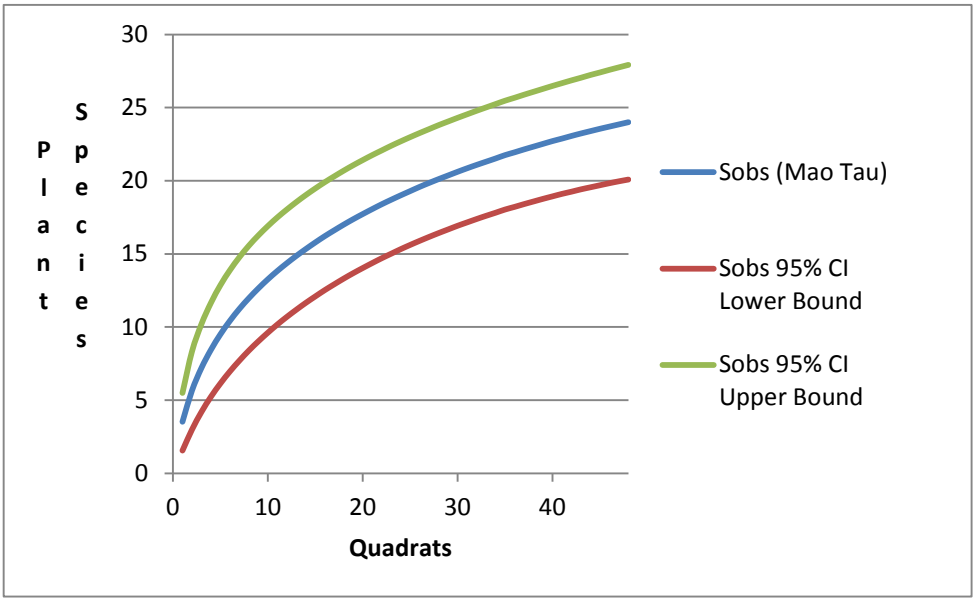

Figure 3. Mao Tau rarefaction curve of plant communities with 95% CIs.

Besides Chinese lespedeza and switchgrass, herb species responsible for at least 15% areal cover at one or more quadrats included heath aster (*Symphyotrichum ericoides*

[L.] G.L. Nesom), horseweed (*Conyza canadensis* [L.] Cronquist), tall fescue (*Schedonorus phoenix* [Scop.] Holub), field thistle (*Cirsium discolor* Muhr. ex Willd.), boneset (*Eupatorium* spp. L.), spreading dogbane (*Apocynum androsaemifolium* L.), teasel (*Dipsacus sylvestris* Huds.), and an unidentified trailing species hereafter referred to as "Weed Y." A few tree species were also present but were not major components of the total plant community: a mature Autumn olive (*Elaeagnus umbellata* Thunb.), Eastern red cedar saplings (*Juniperus virginiana* L.), and Bradford pear saplings (*Pyrus calleryana* Decne. 'Bradford'). Additionally, single-bunch "islands" of switchgrass and cool-season perennial grasses were located at the perimeters of the lespedeza stand as well as short distances within the stand to the south and east. However, only a few small lespedeza individuals were found well within the boundaries of the switchgrass stand, comprising 5% or less of areal cover at associated quadrats. Much of the lespedeza-grass interface zone or mixed plant community was notable for its relatively high area of bare ground and detritus while switchgrass from the interface zone to several sampling points to the west tended to be short in stature and unhealthy looking, possibly indicating that plants at the interface and within the eastern edge of the switchgrass stand were subject to some form of chronic stress (personal observations).

Table 5. Mean (SD) plant community diversity by quadrat

Plant Community	Mean Shannon's Index	Mean Richness	Mean Evenness
Lespedeza-Dominated	0.83 (0.28)[A]	3.20 (0.83)	0.73 (0.20)
Mixed Community	1.06 (0.29)[B]	4.20 (1.48)	0.78 (0.14)
Switchgrass-Dominated	0.52 (0.39)[C]	2.83 (1.25)	0.44 (0.30)
Statistical Significance	A vs. B	$p < 0.10$	
	A vs. C	$p < 0.01$	
	B vs. C	$p < 0.001$	

Values with different superscript letters within a column were statistically significant according to Welch's T-test at p-values listed below. Values without a superscript letter within a column were not significantly different; species richness and evenness could not be evaluated by Welch's T-test.

Table 5 summarizes the plant diversity metrics by community type as calculated from plant percent areal cover values per quadrat. Switchgrass-dominated quadrats scored low in Shannon's diversity because the prairie grass itself restricted community evenness with an average areal cover of over 78%, leaving little space for competitors to grow, a situation previously observed of switchgrass in tallgrass prairie (Baer et al., 2005). However, those plants that managed to grow amongst switchgrass were generally those lacking in the other community types, such as "Weed Y," teasel, and spreading dogbane. In contrast, the mixed communities were most diverse due to high average plant species richness, to which only a small selection of plants failed to contribute, and high evenness near 0.8. The lespedeza-dominated communities were of intermediate diversity because their evenness approximated that of the mixed communities, owing to overlap of

major areal cover classes (e.g. Chinese lespedeza and heath aster), while their species richness approximated that of the switchgrass communities.

Mycorrhizal dependency (responsiveness) relationships between plants can be used to predict when AMF will promote plant community diversity (Van der Heijden, 2002). Most plants that contributed substantially to the vegetation at sampled quadrats were known to be highly mycorrhizal-dependent or belonged to genera with species that were highly-responsive based on a study of tallgrass prairie flora (Wilson and Hartnett, 1998). Plants with the highest mycorrhizal responsiveness included Indian grass (99.5% responsivenss), switchgrass (98.2%), Chinese lespedeza (97%), thistle (*Cirsium vulgare*; 93.5%), and heath aster (aka *Aster ericoides* by old nomenclature in Wilson and Hartnett, 1998; 63.2%). The mycorrhizal-dependency values of boneset, horseweed, spreading dogbane, and teasel were not found in the literature. Cool-season perennial grasses at the site were neutral to moderately-positive in mycorrhizal responsiveness (0.8% for orchard grass and 55.7% for tall fescue, or *Festuca arundinacea* following the old nomenclature) and most minor plant species were positively-responsive. AMF were therefore expected to promote plant diversity at the site because most species, including dominant lespedeza and switchgrass, had similarly-high mycorrhizal-dependencies (Van der Heijden, 2002).

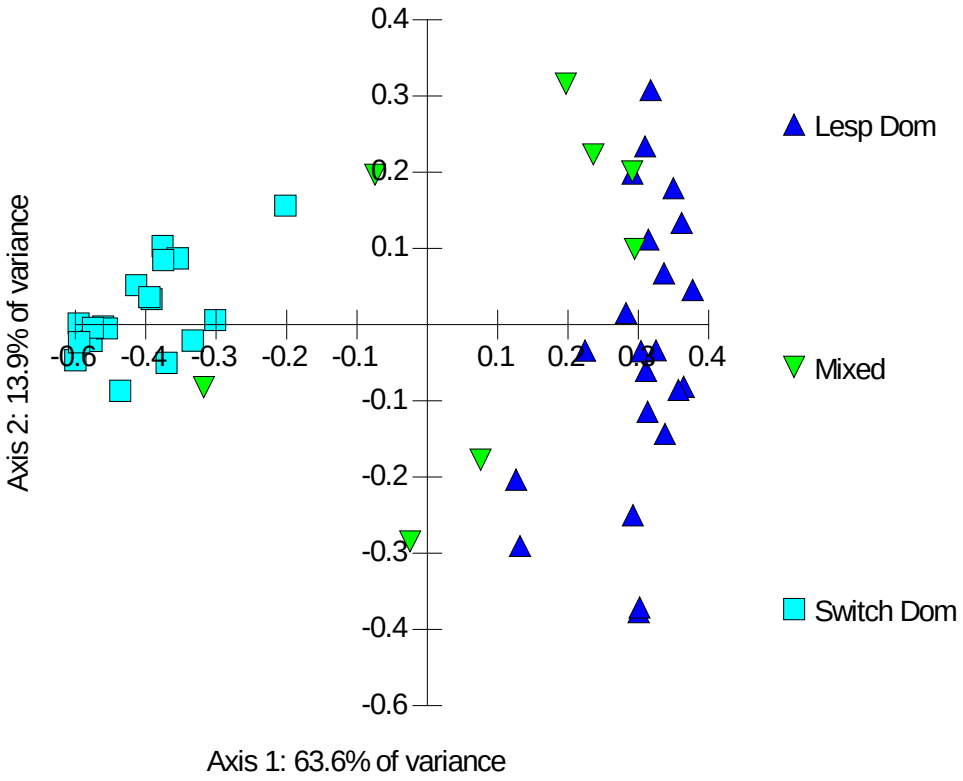

Figure 4. PCO of plant community structures labeled by plant community type.

Figure 4 shows the 2D scatterplot of the plant community structure PCO, which treated each sampling quadrat as a community data point with areal cover of all plant species and bare/littered ground as species variables. Axis 1 clearly separated lespedeza-dominated (Lesp Dom) and switchgrass-dominated (Switch Dom) quadrats while the intermediate-community (Mixed) samples were spread between the two groups but generally closer to one dominant community or the other. Axis 1 explained nearly 64% of the total variance and positive values along the axis indicated greater areal cover of lespedeza and less areal cover of switchgrass. The leftmost two mixed community

quadrats, those for T1Q7 and T4Q9, had only 5-10% lespedeza cover and thus unsurprisingly were placed near the switchgrass-dominated quadrats. Likewise, the rightmost switchgrass-dominated point for T1Q10 was more similar to lespedeza-dominated communities on account of 25% bare ground and the presence of a single lespedeza plant of approximately 5% areal cover. Axis 2 explained an additional 13.9% of the variance, evidently separating quadrats based on secondary plant species composition. Therefore, the PCO thoroughly captured the nuances of the plant community structures since the first two axes explained nearly 78% of the total variance while seven axes explained almost 99% of the variance.

Fungal Community Diversity and Structure from ARISA - (A) Overview

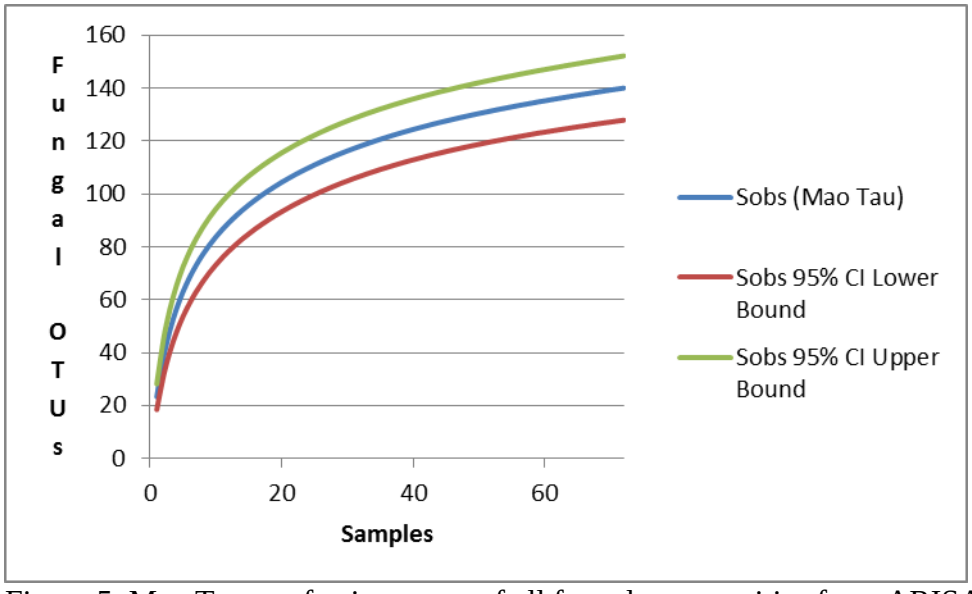

Figure 5. Mao Tau rarefaction curve of all fungal communities from ARISA with 95% CIs.

A total of 157 replicable fluorescent peaks representing fungal OTUs were detected through the ARISA community fingerprinting of 217 PCR replicates derived from over 70 soil and root DNA extracts. Fungal species richness ranged from 8 to 43 OTUs per replicate with sample means (replicate averages) ranging from 12 to 40 OTUs. Only 55 OTUs (35%) were deemed to be "potentially significant" to fungal community structures based on sums of relative abundances (across all replicates) of over 0.89 from 10% or more of all replicates, of which 42 OTUs occurred in over 20% of replicates and only nine (5.7%) were found in over half of all replicates. 15 of the 55 most abundant OTUs differentiated root and soil fungal communities typical of lespedeza and switchgrass (see under Fungal Species Distributions). Remarkably, one OTU, 262.558 (putatively at 262 or 263 basepairs in length), occurred in over 97% of replicates at mean relative abundances of over 5%, representing all fingerprinted samples except T1Q9 Switchgrass Root. Fungal species-saturation was not met for any sample type whether or not samples from both plants were pooled together, but the majority of fungal OTUs were captured in all cases because the rarefaction curves had begun to level-off (see Figure 5 above for rarefaction of all samples; curves by sample type not shown).

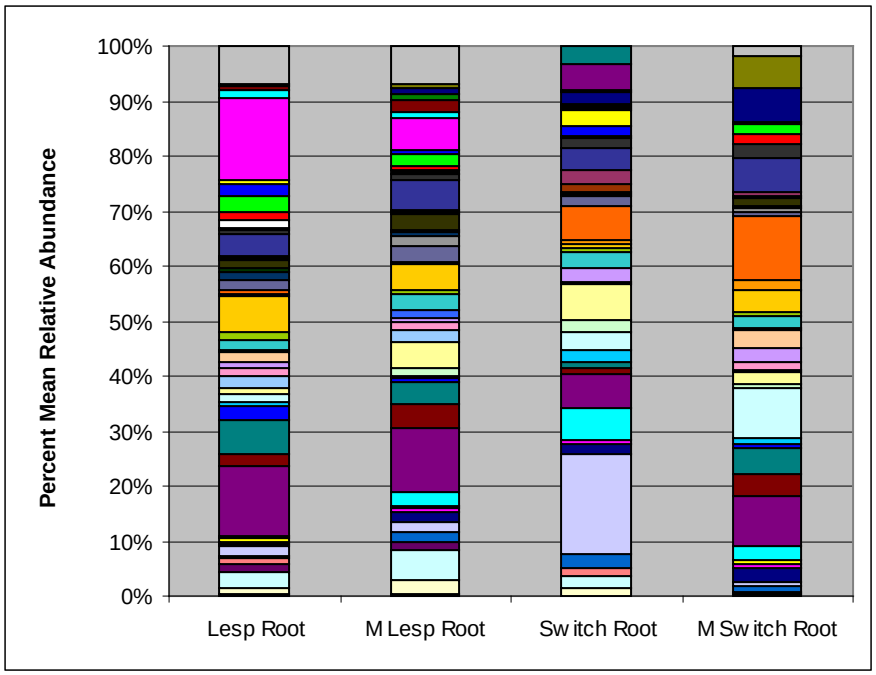

Figure 6. Root fungal OTU profiles from mixed and non-mixed plant communities.

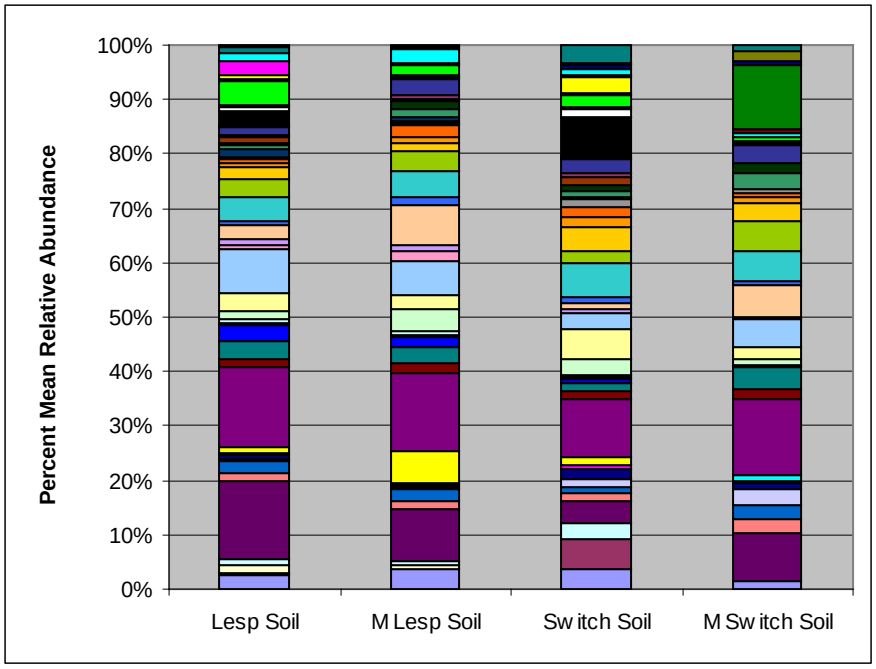

Figure 7. Soil fungal OTU profiles from mixed and non-mixed plant communities.

The average total fungal community structures of root and soil samples are summarized by Figures 6 and 7, respectively, in terms of percent mean relative abundances of fungal OTUs. Bars represent associated plant species (Lesp = lespedeza; Switch = switchgrass) from unmixed (unmarked) and mixed (M)-plant communities and colored bands represent fungal OTUs. Only the 55 most-abundant fungi are included to keep the figures readable and maintain focus on the species most significant to both the overall community structures and the differences in community structures between community types. In order to interpret these results, ARISA data was subjected to Shannon diversity metrics, the multivariate ordination methods of Principal Coordinate Analysis (PCO) and Canonical Correspondence Analysis (CCA), as well as ecological resemblance analysis based on mean Bray Curtis distances.

(B) Fungal Community Diversity

Table 6. Mean (SD) fungal community OTU diversity by quadrat

Fungal Community	Mean Shannon's Index	Mean Richness	Mean Evenness
All Lespedeza Roots	2.81 (0.19)^{A}	24.4 (4.01)	0.88 (0.03)
All Lespedeza Soils	2.95 (0.27)^{A}	26.7 (4.28)	0.90 (0.05)
All Switchgrass Roots	**2.34 (0.64)^{B}**	**19.7 (6.69)**	**0.80 (0.16)**
All Switchgrass Soils	2.93 (0.28)^{A}	28.7 (5.77)	0.90 (0.04)
Unmixed Lespedeza Roots	**2.88 (0.17)^{C}**	**24.7 (3.95)**	**0.90 (0.03)**
Mixed Lespedeza Roots	2.71 (0.17)^{D}	24.0 (4.34)	0.86 (0.02)
Mixed Switchgrass Roots	**2.27 (0.44)^{E}**	**18.7 (5.47)**	**0.79 (0.13)**
Unmixed Switchgrass Roots	2.41 (0.73)^{DE}	20.3 (7.40)	0.80 (0.17)
Unmixed Lespedeza Soils	**2.86 (0.27)^{D}**	**25.6 (3.67)**	**0.88 (0.06)**
Mixed Lespedeza Soils	3.10 (0.24)^{E}	29.0 (5.02)	0.92 (0.03)
Mixed Switchgrass Soils	2.89 (0.34)^{DE}	27.0 (6.07)	0.88 (0.05)
Unmixed Switchgrass Soils	3.09 (0.26)^{E}	29.8 (6.07)	0.92 (0.04)

Statistical Significance	A vs. B	$p < 0.01$
	C vs D and E	$p < 0.05$
	D vs. E	$p < 0.10$

Shannon Index values with different superscript letters within a column were statistically significant at the confidence level indicated by the listed p-values according to two-tailed Welch's T-test. Values within a column and section sharing the same superscript letter or lacking a superscript letter were not significantly different. Bold text emphasizes fungal communities with notably low or high Shannon's Indices.

Diversity analysis of fungal OTUs generally indicated that switchgrass roots were significantly lower in diversity compared to all soils and lespedeza roots, which were approximately equal. As shown in the top section of Table 6 above, only the fungal communities of switchgrass roots significantly differed in Shannon diversity with the

other fungal communities, owing to lower species richness and evenness values, when samples from corresponding dominated, unmixed and mixed-plant communities were pooled together by sample type regardless of plant community structure. However, extended diversity analysis broken down further by plant community type (middle section of Table 6) indicated that the root fungal communities of non-invaded switchgrass were in fact as diverse as the roots of lespedeza in competition with switchgrass. Nonetheless, invaded switchgrass roots were significantly less diverse than both dominant-stand and mixed-community lespedeza roots ($p < 0.05$ and 0.10, respectively). Similarly, while the initial diversity analysis showed that rhizosphere soils were equally diverse, the extended analysis (bottom section of Table 6) indicated that unmixed lespedeza soils were marginally less diverse ($p < 0.10$) than the soils of mixed lespedeza and unmixed, non-invaded switchgrass (yet not those of mixed switchgrass), apparently due to low average species richness. However, the cumulative OTU richness of the samples was evidently not significantly different based on 95% confidence intervals of the rarefaction curves, the right-side confidence intervals and corresponding sampling efforts of which were as follows: 74.8-101.2 from 18 switchgrass roots, 82.28-107.72 from 25 lespedeza roots, 90.96-121.04 from 13 switchgrass soils, and 87.86-114.14 from 18 lespedeza soils. Therefore, the diversity of switchgrass root fungal communities was significantly lower than that of the other sample types based on the DNA fingerprints accumulated so far, but under-sampling of switchgrass roots could not be ruled out as a contributing factor in this determination.

(C) Principal Coordinate Analysis of ARISA Fingerprints

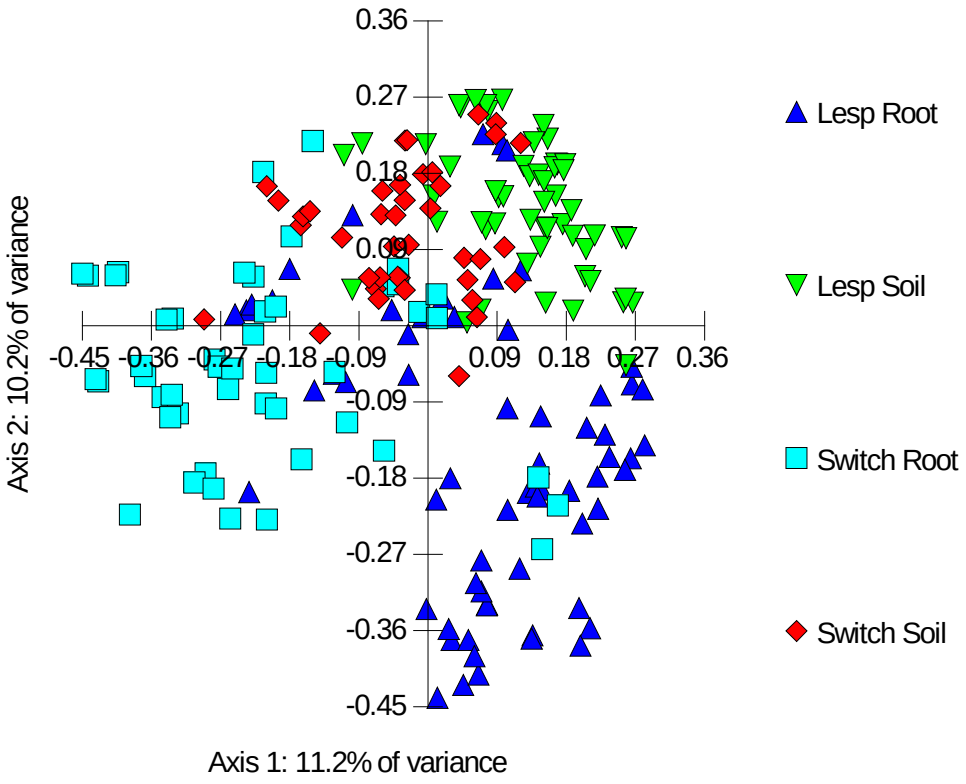

Figure 8. PCO of all fungal communities from ARISA labeled by plant and sample type.

The Principal Coordinate Analysis of all fungal communities, using the relative abundances of the 157 replicable OTUs as variables and the 208 highest-quality ARISA replicates as objects (samples), separated the communities into four largely-distinct but overlapping clusters by plant species (lespedeza/Lesp vs. switchgrass/Switch) and sample type (roots vs. soils). As displayed on Figure 8, the root communities of both plants were largely clustered apart on the opposite ends of the horseshoe-shaped cloud of data points

in the adjacent lower quadrants. The soil community replicates intermingled in the top right, all-positive coordinate quadrant with most of the soil fungal communities of switchgrass to the left and the lespedeza soil fungi to the right. Axis 1, which explained over 11% of the total variance, mostly separated the fungal communities by plant species and Axis 2, which covered an additional 10.2% of the variance between samples, largely divided roots from soils. Axis 3, which explained nearly 6.3% more of the variance, also separated roots from soils based on plots of Axis 3 against the previous axes (data not shown). The first seven principle axes explained a cumulative 44% of the variance and 41 axes were required to cover 100% of the variance, highlighting the complexity of the fungal distribution patterns at the site.

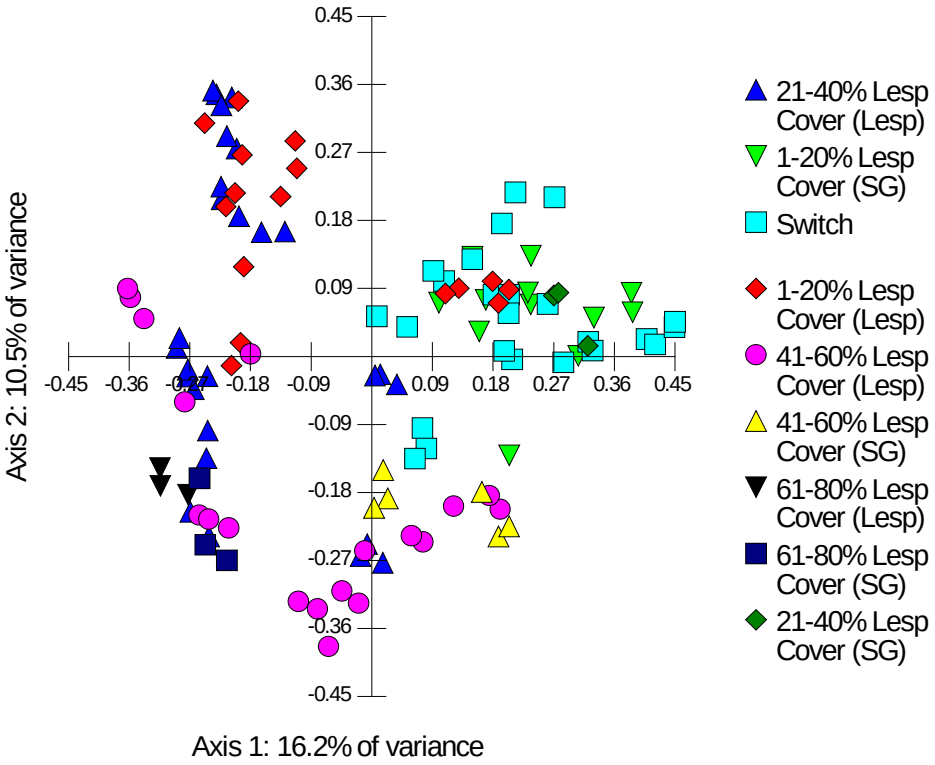

Figure 9. PCO of root fungal communities labeled by percent lespedeza cover.
[Key - Interval of % lespedeza cover (host plant: Lesp = lespedeza, SG = switchgrass); Switch = switchgrass root without lespedeza cover]

The PCO of root fungal communities alone (Figure 9) clustered ARISA replicates largely by associated plant species, but a rough stratification by lespedeza areal cover was also apparent. Axis 1, which explained nearly 16.2% of the variance, was primarily responsible for separating the communities by plant species. Axis 2, which explained a further 10.5% of the variance, appeared to play a role in separating communities by lespedeza cover, with replicates from quadrats of lower lespedeza cover tending to be located higher on the axis. As in Figure 8, there was an overall diagonal orientation of the

scatterplot and fungal communities again separated by lespedeza cover along the slant with communities of lower lespedeza density located toward the top-right and those of higher lespedeza density to the bottom-left. The first seven principal axes explained a cumulative 55.4% of the variance in fungal community structures and 30 axes were necessary to explain 100% of the variance.

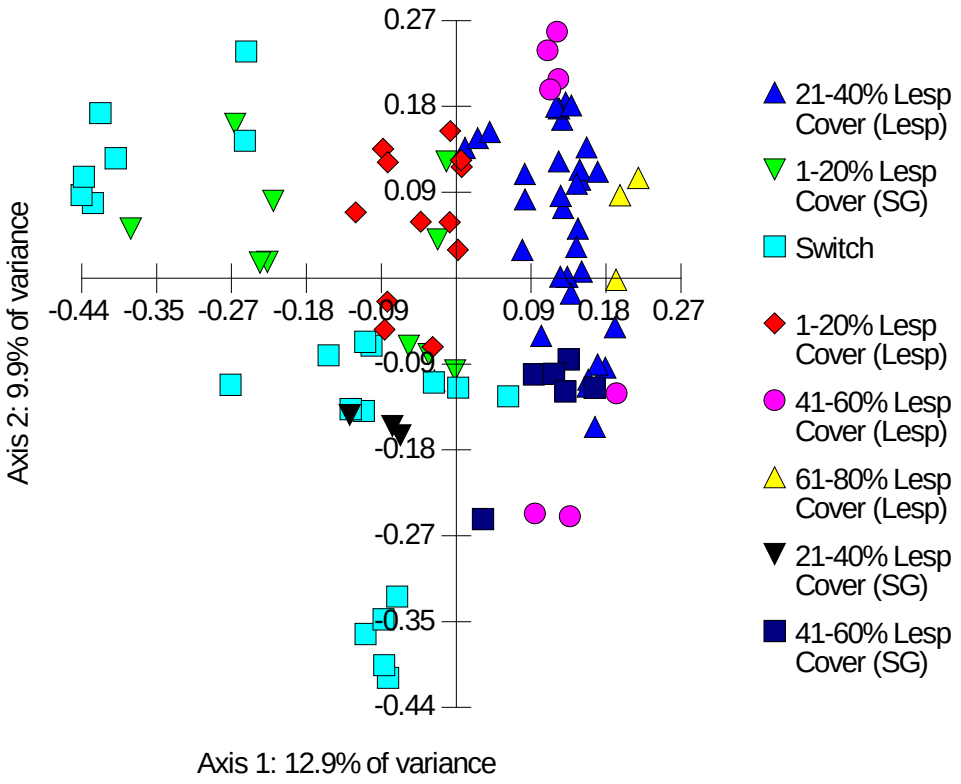

Figure 10. PCO of soil fungal communities labeled by percent lespedeza cover.
[Key - Interval of % lespedeza cover (host plant: Lesp = lespedeza, SG = switchgrass); Switch = switchgrass soil without lespedeza cover]

The PCO of soil fungal communities alone (Figure 10) demonstrated the same general relationships between fungal community structure, host plant, and lespedeza areal

cover as exemplified by the PCO of root fungal communities (Figure 9). Principal Axis 1, which explained 12.5% of the total variance, was again primarily responsible for separating the communities by plant species. Axis 1 was also responsible for most of the separation of soil fungal communities by lespedeza areal cover, with samples from quadrats with higher lespedeza density tending to be located further to the right. Principal Axis 2, which explained an additional 9.5% of the variance, did not appear to reflect the gradient of lespedeza invasion or any obvious characteristics of the plant and fungal communities. Overall, the first seven principal axes explained a cumulative 54% of the variance in soil fungal community structures and 28 axes were required to explain 100% of the variance between samples, indicating that both the soil and root fungal community PCOs from ARISA resolved the multivariate distances between their respective samples about equally.

(D) Canonical Correspondence Analysis of ARISA Fingerprints

The CCAs discussed in this subchapter utilized the following as environmental variables because they significantly differed by plant community (Table 4): soil pH and the areal covers of bare ground, lespedeza, switchgrass, Indian grass, horseweed, heath aster, and unidentified "Weed Y." Analyses were also performed that included "soil moisture state" among the environmental variables based on whether the ground happened to be moist or dry at the time of collection, out of concern for possible sampling effects since the weather had changed during the collection period. However,

the soil moisture biplot vectors proved to be short in all CCAs and clustering of fungal communities based on moisture state was not observed in previous PCO analyses (data not shown), so soil moisture state was dropped from the final analyses.

The option of downweighting rare species in CCA was also taken advantage of to determine which environmental variables correlated more-strongly with common versus rare fungi, based on decreases and increases, respectively, in the combined magnitudes of the Axis 1 and 2 biplot scores when downweighting was put into effect (data not shown). The ecological relevance of this information was that variables with rare species bias might have enhanced fungal diversity and increased differences in fungal community structures at the site. The procedure downweighted all species that occurred in less than one-fifth of the number of samples in which the most common taxon was present, the extent of downweighting based on the species frequency of occurrence (MVSP help file for Correspondence Analysis). It was found that all environmental variables evaluated but soil pH and Indian grass cover responded to species weighting. The couple variables associated with common fungi included bare ground for root fungal communities and horseweed for soil fungal communities. The variables with rare species bias included “Weed Y” in root fungal communities and bare ground, lespedeza, and switchgrass in soil fungal communities. Only heath aster was biased toward rare species in both root and soil samples. The overall results suggested that rare fungi were correlated with major components of plant communities from across the gradient of invasion such that no plant assemblage was distinctive in harboring them, though cover variables associated with

bias for common fungi (bare ground for roots and horseweed for soils) were typically high in quadrats invaded by lespedeza. Thus invasion by lespedeza may have increased the abundances of ubiquitous fungi by impacting the extent of bare ground/detritus and horseweed.

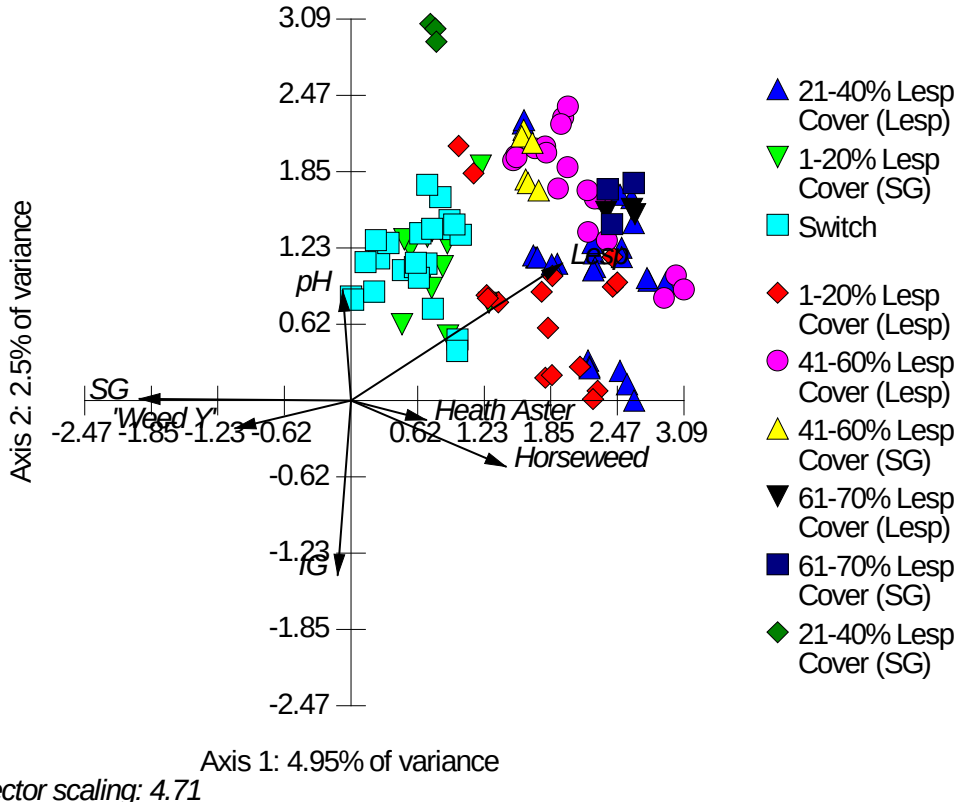

Figure 11. CCA of root fungal communities labeled by percent lespedeza cover.
[Key - Interval of % lespedeza cover (host plant: Lesp = lespedeza, SG = switchgrass); Switch = switchgrass root without lespedeza cover]

As presented in Figure 11, the CCA of root fungi separated fungal community structures by plant species of root sample and percent lespedeza areal cover of the

associated plant communities while displaying (as biplot vectors) the direction and relative magnitudes of greatest change of the environmental factors that correlated with differences in fungal community structure. The first four axes were environmentally constrained and explained a cumulative 9.8% of all variance and over 35% of the cumulative constrained percentage of variance; the cumulative constrained percentage explained did not improve beyond Axis 2. Axis 1 explained 4.95% of the total variance but almost 22% of the cumulative constrained percentage with a species-environmental correlation of 0.905. Chinese lespedeza (Lesp) and switchgrass (SG) dominated Axis 1 with biplot scores of 0.415 and -0.416, respectively, though horseweed (Horseweed), the unidentified herb ('Weed Y'), and heath aster (Heath Aster) also had substantial influences with corresponding scores of 0.304, -0.228, and 0.148. Additionally, the biplot vectors for lespedeza and switchgrass represented a rough diagonal gradient that was responsible for much of the separation of the samples by plant host and plant community in terms of root type and lespedeza areal cover, respectively, and all switchgrass root fungal communities from quadrats of over 40% Lespedeza Cover were ordinated alongside lespedeza roots from similar or identical plant communities (ARISA replicates as yellow triangles and dark blue squares). Axis 2 explained an additional 2.5% of the total variance with species-environment correlations of 0.799. Indian grass (IG) and lespedeza dominated Axis 2 with biplot scores of –0.299 and 0.235, respectively. Soil pH (pH) and horseweed played a relatively minor role in the Axis 2 ordinations with corresponding scores of 0.188 and –0.113. The overall influences of the environmental variables in terms of rounded, combined magnitudes of axis scores were, in decreasing

order, lespedeza (0.65), switchgrass (0.42), horseweed (0.42), Indian grass (0.33), Weed Y (0.28), soil pH (0.21), heath aster (0.18), and bare ground (0.15).

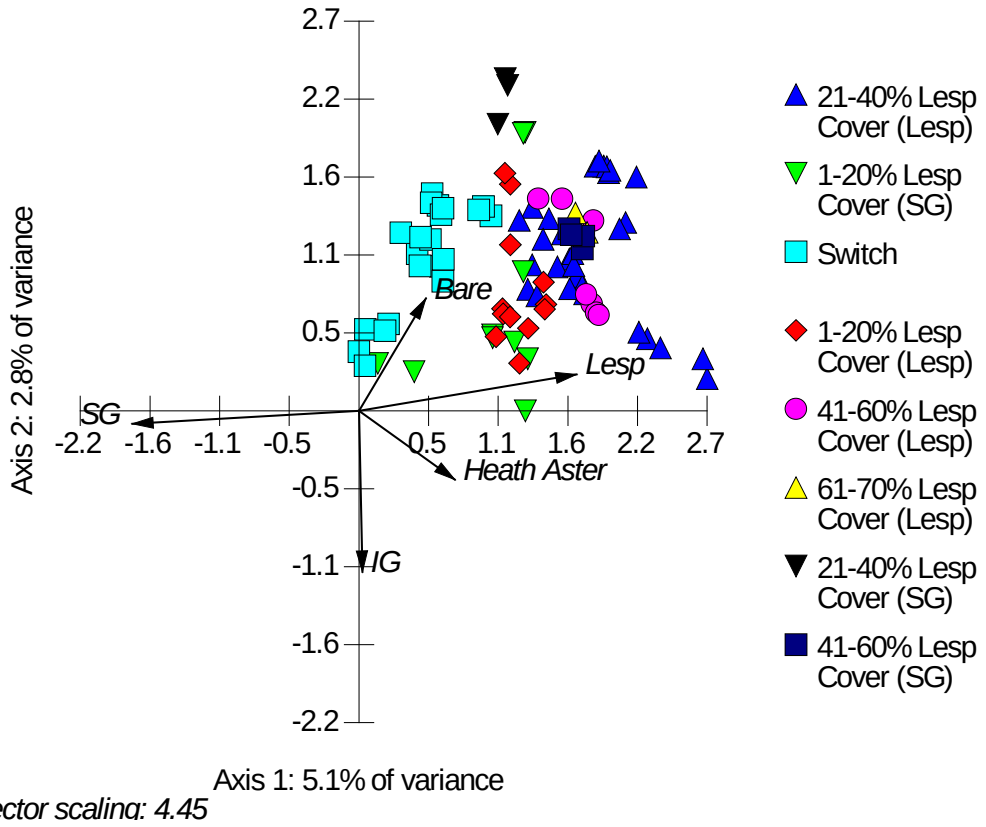

Figure 12. CCA of soil fungal communities labeled by percent lespedeza cover.
[Key - Interval of % lespedeza cover (host plant: Lesp = lespedeza, SG = switchgrass); Switch = switchgrass soil without lespedeza cover]

As presented in Figure 12, the CCA of rhizosphere soil replicates separated fungal community structures by plant species and lespedeza areal cover in the same general manner as the CCA of root fungi discussed above. The first four axes were environmentally constrained and explained a cumulative 10.4% of all variance and about

35.5% of the cumulative constrained percentage of variance. Axis 1 explained over 5% of the total variance and over 22.4% of the cumulative constrained percentage with a species-environmental correlation of 0.932. Chinese lespedeza (Lesp) and switchgrass (SG) dominated Axis 1 with biplot scores of 0.382 and -0.398, respectively, but heath aster (Heath Aster; 0.168), bare ground (Bare; 0.117), and horseweed (Horseweed; 0.107) also contributed. Once again the lespedeza and switchgrass biplot vectors were roughly parallel to the diagonal orientation of the group clusters, showing that the lespedeza-switchgrass environmental gradient was most-directly responsible for separating the fungal communities by associated plant and extent of Lespedeza Cover, and a switchgrass soil fungal community from a quadrat of over 40% Lespedeza Cover was clustered with lespedeza roots from similar or identical plant communities (ARISA replicates as dark blue squares) Axis 2 explained an additional 2.8% of the total variance with species-environment correlations of 0.811. The overall influences of the environmental variables in terms of rounded, combined magnitudes of axis scores were, in decreasing order, lespedeza (0.44), switchgrass (0.42), bare ground (0.29), heath aster (0.28), Indian grass (0.26), horseweed (0.18), Weed Y (0.12), and soil pH (0.03).

Fungal Community Structure from Pyrosequencing – (A) Overview

BLAST queries of the pyrosequencing analysis of 17 fungal community samples yielded 119 species or strains which occurred at 1% or greater relative abundance in at least one community sample (refer to Appendix 3 for a full listing). This only represented

a portion of the actual fungal species richness at the site since the rarefaction curves failed to approach asymptotes whether or not the root and soil samples were pooled together (data not shown; Colwell et al., 2004). Ascomycota was the predominant Phylum of fungi with 47 species, followed by 10 Basidiomycota, four Glomeromycota (AM fungi – all *Glomus* sp.), three Chytridiomycota, and a single Zygomycota. However, 54 hits could not be placed in a Phylum due to insufficient data on the fungi in the nucleotide databanks. Overall 24 hits were matched to described species, 38 were matched to unknown species of a particular Family or genus, and 51 were matched to miscellaneous environmental specimens. Only six fungi had no substantial similarities (>70% identity) with any fungal sequences.

Fungal communities were highly-novel like many previously-characterized endophytic and epiphytic consortia from plants. Approximately 40% of the fungal species detected by pyrosequencing were novel based on their having less than 97% similarity with any NCBI nucleotide entries (Appendix 3: Similarity (%) = Max Identity*[Query Coverage/100]). However, about half of the sequences that did exceed the threshold had their best matches with uncultured environmental fungi but no equally-or-lower-scoring matches of 97% or better similarity with fungi of any confirmed taxonomic level (data not shown). Therefore, upwards of 70% of the fungi sequenced were arguably novel based on a lack of taxonomic information. This level of novelty was comparable to that of *Bouteloua gracilis* roots (69% of all sequences; Porras-Alfaro et al., 2008) and *Phragmites australis* tissues (62% of OTUs sequenced; Neubert et al., 2006).

(B) Principal Coordinate Analysis of Fungal Sequences

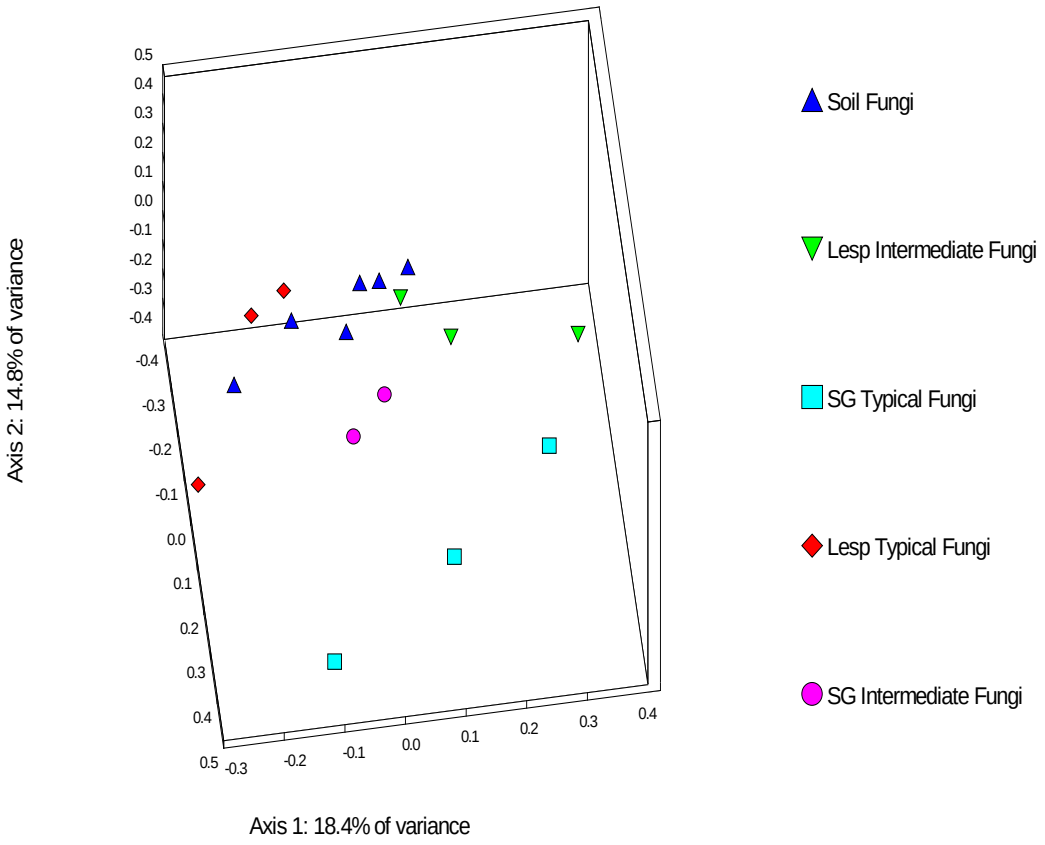

Figure 13. PCO of fungal communities from pyrosequencing labeled by subgroup.

The Principal Coordinate Analysis of the total fungal communities, using the 119 fungi corresponding to fungal sequences as variables and the 17 pyrosequencing profiles as objects (samples), is displayed in Figure 13**.** A 3D scatterplot is presented to emphasize differences in clustering by sample type otherwise obscured in 2D

representations of the first two axes (data not shown). Principal Axes 1, 2, and 3 explained 18.4, 14.8, and 11.1 percent of the variance, respectively, for a cumulative 44.3%, with 100% of the variance covered by 16 axes. The ordination separated the fungal communities into five subgroups by host plant and sample type: (1) three "typical" lespedeza root communities at the top left, (2) three "intermediate" lespedeza root communities at the top right, (3) six combined lespedeza and switchgrass rhizosphere soil communities at the top left-and-center, (4) two "intermediate" switchgrass root communities above the plot's center, and (5) three "typical" switchgrass root communities slanting from right-center to the bottom left. Root samples from mixed plant communities grouped among intermediate fungal communities, with the exceptions of T3Q1 Lespedeza Root with "typical" lespedeza root fungi and T2Q7 Switchgrass Root with "typical" switchgrass root fungi. The rhizosphere soils of both plants had fungal communities that overlapped with those of lespedeza roots based on the three principal axes.

(C) Canonical Correspondence Analysis of Fungal Sequences

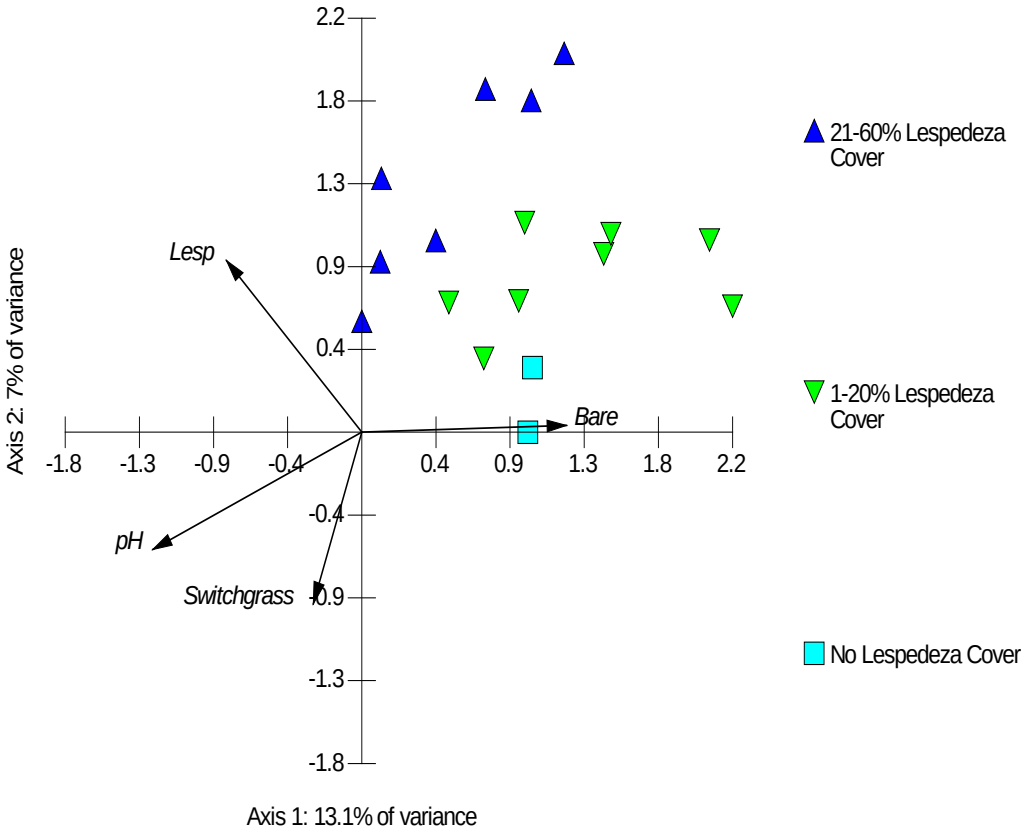

Figure 14. CCA of fungal sequences labeled by percent lespedeza cover.

Figure 14 presents the Canonical Correspondence Analysis of the 17 pyrosequencing fungal community profiles where the 119 fungi were included as species variables correlated with four major environmental variables: Soil pH (pH), Bare Ground Cover (Bare), Lespedeza Cover (Lesp), and Switchgrass Cover (Switchgrass). Rare fungal species were downweighted to reduce the occurrence of spurious species-environment correlations in the limited number of samples; for instance, the hypothetical

case of a misleading correlation with Lespedeza Cover where a fungus was highly-abundant in a single sample purely by chance in a plot with high Lespedeza Cover when all other samples lacked the fungus. The three extracted axes explained a cumulative 21.4% of the variance and the cumulative constrained percentage was 55.1%. Principal Axis 1 explained 13.1% of the variance with species-environment correlations of 0.937 and Principal Axis 2 explained most of the remainder at just over 7% with species-environment correlations of 0.936.

Unlike the CCAs of the ARISA data, here the gradient of plant invasion was largely associated with Principal Axis 2 and other factors were associated with Principal Axis 1. The Axis 1 biplot scores of Soil pH and Bare Ground Cover (-0.309 and 0.302, respectively) were opposite in direction and nearly equal in magnitude with a modest contribution of Lespedeza Cover in the direction of the Soil pH vector (-0.200). The Axis 2 biplot scores of Lespedeza Cover and Switchgrass Cover were exactly equal in magnitude and opposite in direction (± 0.227) and Soil pH contributed modestly in the direction of the Switchgrass Cover vector (-0.155). In terms of overall influence of the environmental variables on fungal community structure, as represented by the sums of the magnitudes of the scores for both axes, Soil pH was the greatest (0.46) followed by Lespedeza Cover (0.43), Bare Ground Cover (0.31), and Switchgrass Cover (0.30).

Ecological Resemblance Analysis of Fungal and Plant Communities

Table 7. Ranking of fungal and plant communities by mean (SD) Bray Curtis distances

Community Group Comparisons	**Distance[1]**	**Significance[2]**
Root Fungal Communities		
		A vs. C:
Lespedeza Root: Switchgrass Root	0.84 (0.07)^{A}	$p < 0.02$
Mixed Switchgrass Root: Switchgrass Root	0.84 (0.11)^{AB}	
Mixed Lespedeza Root: Switchgrass Root	0.83 (0.07)^{AC}	*AB vs. C*:
Mixed Lespedeza Root: Mixed Switchgrass Root	0.81 (0.12)^{C}	$p < 0.05$
Mixed Switchgrass Root: Lespedeza Root	0.81 (0.12)^{C}	
Mixed Lespedeza Root: Lespedeza Root	0.70 (0.12)^{D}	*D vs. all*:
		$p < 0.001$
Soil Fungal Communities		
		A vs. B vs. C:
Mixed Switchgrass Soil: Switchgrass Soil	0.70 (0.07)^{A}	$p < 0.01$
Mixed Lespedeza Soil: Switchgrass Soil	0.69 (0.08)^{A}	
Lespedeza Soil: Switchgrass Soil	0.69 (0.07)^{A}	
Mixed Switchgrass Soil: Lespedeza Soil	0.63 (0.07)^{B}	
Mixed Lespedeza Soil: Mixed Switchgrass Soil	0.63 (0.08)^{B}	
Mixed Lespedeza Soil: Lespedeza Soil	0.57 (0.07)^{C}	
Plant Communities		
		A vs. B vs. C:
Lespedeza-Dominated: Switchgrass-Dominated	0.90 (0.09)^{A}	$p < 0.0001$
Mixed Community: Switchgrass-Dominated	0.72 (0.18)^{B}	
Mixed Community: Lespedeza-Dominated	0.53 (0.17)^{C}	

[1]Means and standard deviations from Bray Curtis distances, aka Odum's percentage differences, calculated by pairwise comparisons according to the following formula: $\sum_i(|X_{1i} - X_{2i}|)/\sum_i(X_{1i} + X_{2i})$, where X_1 and X_2 are corresponding relative abundances of a fungal OTU, or percent areal covers of bare ground or a plant species, (i) belonging to the two fungal or plant community groups (1, 2) being compared (Equation 7.57, Legendre and Legendre, 1998).

[2]Values with different superscript letters within a column (separately by community type: root fungi, soil fungi, or plant) were statistically significant at the confidence level indicated by the listed p-values according to two-tailed Welch's T-test. Values sharing the same superscript letter were not significantly different.

Table 7 presents the results of the ecological resemblance analysis of the fungal and plant communities where Bray Curtis community distances between pairs of groups indicated the total proportion of dissimilarity between them without indication of which variables contributed to the results. Therefore, significantly different values simply meant that one set of groups was more unlike each other than the groups composing another set, the ecological meaning of which must be determined using logical inference. For instance, the significantly greater community distances between the fungi of switchgrass roots from dominated plots and all other root fungal communities (Lespedeza Root: Switchgrass Root, Mixed Switchgrass Root: Switchgrass Root, and Mixed Lespedeza Root: Switchgrass Root) compared to the distances between the fungal communities of lespedeza roots harvested from the main stand and invasion front (Mixed Lespedeza Root: Lespedeza Root) indicated that lespedeza clearly possessed the most cohesive of the root fungal communities whereas uninvaded switchgrass roots had the most distinctive fungi. The same relationships held for the soil fungal communities. Moreover, for both roots and soils, the intermediate community distances between Mixed Switchgrass and both unmixed Lespedeza and Mixed Lespedeza compared with the significantly greater distances between Mixed Switchgrass and unmixed Switchgrass implied that the fungal communities of switchgrass became more “lespedeza-like” following invasion. Additionally, the rank-order of the plant community comparisons paralleled those of the fungal communities, inferring that greater differences in plant community structures were associated with greater differences in the underlying fungal communities.

Fungal Species Distributions

(A) Fungal OTUs Associated with Host Plants and Their Responses to Invasion

Table 8. Fungal OTUs by plant affinity and their responses to invasion

Plant Affinity	Fungal Community	OTU (bp[1])	Avg. Response to Invasion
Lespedeza (9)[2] *Lespedeza cuneata*	Root & Soil (3)	**271.7***……	Increased abundance in switchgrass rhizospheres **[p < 0.05]** and lespedeza rhizospheres with increasing lespedeza density; stable in lespedeza roots.
		264.9*……	Increased abundance in lespedeza roots and rhizospheres with increasing lespedeza density.
		262.56*……	Stable in roots, rhizospheres.
	Root (4)	283.69*……	Increased abundance in lespedeza roots with increasing lespedeza density.
		314.4*, 318.84, 494.7*……	Stable in lespedeza roots.
	Soil (2)	246.74*……	Increased abundance in lespedeza rhizospheres with increasing lespedeza density.
		265.9……	Stable in lespedeza rhizospheres.
Switchgrass (6) *Panicum virgatum*	Root (6)	**255.12**, 306.7……	Decreased abundance in switchgrass roots **[p < 0.10]** with decreasing switchgrass density.
		266.8, 269.4, 354.87, 365.7……	Scarce in switchgrass roots by lespedeza; abrupt decline.

[1]Fungal OTU designations are estimated basepair lengths of PCR amplicons as recorded during capillary electrophoresis. All OTUs had significant ($p < 0.05$; Mann-Whitney U test) plant affinities within the fungal community types listed to the left of the OTUs.

[2]Number of pertinent OTUs by plant affinity and fungal community in parentheses and significances of average responses to invasion by fungal community in brackets (Bonferroni pairwise contrasts between average OTU abundances from switchgrass samples of mixed and unmixed plant community quadrats). OTUs that experienced significant overall changes in abundance during invasion in bold.

*OTU responsible for making individual switchgrass fungal communities in mixed plant community quadrats more "lespedeza-like" based on unusually high abundances in the switchgrass samples.

A total of 15 fungal OTUs were found to underlie most differences between fungal communities and they responded to the progression of invasion in several ways (Table 8). OTUs 318.84 and 246.74 best-distinguished lespedeza root and soil fungal communities, respectively, from those of switchgrass in terms of abundance and frequency of occurrence. Four of the nine lespedeza-associated OTUs (246.74, 264.9, 283.69, and 271.7) increased in relative abundance with lespedeza cover within their respective fungal communities, OTU 271.7 being distinguished in also having increased its average abundance in adjacent switchgrass rhizospheres throughout the invaded field ($p < 0.05$). All other lespedeza associates, and OTU 271.7 in lespedeza roots, remained significantly more abundant in samples from lespedeza after invasion but at a relatively constant level independent of lespedeza density. In contrast, OTU 255.12 largely differentiated switchgrass roots and no single OTU typified switchgrass soils; instead various fungi would occur in concert in switchgrass rhizospheres at relatively low abundances (data not shown). Furthermore, all six switchgrass root-associated OTUs failed to respond to lespedeza cover in a linear fashion but instead seemed to abruptly decline when lespedeza was present (data not shown: almost all points on scatterplots of Lespedeza Cover vs OTU abundance would lie directly on the X and Y axes). However, two switchgrass root fungi (255.12 and 306.7) demonstrated positive relationships with switchgrass cover that were nonetheless stronger when lespedeza samples were included in the regressions ($R^2 = 0.27$ vs 0.23 and 0.29 vs 0.25 for regressions of OTUs 255.12 and 306.7, respectively, with vs without lespedeza root samples)**.** Indeed, a density-dependent effect of switchgrass on OTUs 255.12 and 306.7 in lespedeza roots was also apparent, but

only in regressions that excluded samples with zero-abundances (R^2 = 0.71 and 0.87 for 255.12 and 306.7 in lespedeza roots, respectively). Therefore, Switchgrass Cover had an apparent modest effect in spreading these OTUs to lespedeza roots, but this was not significant at the field-scale. In fact, OTU 255.12 seemed to be more heavily-impacted by its host's competition with lespedeza since its abundances were significantly lower in switchgrass roots throughout the invaded portions of field ($p < 0.10$) and the OTU was highly-abundant only in switchgrass roots from non-invaded, high-density stands.

The overall effect of lespedeza invasion on the switchgrass fungal communities was a decrease in distinctive root fungi in the grass offset by a gain of less-distinctive root and rhizosphere fungi from lespedeza. As denoted by asterisks on Table 8, seven OTUs were observed to "crossover" from lespedeza to individual switchgrass communities in mixed plant assemblages and thereby contribute to shifts in fungal community structures, albeit most of these occurrences did not amount to significant changes at the field-scale. Additionally, switchgrass root fungal communities were often more "lespedeza-like" through an absence of normally switchgrass-associated OTUs alongside the presence of lespedeza-associated fungi. On the contrary, switchgrass-associated fungi were never very abundant in the roots of neighboring lespedeza and "switchgrass-like" T2Q7 Lespedeza Root was characterized by greater abundances of minor OTUs found in some switchgrass samples relative to lespedeza-typical fungi, not a preponderance of switchgrass-typical fungi (data not shown).

(B) Fungal Sequences by Plant Host and Plant Community

Table 9. Representative samples and associated sequences of fungal community groups.

Fungal Group from PCO Representative Samples	Group-Associated Fungi[1]	Associated Variables[2]
Lespedeza Typical Roots		
T2Q3 Lespedeza Root	***Aspergillus flavipes***	**Bare Ground Cover***
T3Q1 Lespedeza Root	***Fusarium* sp. 5/97-45**	**Lespedeza Cover**
T4Q3 Lespedeza Root	***Fusarium oxysporum* BD**	**Bare Ground Cover***
	Nectria haematococca	**Lespedeza Cover***
	Uncultured *Fusarium* sp.	**Lespedeza Cover**
Intermediate Lespedeza Roots		
T1Q6 Lespedeza Root	*Aspergillus flavipes*	Bare Ground Cover
T1Q7 Lespedeza Root	***Aureobasidium pullulans***	**Lespedeza Cover**
T2Q7 Lespedeza Root	***Bionectria ochroleuca***	**Lespedeza Cover**
	Contig2815_1306	Switchgrass, Bare Cover
	Fusarium oxysporum BD	Bare Ground Cover
	Uncultured *Mortierella*	**Bare Ground Cover***
	Unmatched Fungus H	Bare Ground Cover
Intermediate Switchgrass Roots		
T3Q1 Switchgrass Root	*Alternaria alternata*	Bare Ground Cover
T4Q8 Switchgrass Root	Soil Fungus 138-35	Lespedeza Cover
Switchgrass Typical Roots		
T1Q8 Switchgrass Root	**Contig2815_1306**	**Switchgrass, Bare Cover**
T2Q7 Switchgrass Root	**OTU#3267_1024**	**Switchgrass Cover**
T3Q8 Switchgrass Root	Ascomycete 5/97-81	Bare Ground Cover
	Epicoccum nigrum	Lespedeza Cover
Rhizosphere Soils (Approx. Lespedeza Roots)		
T1Q2 Lespedeza Rhizosphere	*Epicoccum nigrum*	Lespedeza Cover
T1Q7 Lespedeza Rhizosphere	*Fusarium oxysporum* BD	Bare Ground Cover
T2Q7 Lespedeza Rhizosphere	***Fusarium oxysporum* F-X**	**Bare, Lespedeza Cover***
T1Q8 Switchgrass Rhizosphere		
T2Q7 Switchgrass Rhizosphere		
T4Q8 Switchgrass Rhizosphere		

[1]Fungal designations based on NCBI accession numbers (refer to Appendix 3 for complete listing of fungi). Bold text indicates first occurrences of fungi with strongest support for group membership.

[2]Environmental variables strongly-associated with fungus listed to left based on high variable-score rankings against CCA biplot vectors. Most strongly-associated variables for a fungus listed first within row. *Association with sample group significant at $p < 0.15$ according to Bonferroni pairwise contrasts.

Table 9 presents the results of a composite analysis of the pyrosequencing-based PCO and CCA ordinations (details under Materials and Methods: Statistical Analysis). 11 of the 16 evaluated fungi were "core species" with statistical support for group membership, including all fungi associated with Lespedeza Typical Roots yet none thought associated with Intermediate Switchgrass Roots. Furthermore, eight of the 11 core fungi had a strong affinity with lespedeza as a host whereas only two had such with switchgrass. Additionally, only three fungi were linked with Switchgrass Cover compared to nine for Lespedeza Cover and 12 for Bare Ground Cover. The prevalence of Lespedeza Cover and Bare Ground Cover influences in all fungal groups, including associations with all but one fungus of Switchgrass Typical Roots, may have indicated that Switchgrass Cover had minimal effect on fungal community structure at the site. Thus invasion by Chinese lespedeza (i.e. Lespedeza Cover) and stress leading to loss of vegetation or reduced germination (Bare Ground Cover as a stand-in for drought, disease, and herbivory) appeared to have been the overriding processes affecting the fungal communities of the 17 pyrosequenced samples. Five species did not appear to be strongly-associated with any group, so their placements were dubious but may yet prove real following the pyrosequencing of more samples: Unmatched Fungus H, *Alternaria alternata*, Soil Fungus 138-35, Ascomycete 5/97-81, and *Epicoccum nigrum*.

Fungal Colonization of Roots

Table 10. Mean (SD) percent fungal colonization of root samples

Plant Community (Root[1])	Arbuscules[2]	Vesicles	AM Hyphae	DSF
Lespedeza-Dominated (L)	70.6 (17.1)	29.8 (10.9)^{A}	95.3 (5.5)	32.9 (20.4)^{C}
Mixed Community (L)	60.3 (11.3)	24.4 (17.0)^{AB}	95.8 (5.1)	25.0 (18.6)^{CD}
Mixed Community (SG)	63.0 (11.4)	17.9 (13.2)^{AB}	88.1 (21.0)	70.9 (26.3)^{E}
Switchgrass-Dominated (SG)	64.9 (29.6)	14.1 (11.2)^{B}	92.6 (8.1)	71.9 (14.7)^{E}

Statistical Significance		
	A vs. B	$p < 0.005$
	C vs. E	$p < 0.005$
	CD vs. E	$p < 0.0001$

Values with different superscript letters within a column were statistically significant according to Kruskal-Wallis test with Bonferroni pairwise contrasts. Values sharing or lacking a superscript letter had no statistically significant difference with each other or other values in the column, respectively.

[1](L) *Lespedeza cuneata* root sample; (SG) switchgrass or *Panicum virgatum* root sample.

[2]Means and standard deviations for Arbuscules derived without corresponding values from Transect 1 samples (due to irregularities in quality of early root stains); means and standard deviations were originally as follows from top to bottom of column: 58.3 (27.3), 51.4 (25.2), 45.6 (31.2), and 41.7 (39.7); none of these group means were significantly different at $p < 0.05$.

As illustrated by Table 10, both lespedeza and switchgrass roots were highly colonized by AM and DSF concurrently. In particular, all roots were heavily colonized by AM hyphae, with 70-100% of intersections crossed by stained hyphae per sample, and no differences between group means were significant. The presence of arbuscules, which generally occurred at 50-80% of intersections per sample, also did not differ significantly between groups. However, AM vesicles were found to be significantly more abundant in unmixed lespedeza roots than unmixed switchgrass roots, but unmixed and mixed-stand lespedeza roots and mixed switchgrass roots were not significantly different from one

another. Interestingly, there was a decreasing average in intersections with vesicles across the gradient of plant invasion from lespedeza to switchgrass, and the differences in vesicle abundance were significantly different with the exception of mixed lespedeza roots and mixed switchgrass roots. Finally, the most striking difference in root colonization between lespedeza and switchgrass was the disparity in the number of intersections with DSF structures (brown hyphae and microsclerotia), with the grass having on average about 40% greater (i.e. twice as much) colonization than Chinese lespedeza at $p < 0.005$.

DSF colonization may have been antagonistic to AM vesicle formation in lespedeza roots but not necessarily in switchgrass roots, where DSF levels were consistently high. Linear regressions of percent colonization of AM vesicles vs. DSF (plots not shown) demonstrated modest negative correlations in lespedeza roots and all roots pooled together but not when switchgrass roots were analyzed separately (R^2 = 0.164, 0.139, 0.347, and 0.233 for all lespedeza, dominant-stand lespedeza, mixed stand lespedeza, and all roots, respectively; switchgrass root regressions were non-linear). Conditions at the invasion front appeared to have led to the strongest level of negative correlations (0.347) between AM vesicle formation and DSF in lespedeza roots. Note that DSF colonization did not appear to have an overall negative effect on AMF colonization in either plant based on high average extent of AM arbuscules and hyphae regardless of DSF levels.

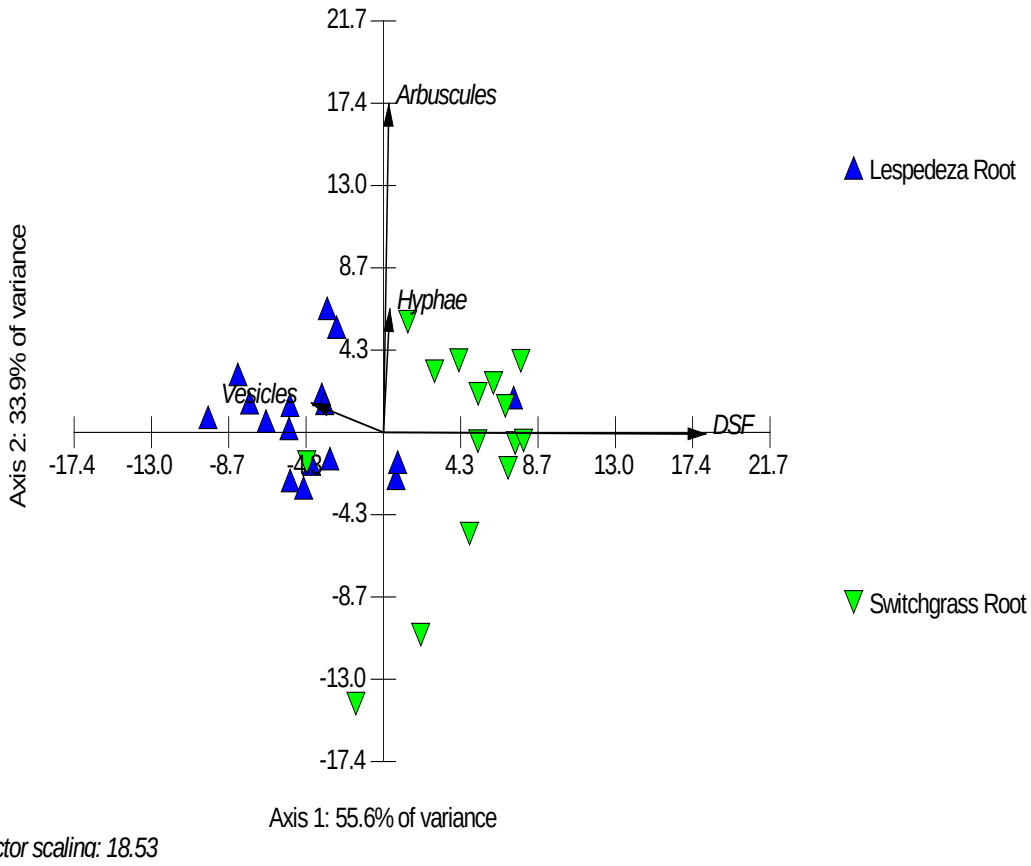

Figure 15. PCA of fungal root colonization with samples labeled by host plant.

Figure 15 displays the Principal Component Analysis results of the fungal root colonization data, with Transect 1 root samples excluded in order to eliminate inaccuracies due to the uncertain identification of putative arbuscules in the earliest-analyzed roots. The PCA agrees with the Kruskal-Wallis analysis of the same data discussed above (Table 10) in that lespedeza and switchgrass roots were differentiated by group primarily by DSF, the biplot vector of which provided a variable loading of 0.976 toward Axis 1, which accounted for 55.6% of the variance. Similarly, AM vesicles supplied an additional Axis 1 variable loading of –0.217 and the biplot vector's

orientation away from the switchgrass cluster toward the lespedeza roots indicated a modestly greater vesicle frequency in lespedeza, again in line with the Kruskal-Wallis analysis. Although Axis 2 accounted for nearly 34% of the variance for a cumulative 89.5%, it played little to no role in separating the root samples by plant despite arbuscules and AM hyphae supplying variable loadings of 0.933 and 0.349, respectively. However, this is understandable in light of the statistically insignificant group differences in root colonization by arbuscules and stained hyphae displayed in Table 10. The high amount of variance explained by the principal axes indicates that Figure 15 is a good stand-alone representation of the results.

The two samples seemingly misplaced with those of the other plant species are Transect 2 Quadrat 4 - Lespedeza Root (as blue triangle among green triangles) and Transect 3 Quadrat 8 - Switchgrass Root (as green triangle among blue triangles), owing mostly to unusually high and low colonization by DSF, respectively. Unusual plant community structure may have been responsible for the uncharacteristic DSF frequencies. For instance, T2Q4 had the highest total areal cover for secondary plant species at 45% (for horseweed and field thistle combined) and T3Q8 had a slightly "mixed" plant community with several small lespedezas providing 10% areal cover alongside 70% switchgrass. Interestingly, the lespedeza roots from T3Q8 had less than half as many intersections with DSF and AM vesicles (11 and 9%, respectively) than the adjacent switchgrass (29 and 30%), meaning that switchgrass at the quadrat was actually

"normal" in having more DSF than its competitor yet "unusual" in having substantially more vesicles.

Endophytic Fungi: Micrographs of Stained Roots

The morphology of AM and DSF observed in cleared and stained roots was similar in both plant hosts. All AM had *Arum*-type (linear-VAM) growth distinguished by predominantly longitudinal, intercellular hyphae and distinct intracellular arbuscules (Figure 16), though intracellular hyphal coils that indicated colonization events were common (Figure 17; Brundrett, 1999). Most AM hyphae and arbuscules were relatively thick and well-stained (Figures 17-19), though faintly stained forms were sometimes present. AM vesicles were typically relatively large and ovular and sometimes contained visible lipid globules (Figure 20), but smaller and/or more spherical vesicles were also frequent. DSF hyphae varied in relative size and darkness but tended to be thicker and darker than not, though they were thinner than most AM hyphae. AM and DSF intimately coexisted in the roots of lespedeza and switchgrass, the hyphae of both fungal types frequently growing side-by-side in the same intercellular spaces (not pictured). Microsclerotia were often present in root cortex cells (Figure 21), and occasionally vascular tissue (not pictured), and putative DSF spores or vesicular bodies were often abundant in root cortex (Figure 22), though many examples of free-floating brown bodies outside roots were encountered (not pictured). Putative "brown spores" tended to be large and ovular to spherical without visible internal structures. While AM were almost wholly

observed within plant roots, DSF hyphae also tended to loosely coat the root epidermis (Figure 23) and sometimes externally formed dense mat-like clumps or low mounds, as observed by authors such as Peterson *et al.* (2004; not pictured). External congregations of DSF often appeared to have exuded a substance that gave the clumps a dirty or slimy appearance, giving the impression of a fused, biofilm-like growth (not pictured). In fact, the presence of this brownish grime on thoroughly-washed roots greatly enhanced the visibility of DSF under magnification since melanized hyphae invariably occurred within a short distance of the substance. Switchgrass roots were also found containing a variety of distinctive fungal structures like masses of spiny (echinulate) brown spores that may have belonged to *Periconia macrospinosa* (Watanabe, 1994; Figure 24) and stalked, external conidiophores (Figure 25).

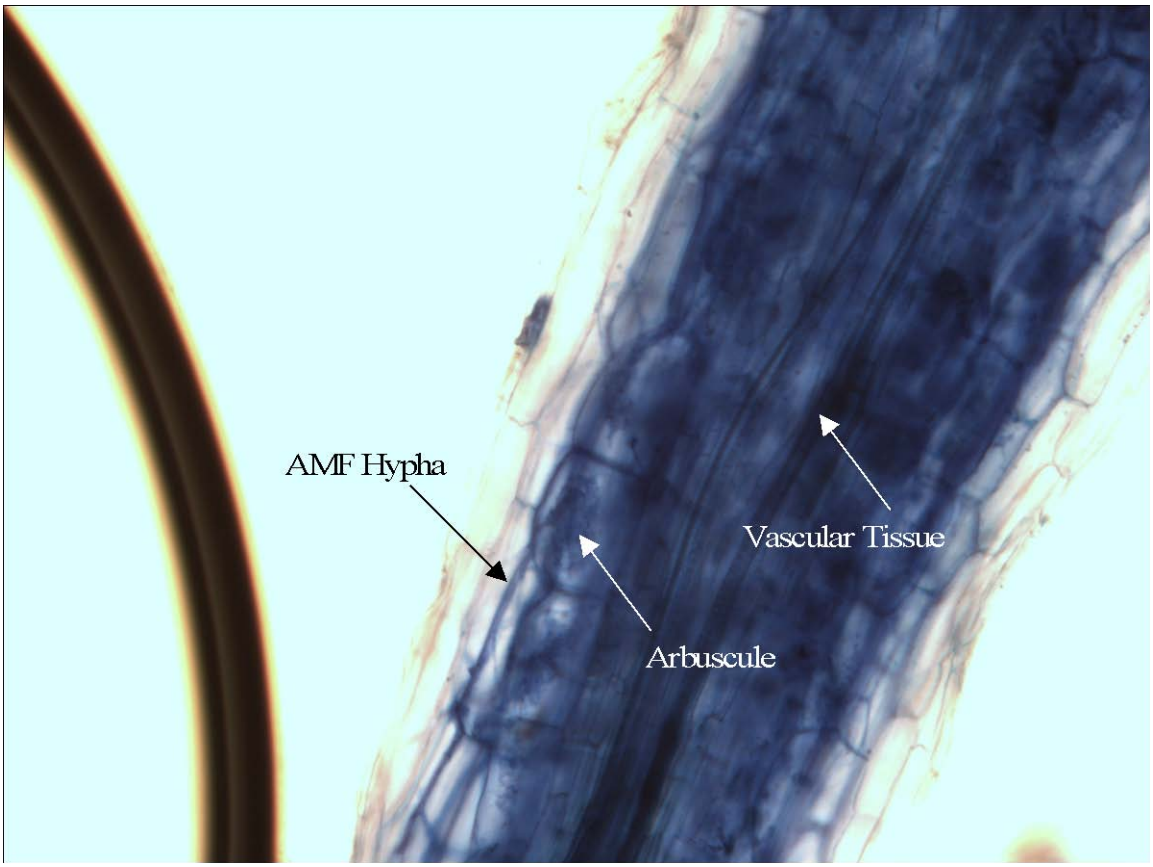

Figure 16. Fine root filled with AMF hyphae and arbuscules (lespedeza root, Trypan blue stain).

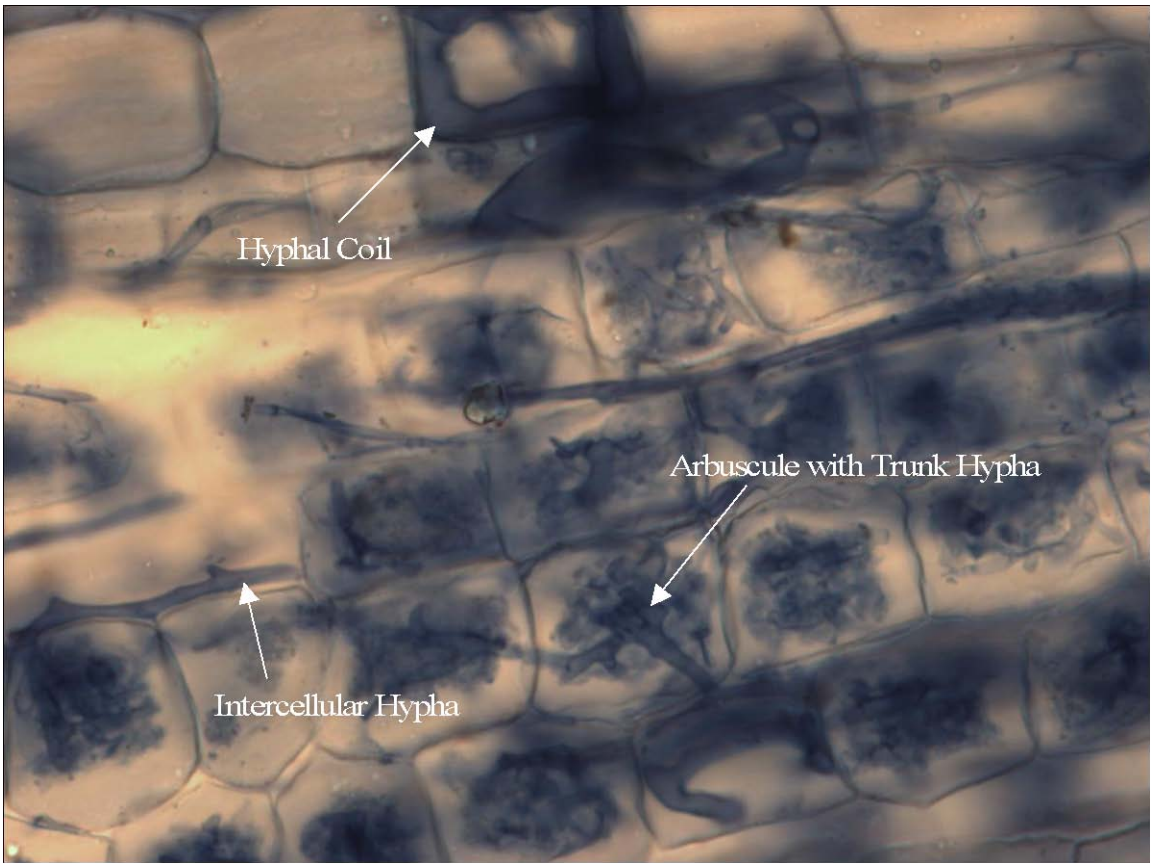

Figure 17. Arbuscules in root cortex near hyphal coils (lespedeza root, Trypan blue stain).

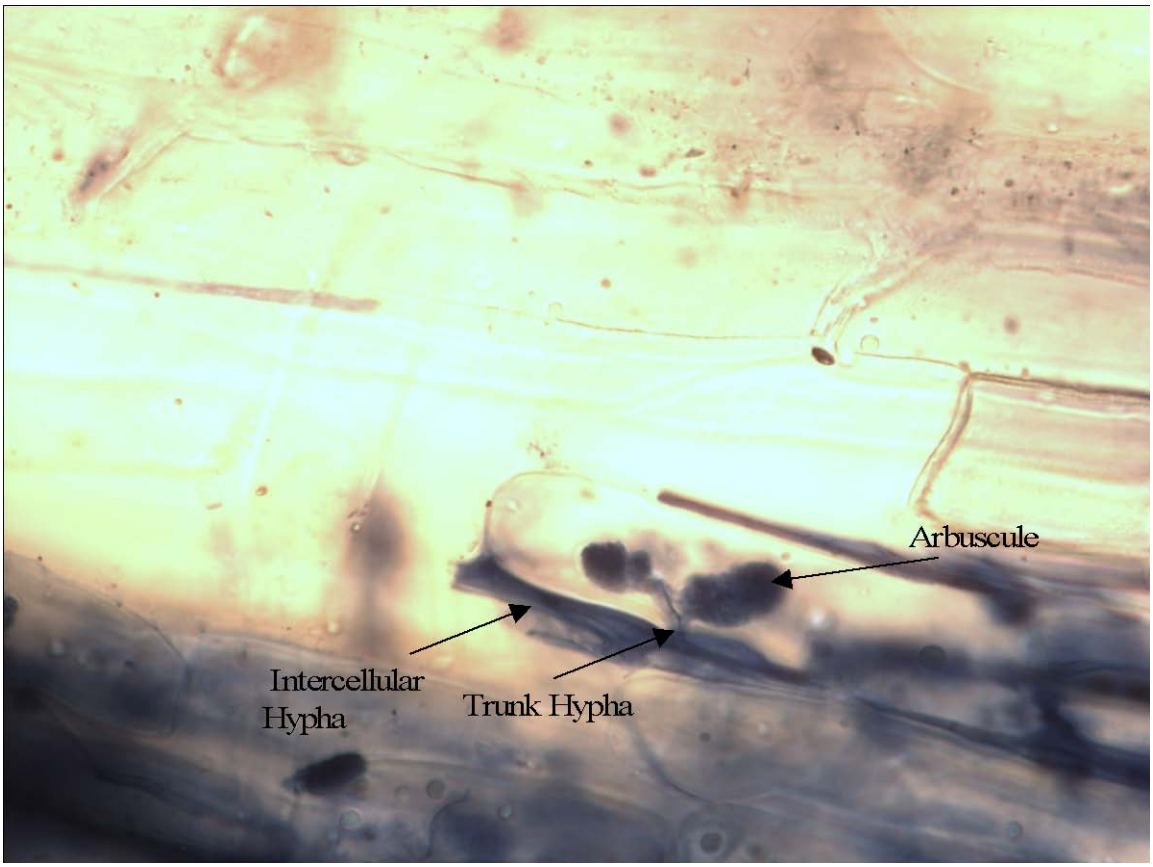

Figure 18. Double-lobed arbuscule in a root cortex cell (switchgrass root, Trypan blue stain)**.**

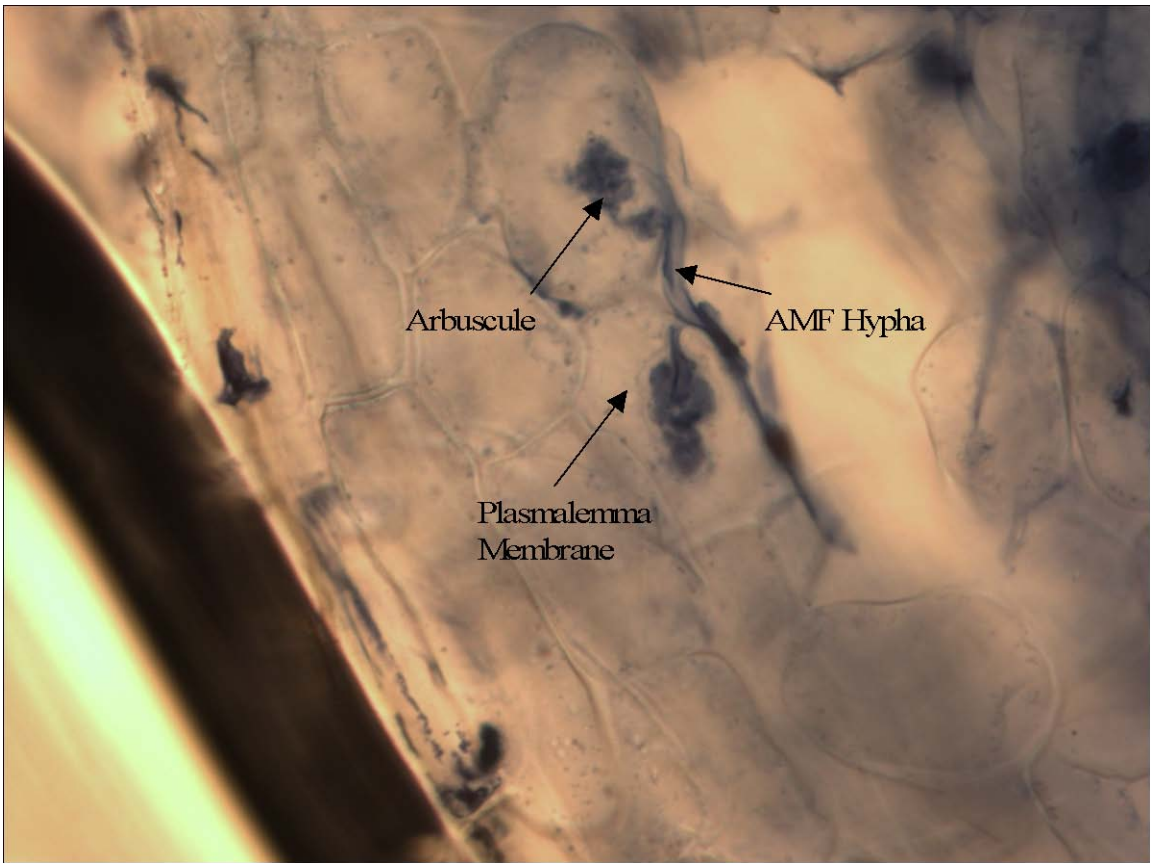

Figure 19. Single-lobed arbuscules in adjacent root cortex cells, with visible plasmalemma (switchgrass root, Trypan blue stain).

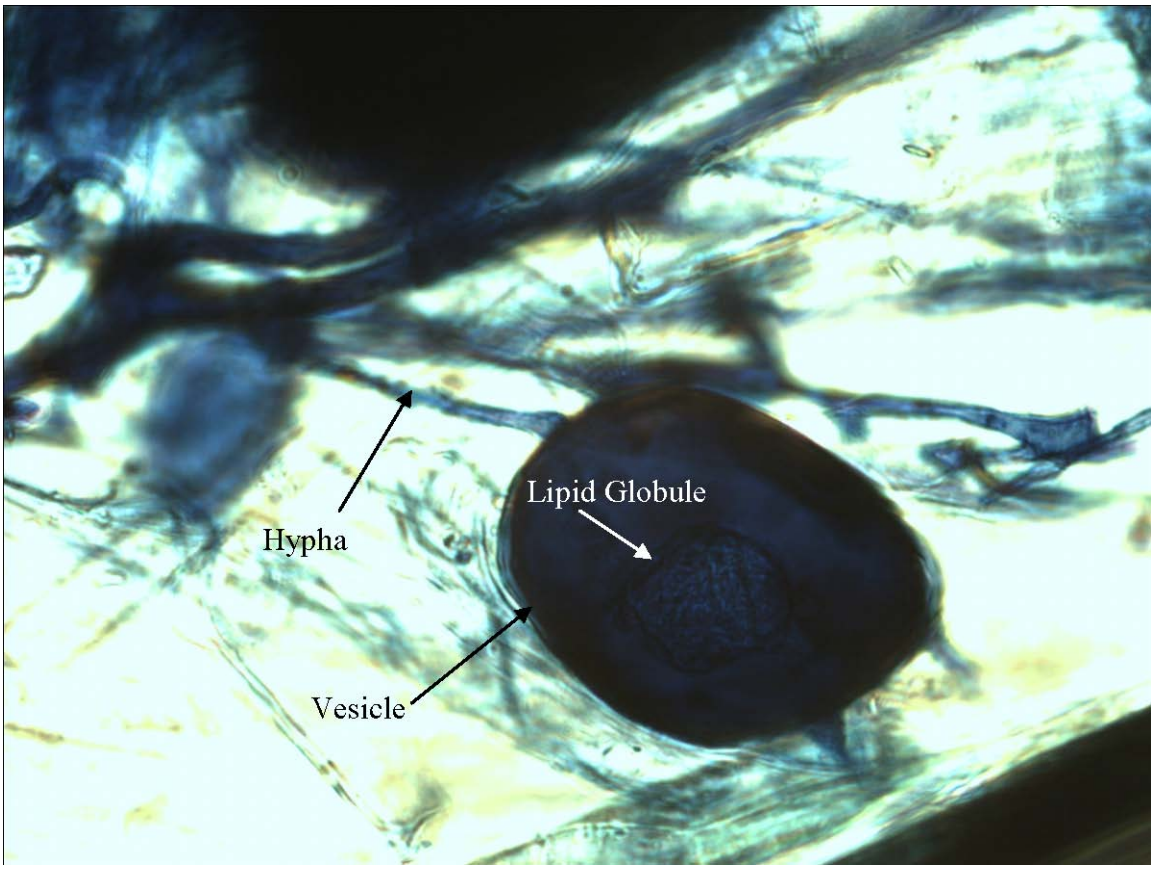

Figure 20. AMF vesicle with visible lipid globule (lespedeza root, Trypan blue stain).

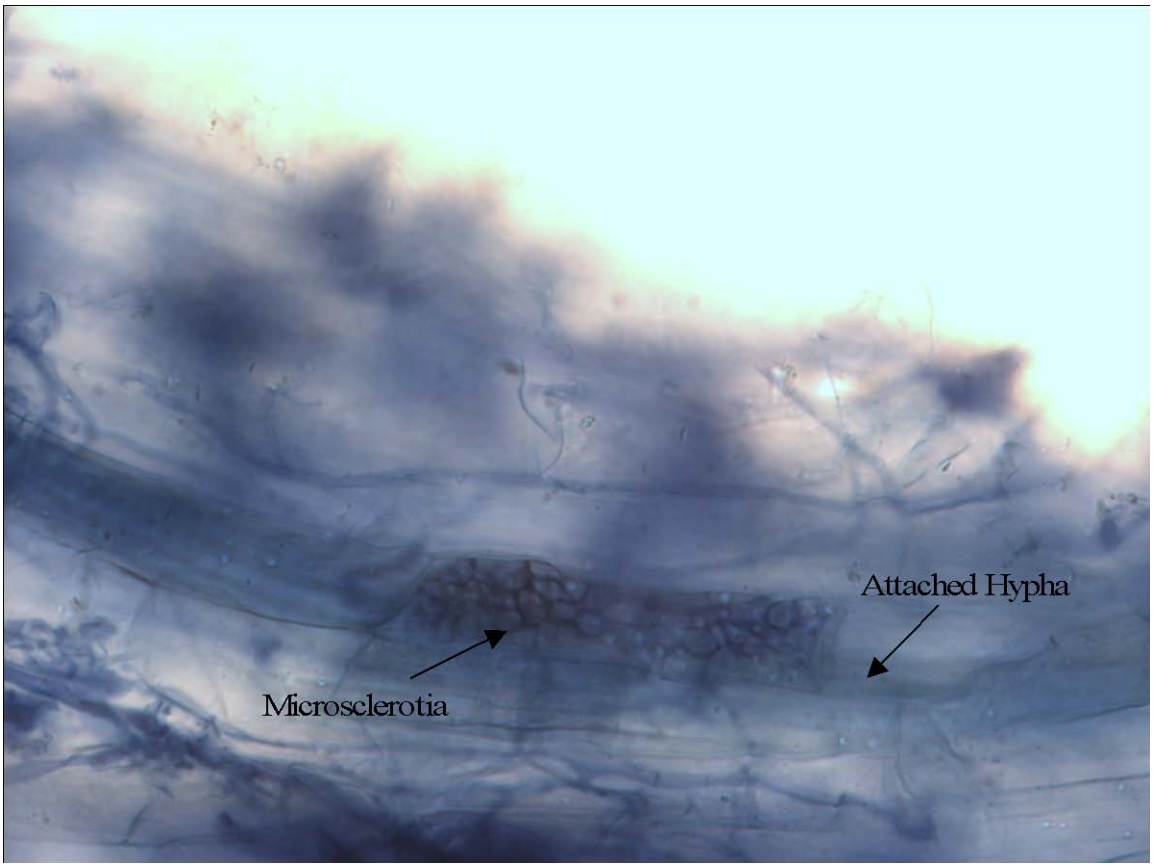

Figure 21. DSF microsclerotia in root cell (switchgrass root, Trypan blue stain).

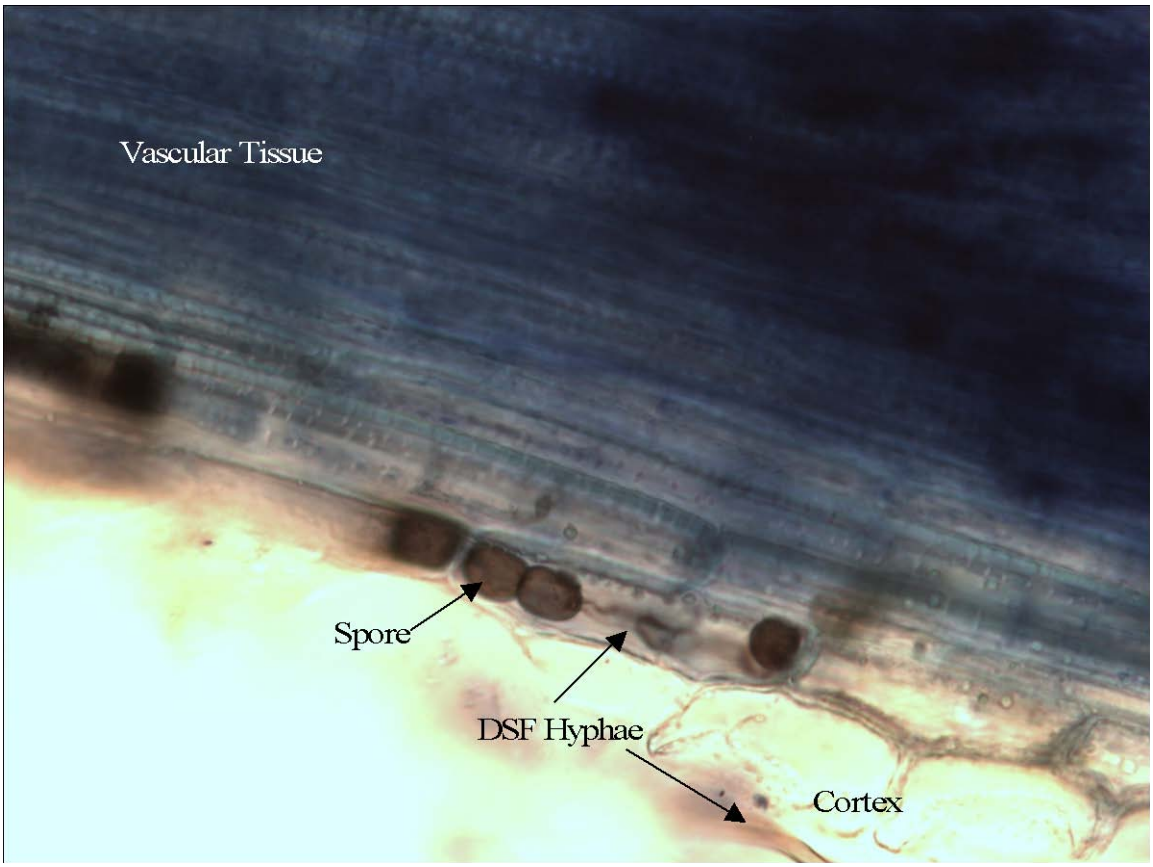

Figure 22. Melanized putative resting spores near vascular tissue (switchgrass root, Trypan blue stain).

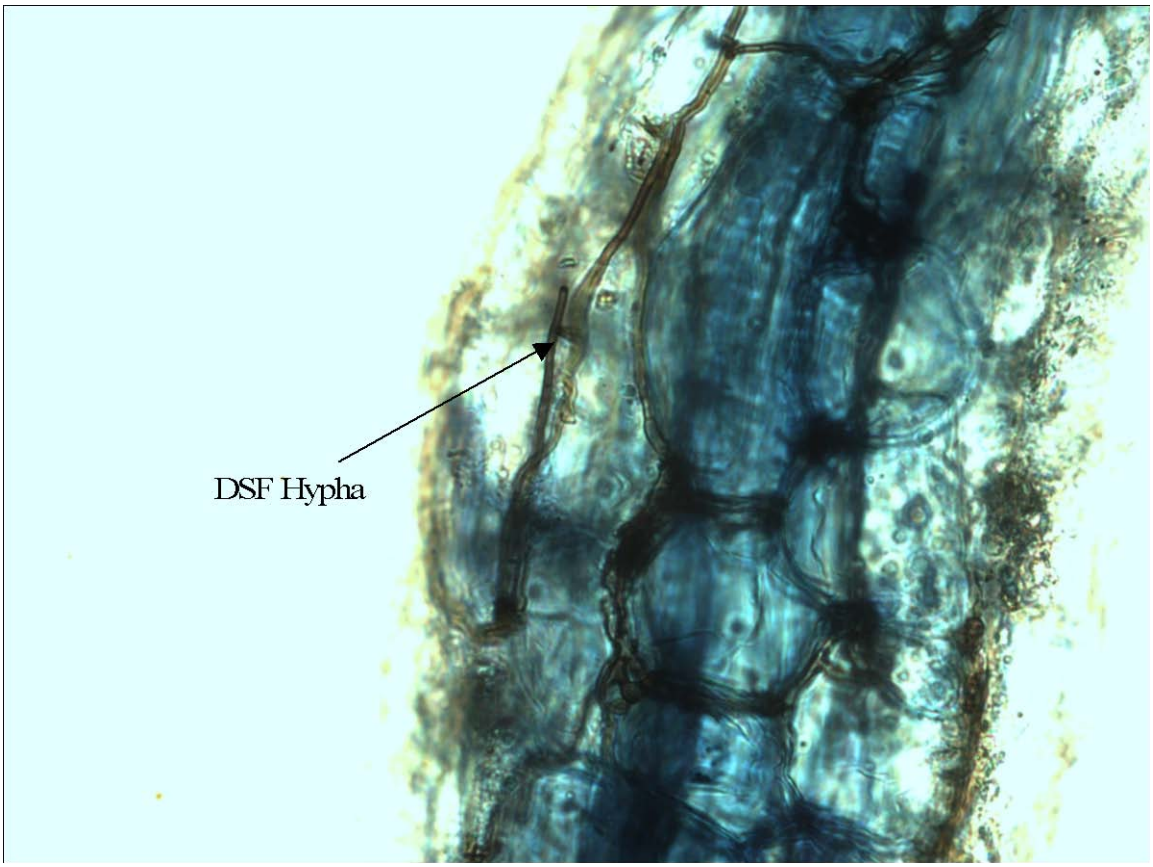

Figure 23. DSF hyphae coating fine root (lespedeza root, Trypan blue stain).

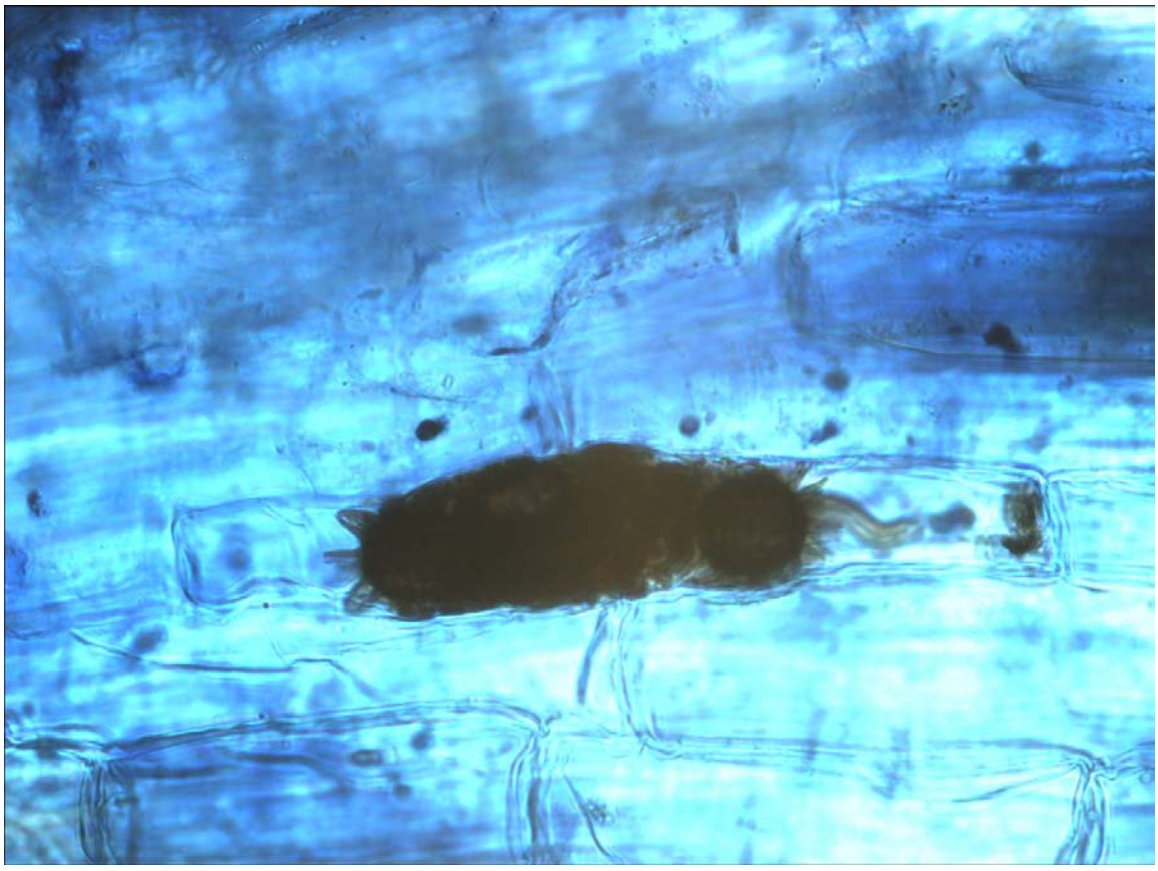

Figure 24. Formation of spiny, melanized structures within root cortex (switchgrass root, Trypan blue stain).

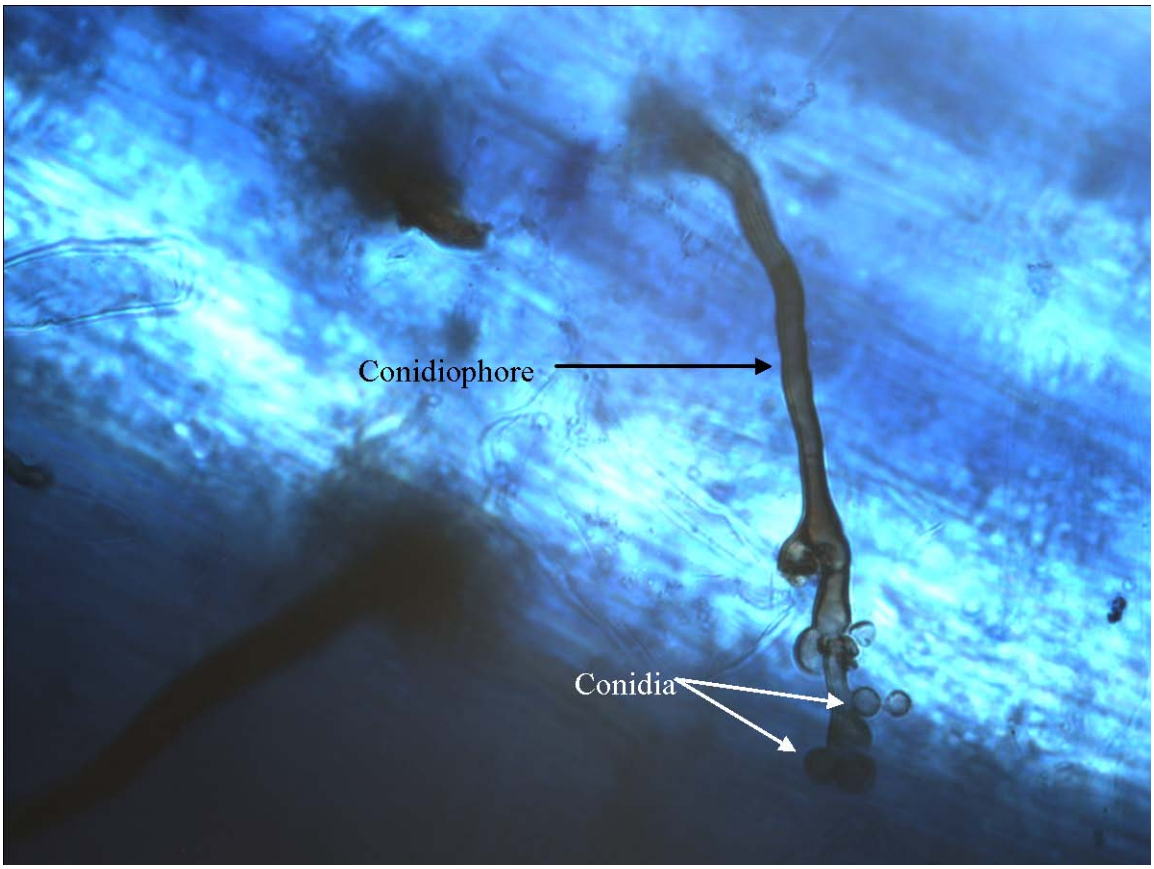

Figure 25. Detail of budding, melanized, septate conidiophore emerging from a root (switchgrass root, Trypan blue stain).

4. Discussion

Characterization of Fungal Communities

The fungal taxa encountered at the site (Appendix 3) were broadly consistent with those found in plants and soils by other researchers. Taxonomic diversity by Phylum was not unusual for root and soil fungal communities, with Ascomycota having the greatest number of fungal sequences followed by Basidiomycota, Glomeromycota, Chytridiomycota, and Zygomycota in decreasing order. Many abundant fungi belonged to genera that have been isolated from soils worldwide with cultivation methods, including *Mortierella*, *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* (Watanabe, 1994). Fungi widely isolated from the roots and/or leaves of live plants in Utah and Europe also included many species and genera found here: *Alternaria alternata*, *Epicoccum nigrum*, *Aureobasidium pullulans*, *Cladosporium* sp., *Aspergillu*s sp. (including *A. flavipes*), *Fusarium* sp. (including *F. oxysporum* and *F. solani*), *Preussia* sp., *Penicillium* sp., *Lachnum* sp., and *Leptosphaeria* sp. (Belnap and Phillips, 2001; Macia-Vicente et al., 2008; Marquez et al., 2008; Wilberforce et al., 2003). A molecular analysis of the roots and shoots from *Phragmites australis* in Germany (Neubert et al., 2006) yielded an ITS sequence profile with many similarities to the overall W&OD Trail results: dominance by a *Cladosporium* and an uncultured Ascomycete with minor species including

Aureobasidium pullulans, Stachybotrys elegans, Cryptococcus sp., *Glomus* sp., *Sebacina vermifera,* and an *Exophiala*. Seemingly distinctive fungi at the study site, excluding candidates from the large selection of environmental sequences, included *Leptosphaerulina* sp., *Myrmecridium* sp., putative *Kappamyces* sp., and a putative relative of *Magnaporthe grisea*.

Both lespedeza and switchgrass hosted a wide variety of fungi in their roots and soils but each plant was distinguished by a small number of species. Lespedeza roots were particularly associated with *Fusarium* sequences and *Aspergillus flavipes* (Table 9) and high abundances of OTUs 271.7, 314.4, 318.84, and 494.7, among others (Table 8). Switchgrass roots were distinguished by at least three uncultured environmental sequences putatively belonging to Ascomycete order Pleosporales (Table 9 and supporting text) and high abundances of OTUs including 255.12, 266.8, 354.87, and 365.7 (Table 9). Notable sub-dominant fungal sequences of lespedeza roots included *Aureobasidium pullulans, Bionectria ochroleuca, Leptosphaeria* sp., *Myrmecridium* sp., and "Unmatched Fungus H," whereas additional sequences from switchgrass roots included *Alternaria alternata, Epicoccum nigrum,* and Phaeosphaeriaceae sp.. Rhizosphere soils from both plants were similar but generally distinguished from roots by a limited selection of fungi including *Fusarium oxysporum* strain F-X.1.7 along with OTUs 223 and 246.74. Most fungal communities, regardless of sample type, had substantial abundances of *Cladosporium* sp. F40 and Uncultured Ascomycota clone 374 along with OTU 262.56.

Evidence of Fungal Community Shifts Following Lespedeza Invasion

The results of this study provide abundant evidence that invasion by Chinese lespedeza (*Lespedeza cuneata*) shifted both the root and soil fungal communities of switchgrass (*Panicum virgatum*) at the Washington and Old Dominion Trail site, with the extent of lespedeza cover (and hence progress of invasion) correlated with shifts in switchgrass fungal communities to a more "lespedeza-like" state.

The multivariate ordinations of the ARISA profiles through PCO showed that lespedeza density was indeed a primary driver of fungal community structure at the site, especially since there was an apparent threshold of approximately 40% lespedeza areal cover at which root and soil fungal communities of switchgrass would more-closely resemble those typical of lespedeza. Examining the root fungal PCO displayed on Figure 9, the three switchgrass root samples with "lespedeza-like" fungal communities came from quadrats with over 40% lespedeza areal cover, shown as yellow triangles and dark blue squares. Likewise, switchgrass soils from quadrats of over 40% lespedeza cover with fungal consortia typical of competing lespedeza appear as dark blue squares on scatterplot Figure 10. Conversely, two lespedeza roots with fungal communities similar to those of switchgrass roots (red diamonds in a cluster of mostly pale blue squares on Figure 9) were collected from quadrats of under 21% lespedeza areal cover intermixed with higher densities of switchgrass, whereas lespedeza soils from quadrats with less than

21% lespedeza cover were generally more similar to the soils associated with uninvaded switchgrass (red diamonds in intermediate position between main clusters of switchgrass and lespedeza samples on Figure 10). Overall, there was a positive relationship between the density of lespedeza and the likelihood that both resident lespedeza and switchgrass fungal communities exhibited “lespedeza-typical” structure. On the other hand, there was no apparent relationship between the density of switchgrass and fungal community status revealed by classing the data points in intervals of percent Switchgrass Cover (data not shown). This implied that changes in the density of lespedeza, but not necessarily that of switchgrass, were responsible for the observed shifts in fungal community structure.

Both the root and soil fungal community CCAs derived from the ARISA profiles (Figures 11-12) reflected the <21/>40% Lespedeza Cover thresholds for community shifts shown by the corresponding PCOs (Figures 9-10). Lespedeza root fungi from plots of less than 21% lespedeza clustered at the edge or center of the main switchgrass cluster (represented by blue squares) whereas both lespedeza and switchgrass samples from plots of over 40% lespedeza cover clustered near or within the lespedeza cluster. Similarly, both lespedeza and switchgrass soil fungal communities from plots of over 41% lespedeza cover grouped among the core lespedeza samples (Figure 12). Likewise, soil fungal communities from quadrats of 1-20% lespedeza cover seldom clustered among core switchgrass samples regardless of plant origin, indicating that any amount of exposure to lespedeza could potentially alter the fungal communities of switchgrass rhizosphere. The gradient of plant invasion, represented by the opposing biplot vectors of

Lespedeza Cover and Switchgrass Cover, was largely associated with Principal Axis 1 in both ARISA-CCAs, indicating that the spread of lespedeza at switchgrass' expense was most responsible for observed shifts in fungal community structures.

Environmental variables other than Lespedeza Cover and Switchgrass Cover had modest effects on the results of the ARISA-CCAs. Bare Ground Cover, Soil pH, and minor plant cover classes accentuated differences in fungal communities along the gradient of invasion in some cases (biplot vectors more parallel to Lespedeza-Switchgrass Cover) and otherwise altered fungi independently of lespedeza and switchgrass (biplot vectors perpendicular to Lespedeza-Switchgrass Cover). Moreover, with the exception of a virtual tie between Switchgrass Cover and Horseweed Cover in the ARISA-CCA of roots (Figure 11 and supporting text), Lespedeza Cover and Switchgrass Cover were consistently the most influential environmental variables based on combined Axis 1 and Axis 2 scores. Individually the others variables only scored higher for the Axis 2 scores, "pushing" fungal communities largely perpendicular to the gradient of invasion on the ordinations and thus influencing fungi in a manner of little consequence to testing the research hypotheses. Horseweed (*Conyza canadensis*) was no exception because the Horseweed Cover vector angled away from that of Lespedeza Cover in the root analysis, the result of an additive effect with lespedeza along Axis 1 being balanced by a modest opposing influence along Axis 2.

The fungal community subgroups from the pyrosequencing-based PCO in Table 10 roughly mirrored the clustering by host plant and sample type seen in the ARISA-based all-sample PCO (Figure 8), albeit the pyrosequencing dataset was far smaller and biased toward fungal communities from "mixed" plant communities at the invasion front. Therefore, it was surprising that the axes of the pyrosequencing-based PCO actually explained more of the variance than did those of the three ARISAs (Figures 8-10). The most likely reason for the greater proportion of variance explained in the pyrosequencing analysis was the identification of sequences by species and not just basepair lengths, which allowed amplicons of multiple sizes belonging to a single fungus to be merged in the dataset prior to analysis and allowed the differentiation of fungi that had produced equally-sized amplicons of varying sequence order (refer to Effects of Invasion by Chinese Lespedeza on Fungal Consortia for further discussion of technical issues with the community profiling methods). In short, the pyrosequencing data was better resolved by the dimension-reduction procedures of PCO than the ARISA profiles despite the smaller size of the sequenced dataset.

In agreement with the results of the ordinations discussed above, labeling the sample points of the pyrosequencing-based CCA by intervals of percent Lespedeza Cover (Figure 14**)** revealed a gradient of fungal community structures parallel to the Lespedeza Cover vector and corresponding with positions along the gradient of invasion, regardless of sample type and in spite of the other environmental variables. However, contrary to the ARISA-CCAs, Principal Axis 1 was associated with Soil pH and Bare Ground Cover

whereas Principal Axis 2 was associated with Lespedeza Cover and Switchgrass Cover. Therefore, environmental stress appeared to have the greatest influence on fungal community structure, with stress as greater soil acidity and exposed ground increasing to the right on Axis 1, whereas Axis 2 represented the comparatively modest effects of lespedeza invasion on fungal communities (greater lespedeza density being higher on Axis 2). This contrast with the ARISA results was likely due to the aforementioned data bias in the limited selection of samples analyzed by pyrosequencing; the 17 fungal communities of predominantly invasion front origin were affected by changes in Soil pH and Bare Ground Cover to a greater degree than the over 70 fungal assemblages with over 200 replicates from across the whole site that were analyzed by ARISA, so differences in fungal community structure appeared to more strongly-correlate with the stresses typifying the mixed-plant communities than changes in lespedeza density following invasion captured by the sampling transects. It was probable that the assignment of environmental variables to the first two principal axes would have been switched had a much larger, more-representative selection of fungal communities been sequenced.

While some fungal communities of lespedeza and switchgrass were comparatively similar in structure to those of the competing plant species based on the ordinations, the fungal consortia of any two samples were never identical and similarities were modest. Sample points that overlapped on a scatterplot could not be assumed to represent identical community structures where the principal axes explained a low

amount of variance, especially because only two or three axes could be plotted at a time. In other words, clusters of points on the scatterplot represented fungal communities similar to one another to a limited extent according to the principal axes on the plot. For instance, the root fungal communities from sympatric lespedeza and switchgrass plants in Transect 3 Quadrat 2 (T3Q2) on Figure 9, symbolized as blue squares and black triangles in the bottom-and-leftmost region of the scatterplot, respectively, possessed "very similar" fungal communities based on their ordination along Principal Axes 1 and 2. However, T3Q2 Lespedeza Root and T3Q2 Switchgrass Root shared 14 of 30 axes for a hypothetical community similarity of only 32.1%, the similarity between two communities being here-defined as the sum of the variation explained by all axes in which the average case scores of the communities fell within ±0.05 of each other (just over a half of a tick interval in the figure). Therefore, the PCO displayed small overall similarities between root fungal communities, the distinguishing pattern of fungal community structures on the 2D scatterplot labeled by plant community being the largest single "needle" of order in a "haystack" of randomness spread over all axes, with community similarities in the later axes offering diminishing returns in the variance explained.

The failure of the ordination methods to adequately resolve fungal community structures to a few principal axes meant that further analysis was required to determine the significance of observed differences between samples. Thankfully, the ecological resemblance analysis (Table 7) showed that invasion by Chinese lespedeza significantly

altered fungal communities and turned the switchgrass root and rhizosphere fungal communities to more “lespedeza-like” communities. As hypothesized, invasion altered soil fungal communities as supported by the non-equivalence of distances between unmixed stand and invasion front soils ($p < 0.01$; Lespedeza Soil: Switchgrass Soil greater than Mixed Lespedeza Soil: Mixed Switchgrass Soil). Additionally, as hypothesized, mixed switchgrass soil fungi more closely-resembled the soil fungi of lespedeza than those of “pure” switchgrass ($p < 0.01$; Mixed Switchgrass Soil: Switchgrass Soil greater than Mixed Switchgrass Soil: Lespedeza Soil). The same relationships held for the significant alteration of root fungal communities with invasion ($p < 0.02$; Lespedeza Root: Switchgrass Root greater than Mixed Lespedeza Root: Mixed Switchgrass Root) and the shift of invaded switchgrass root fungi to a more lespedeza-like state ($p < 0.05$; Mixed Switchgrass Root: Switchgrass Root greater than Mixed Switchgrass Root: Lespedeza Root). It was not obvious whether lespedeza invasion had greater impact on the fungal communities of switchgrass roots or those of switchgrass soils because the ranges of average Bray Curtis distances were nearly equal by community type (0.14 for root fungi and 0.13 for soil fungi).

Shifts in fungal community structure correlated with differences in overall plant community structure since the rank-orders of the fungal community distances (Table 7) followed a rough pattern that mirrored changes in the plant community along the gradient of invasion: lespedeza vs. mixed lespedeza (least different) < mixed lespedeza vs. mixed switchgrass < both mixed vs. switchgrass < lespedeza vs. switchgrass (most different).

The non-invaded switchgrass stand's greater ecological distance from other types of plant community ($p < 0.0001$) appeared to have been responsible for the underlying fungi being significantly more distant from all other fungal communities of roots ($p < 0.05$) and soils ($p < 0.01$). As such, the root and soil fungal communities of non-invaded switchgrass were structurally distinct from those of all invaded plots in terms of cumulative differences in fungal abundances, despite any overlap in composition. Conversely, lespedeza-dominated quadrats, having been most similar to mixed plant communities ($p < 0.0001$), supported less-distinct fungal assemblages by reason of the associated fungi having significantly lower distances from the other fungal communities of roots ($p < 0.001$) and soils ($p < 0.01$). Unfortunately, the specific effects of lespedeza cover on the distances between fungal communities could not be disentangled from the influence of the other components of the plant community because the invasive plant and competitor species contributed to all calculations of Bray Curtis distances between groups. However, as with overall plant community structure, the rank-order of the pairwise comparisons suggested that the degree of difference in lespedeza cover between samples mirrored the extent of differences in their fungal community structures.

Effects of Invasion by Chinese Lespedeza on Fungal Consortia

Invasion by Chinese lespedeza impacted the fungal communities of switchgrass by increasing the abundance of seven fungal OTUs associated with the invader while decreasing the abundance of six fungal OTUs of switchgrass (Table 8). Likewise, eight

and two fungal sequences had statistically-supported associations with lespedeza roots and switchgrass roots, respectively, and only one sequence with rhizosphere soils as a whole that nonetheless seemed to be associated with lespedeza (Table 9). Therefore, invasion impacted only about 10% of fungal species detected and those fungi associated with lespedeza were seemingly favored. Surprisingly, a greater number of root fungi were affected in the process than soil fungi and the most significant shift, lespedeza-associated OTU 271.7's expansion in switchgrass rhizospheres ($p < 0.05$), involved a predominantly root-associated fungus. As such, shifts in fungal community structure in the wake of invasion appear to have been driven by lespedeza's endophytes at the expense of switchgrass' own.

A similar number of fungi were responsible for widespread similarities between fungal assemblages as were responsible for differences. Only 13 OTUs (8.3%) were shared between fungal communities to a substantial extent without host-discrimination: amplicons (OTUs) 223, 240.25, 241.06, 249.5, 252.49, 267.7, 270.72, 277.6, 280, 281, 282, 289.44, and 295.77. The selection of sequenced fungi was far leaner – most similarity was due to ubiquitous *Cladosporium* sp. F40 and Uncultured Ascomycota clone 374, though Ascomycete sp. 5/97-81, *Alternaria alternata*, *Epicoccum nigrum*, and *Fusarium oxysporum* occurred in both plants frequently enough to reduce differences between many individual samples. The nearly 80% of remaining OTUs and sequenced fungi occurred seemingly randomly and infrequently, thereby failing to contribute to

patterned differences between samples that could be meaningfully resolved by ordination techniques.

Matching fungal OTUs by estimated amplicon length (ARISA data) to the corresponding sequences of known lengths (pyrosequencing data) allowed the hypothetical extrapolation of fungal typing to samples not yet sequenced and a better-understanding of samples that were, but technical issues kept the scope very limited and the conclusions tentative. One hurdle was the great redundancy in amplicon lengths shared by very different fungi (refer to Appendix 3 for a list of basepair lengths by fungus). The widespread but mostly lespedeza-associated OTU 262.56 was a pertinent case: many fungi yielded amplicons 261-263 basepairs in length, including *Kappamyces* sp. (a Chytrid), an uncultured *Mortierella* (a Zygomycete), and *Fusarium oxysporum* (an Ascomycete). As a result, the ARISA peak likely represented multiple species that co-occurred in many samples (data not shown), diminishing the ecological relevance of the OTU and preventing a one-to-one match between the two sets of molecular data. An unfortunate consequence was that *Mortierella* and *F. oxysporum* certainly contributed to OTU 262.56 and its association with lespedeza, but the extent of each fungal species' contribution was unknown and other fungi of equal amplicon-length that lacked real associations with lespedeza may have affected the results by reducing the calculated plant affinities and species-environment correlations. Another issue was that some fungi, such as *Alternaria alternata* and *Aureobasidium pullulans*, each yielded amplicons of various lengths that overlapped in size with those of other fungi (e.g. *Fusarium* sp. 5/97-45 and

A. alternata at 266 bases), further complicating the task. Third, the longest pyrosequencing read was 307 bases whereas nearly half of the significant OTUs listed on Table 8 were longer, meaning that a large proportion of OTUs could not be matched to sequences even had there been single correspondences between amplicon lengths and fungal species. Lastly, exact basepair length matches with sequenced amplicons were not found for some OTUs, like OTU 223, possibly because the software had committed "rounding errors" in merging the signals of fluorescent peaks spread within intervals of a few bases over different ARISA profiles or the sequence simply may not have been amplified. These problems with ARISA and pyrosequencing are characteristic of the techniques and have been encountered by other authors (e.g. Gillevet et al., 2009). Nonetheless, the fungal community fingerprints made using both methods presented similar outcomes to the invasion of the switchgrass field by lespedeza despite their imperfect complementation.

A collection of fungal sequences appeared to correspond with OTUs responsible for shifts in fungal communities. The most pertinent contributor to OTU 271.7 was likely lespedeza-associated *Bionectria ochroleuca* at 272 bp length, though *Cladosporium* sp. F40 and an uncultured Basidiomycete may have also played a role. As mentioned above, OTU 262.56 likely subsumed lespedeza-associated sequences for *Fusarium oxysporum* strain BD and *Mortierella* sp. with possible "interference" from a putative *Kappamyces*. Other members of *Fusarium* were probable matches with OTUs 264.9 and 265.9 in connection with the invader. Finally, both the amplicon size and response to invasion of

switchgrass-associated OTU 255.12 were in accord with the distribution of environmental fungus Contig2815_1306, though Ascomycete 5/97-81 probably contributed.

Ecological Implications: Potential Facilitation of Invasion by Endophytes

Most fungi encountered in the roots of switchgrass and Chinese lespedeza were also found in rhizosphere soils. Contamination of root samples by soil fungi was avoided by thoroughly washing fine roots of dirt followed by the repeated washing of ground roots to remove external fungi (Torzilli et al., 2006), ensuring that the root-associated fungi reported here were predominantly, if not exclusively, endophytes. Furthermore, care was taken to avoid saprotrophic fungi by harvesting living, fine roots with intact arbuscules, as verified microscopically after the time of harvest. Few abundant fungi occurred solely in rhizosphere samples (data not shown), so the contribution of non-endophytic species to any fungal community appears to have been minor. Thus hyphae and spores that issued from root systems provided most fungal DNA to the rhizosphere samples and the rhizosphere acted as a fungal propagule source for the roots of both switchgrass and lespedeza. The ecological significance is that most fungal species detected in this study could have impacted plant growth as endophytes, so any significant shifts in fungal community structure with invasion by lespedeza may have affected the competition between host plants or plant physiology. Furthermore, fungal propagules

deposited in the soils could have acted as a “seed bank” with the potential for affecting the plant community over the long term.

This study revealed several lines of evidence that residues from lespedeza and switchgrass were a possible cause of changes in fungal community structure. First, the correlations between lespedeza density (areal cover) and shifts in fungal community structures suggested that greater biomass and concomitant release of metabolites from the roots and/or leaf litter likely influenced the fungi which, in turn, may have affected the growth of neighboring plants. This is plausible since *Lespedeza cuneata* is already established as a source of allelopathic substances (Kalburtji and Mosjidis, 1993; Lindroth et al., 1986), allelochemicals in general can have antimicrobial properties (Callaway and Maron, 2006), and tannins in particular have known antifungal properties (Chung et al., 1998). Second, fungal taxa encountered in this study included genera with demonstrated effects on plants and their endophytes and may have served as a source of secondary metabolites. For instance, *Rhodotorula* yeast detected in switchgrass may have produced AMF-bioactive rhodotorulic acid in the rhizosphere or the plant growth-promoting indole-acetic acid (IAA) when present as an endophyte (Scervino et al., 2008; Xin et al., 2009). Likewise, fungal genera such as *Alternaria*, *Aspergillus,* and *Fusarium* include species known to produce diverse metabolites that affect both plants and other endophytes in a variety of ways, including the suppression of fungi (Suryanarayanan et al., 2009). Hypothetically, antifungal compounds produced by lespedeza-associated *Fusarium* could have suppressed *Rhodotorula* in the roots or soils of switchgrass,

whether released by invading hyphae or leached through the soil matrix, thereby harming the native grass through the loss of one or more growth-promoting metabolites. Lastly, the author found indirect evidence of volatiles being released by both dominant plants in the form of distinctive odors that may have belonged to chemicals with established fungal bioactivity (refer to Results: Soil Characteristics). Briefly, the chicory-like aromas of lespedeza roots and soils could have been due to alkenols with antifungal properties whereas the unpleasant odors of switchgrass roots and soils may have been due to indole or bioactive sulfurous compounds (Bais et al., 2003). However, the author has not found other sources that mention a foul odor associated with switchgrass, which may have been unique to the switchgrass population at the time of sample collection.

Alternative density-dependent mechanisms by which lespedeza could have influenced fungal communities included (1) shading, a trait particularly important to the competiveness of *Lespedeza cuneata* in the field (Allred et al., 2009; Brandon et al., 2004), (2) changes in organic substrates deposited in the soils, and (3) an expanding "myco-rhizosphere" connected with the root systems (Coleman, 2008). Shading may have enhanced fungal growth indirectly by helping the affected soils retain moisture during the drought, but this was doubtful since the return of precipitation during the latter part of the sampling period failed to have any discernable effect on the ARISA fingerprints (refer to Results: (D) Canonical Correspondence Analysis of ARISA Fingerprints). Furthermore, quadrats from the lespedeza-dominated stand and invasion front had significantly greater Bare Ground Cover than did non-invaded quadrats ($p <$

0.005; Table 4), so it was unlikely that Lespedeza Cover would have led to significantly greater soil moisture-retention when Switchgrass Cover was responsible for greater shading and direct vegetative cover. In contrast, organic litter deposition including the shedding of root tissues by the plants were likely to have favored the growth of some fungi over others through altering the available "food" sources to decomposers and even improving the physical properties of the soil (Walker et al., 2003; Wolfe and Klironomos, 2005). Lastly, mycorrhizae could have grown directly from expanding root systems as part of an expanding "myco-rhizosphere" where hyphae weaved through the soil matrix and greatly enhanced the effective root zone of the host plant (Coleman, 2008), possibly leading to hyphal connections between the roots of different plant species. Some plants could have benefitted more than others once connected to the growing mycorrhizal network (Van der Heijden, 2002) and "parasitic" interactions have been observed elsewhere (Carey et al., 2004; Marler et al., 1999).

Arbuscular-mycorrhizae were certainly important to the growth and competitiveness of lespedeza and switchgrass, but AMF may have found a superior partner in *Lespedeza cuneata*. There was a significantly greater presence of AMF vesicles in unmixed lespedeza roots than uninvaded switchgrass roots ($p < 0.005$; Table 10) but not between mixed-community roots of both plants. This likely indicated differences in host plant health, but not necessarily AMF composition, since vesicles are energy-storing organs widespread among AM species and the fungus receives its food as an investment from the host (Peterson et al., 2004). Mature stands of lespedeza may have better-

supported resident AMF as an indirect consequence of nitrogen-fixation augmenting plant growth and attendant photosynthesis, thereby promoting greater accumulation of fat-storing vesicles within the main lespedeza stand, whereas competition between plants and/or younger age of lespedeza at the invasion front may have reduced vesicle abundances by limiting the host plant's excess energy stores. On the other hand, both plants were highly mycorrhizal-dependent (Wilson and Hartnett, 1998), so "equitable" nutrient transfer through mycelial conduits may have occurred between otherwise competing lespedeza and switchgrass (Van der Heijden, 2002), thereby closing-the-gap in the vesicle numbers of plant roots through interspecies resource-sharing. Interestingly, evidence of a negative linear relationship between the frequencies of AM vesicles and DSF in mixed-community lespedeza roots but not switchgrass roots (refer to Results: Fungal Colonization of Roots) may have indicated that an AM-DSF interaction specific to the invader was responsible for the depressed vesicle levels at the invasion front. This could have been a case where lespedeza had devoted more energy to resident DSF at the expense of AM or outright antagonism between the fungi, albeit AMF levels remained high in the invader so any negative effects on the mycorrhizae must have been subtle.

Switchgrass roots were distinctive in terms of their ecological distance from all other fungal communities examined (Table 7), relatively low fungal diversity and species evenness (Table 6), high colonization by dark-septate endophytic fungi or DSF (Table 10), and resident fungi that included a large contribution from unidentified environmental sequences and uncultured Ascomycetes (data not shown). This suggested that *Panicum*

virgatum hosted a root endophyte community distinguished from that of *Lespedeza cuneata* in part due to an assortment of largely unstudied DSF in fungal assemblages dominated by relatively few species. Unfortunately, the fact that switchgrass roots had nearly twice as many intersections with DSF compared to lespedeza ($p < 0.005$; Table 10) did not allow the identification of OTUs and sequences with the DSF responsible since it was unknown how percent root colonization translated into relative abundances of DNA amplicons. *Periconia macrospinosa* was one of the few known DSF detected at the W&OD site in switchgrass but never frequently or in great abundance (Mandyam and Jumpponen, 2005).

DSF are common endophytes of grasses that often demonstrate beneficial growth-effects on their hosts and could one day be shown to function as mycorrhizae (Mandyam and Jumpponen, 2005). As a consequence, switchgrass may have benefitted from interspecies nutrient transfer through networks of dark-septate hyphae by mechanisms similar to AMF (Green et al., 2008). Additionally, exudation of secondary metabolites from DSF can modify the activity of co-occurring AMF (Scervino et al., 2009), so a greater abundance of DSF in switchgrass than lespedeza could have indicated that the equally-abundant mycorrhizae functioned differently depending on the host. Furthermore, the greater levels of DSF colonization in switchgrass could have meant that the fungi were vital to maintaining the dominance of grasses in the field. Note that while changes in plant community structure did not affect the magnitudes of root colonization by DSF at the invasion front (Table 10), individual DSF species may have been affected by

lespedeza invasion even though they could not be readily distinguished from one-another under the microscope.

Distinguished among endophytes for their destructiveness, plant-pathogenic fungi may have facilitated invasion by lespedeza through disproportionately harming switchgrass had the invader served as an effective vector. As noted earlier, *Alternaria alternata* and *Fusarium oxysporum* possess infectious strains but some can grow asymptomatically in plants (Macia-Vicente et al., 2008). Other potential disease fungi encountered in this study included yet more *Fusaria*, *Bionectria ochroleuca*, *Epicoccum nigrum*, and an 85% match with *Magnaporthe grisea*. While the effects of fungi on their hosts were not explored in this study for lack of growth experiments and detailed observations of plant health in the field, much of the switchgrass at the invasion front had looked dry and discolored in comparison to both neighboring lespedeza and switchgrass well-within the grass stand, so disease may have played a major role, especially given that switchgrass is robust and drought-tolerant (Baer et al., 2005; Keshwani and Cheng, 2009). Furthermore, few fungal diseases are known to affect Chinese lespedeza, namely *Erysiphe beta*, *Erysiphe glycines*, and *Uromyces lespedezae-procumbentis* (Zheng et al., 2004).

To the author's knowledge, no other study has focused on the effects of lespedeza invasion on fungi or any other microorganism. Although *L. cuneata*'s relationships with rhizobial bacteria and mycorrhizae have been a subject of agricultural research (e.g.

Wilson, 1988; Yao et al., 2002), the lack of microbial-ecological foci in studies of lespedeza invasiveness was surprising (e.g. Allred et al, 2009; Blair and Fleer, 2002; Sanders et al., 2007). In fact, all or most traits thought responsible for lespedeza's invasiveness could be indirectly controlled by symbiotic relationships by virtue of endophytes affecting their host's overall growth and survival. In the context of lespedeza incursions into native tallgrass prairie, previous studies suggested that the invader could exploit openings in grassland due to disturbances such as fire and mowing based on a suite of characteristics that made lespedeza a tough competitor with native species (Allred et al., 2009; Smith and Knapp, 2001). Changes in fungal communities accompanying the spread of lespedeza may facilitate invasion in the wake of a disturbance and thereafter solidify the invader's gains, whether in the prairie or in similar plant-communities like those along the W&OD Trail (Wilson and Hartnett, 1998). Alternative scenarios where fungi could have played a significant role in the invasion by *Lespedeza cuneata* include spread under low-light, low-competition conditions beneath pine canopies (Mays and Bengtson, 1985; Pitman, 2006) and in full-sunlight fields dominated by cool-season perennial grasses (personal observations in Northern VA region since 2005; VDCR, 1992). *Lespedeza cuneata* has been repeatedly shown as a tough and versatile colonizer of habitats within the continental United States and much of its success may have been facilitated by mycorrhizae and other fungal endophytes.

Considerations for Further Study

This study focused on one site in order to characterize changes in fungal community structure comprehensively over a small-scale, with plant areal cover used in lieu of individual plant counts and dry weight biomass as descriptors of plant community structure to be correlated with the underlying fungi. The main hurdle to the determination of significant shifts in the fungal communities was the relatively low availability of "mixed plant community" samples at the site compared to the number of "dominant or unmixed plant community" samples, a consequence of applying a transect-based sampling scheme to a field with a narrow invasion front. Since the author has observed that this situation is typical of well-defined lespedeza stands in the region, where it is rare to encounter stand boundaries of considerable width and substantial admixture with co-dominant plants, future studies may need to follow Batten *et al.* (2006) in collecting set numbers of samples from stand centers and edges. Additionally, the replication of the study over more sites and with other native species as competitors, especially within tallgrass prairie invaded by Chinese lespedeza, is necessary to verify that the observed shifts in fungal communities are typical influences of the invasive plant.

Regarding the choice of environmental variables, plant areal cover is easy to record at the time of sample collection and minimizes the amount of disturbance to the study site compared to the extensive harvest of vegetation in a quadrat, but plant biomass could serve as a better indicator of a species' performance, contribution to the plant

community, and impact on resident fungi. Likewise, soil pH is easy to determine and has well-defined effects on plant growth and physiology, but the inclusion of other soil variables such as organic matter content and nitrate levels would offer more opportunities to detect fungal species-environment correlations through CCA. Furthermore, measuring a greater variety of soil variables would allow the evaluation of plant species-environment correlations if, prior to CCA, soil characteristics were substituted for plant areal covers in the environmental dataset and plant areal covers converted to species data. The converse treatment of fungal abundances as environmental data affecting plant occurrence or soil characteristics would be difficult to justify without prior knowledge of significant interactions (e.g. known soil contamination with an aggressive root-rot fungus), thus necessitating the inclusion of more physical-chemical soil data to explore better-understood determinants of plant community structure. Thereafter the comparison of plant-fungal CCAs with corresponding soil-plant CCAs may reveal situations where underlying fungi structured the overlying plant communities. For instance, correlations between plant cover and a fungus in one CCA may indicate such a relationship if the fungus-correlated plant species has a very patchy distribution implying resource-limitation yet lacks the expected correlations with limiting soil variables in the other CCA. On the other hand, agreements in plant-fungal and soil-plant correlations may indicate functional relationships between putative mycorrhizae, soil nutrient limitation, and host plant distribution.

Less than 20% of the detected fungi contributed to fungal communities in a meaningful manner such that the ARISA and pyrosequencing-based ordinations of fungi (Figures 8-14) failed to explain the majority of variation between samples in the first few principal axes, unlike the ordinations of the smaller and less-complex data sets of plant community structures and root colonization tallies (Figures 4 and 15, respectively). Therefore, the easiest way to improve the effectiveness of the ordination techniques in a case such as this is to analyze a smaller selection of relevant species rather than increasing the number of samples (for all methods) or expanding the environmental data set (for CCA). Given the contribution of *Fusarium* to the fungal communities of *Lespedeza cuneata* and soils in general, molecular analysis of the translation elongation factor-1α (TEF-1α) region with sequence-comparison against the FUSARIUM-ID database would be an ideal future approach to the analysis of the Washington and Old Dominion Trail site (Geiser et al., 2004; Macia-Vicente et al., 2008). Similarly, the utilization of primers specific to AMF or Basidiomycetes would have greatly improved the resolution of the fungal communities. Nested-PCR, where a more-specific primer set is used to amplify certain DNA from an earlier PCR reaction (Martin and Rygiewicz, 2005), would be a reasonable approach for the analysis of either plant if the total fungal community structure were still of interest or the desired DNA were scarce in the environmental samples, as with the case here of known AMF. DSF would have to be targeted with custom-designed primers since the Ascomycetes as a whole were likely responsible for most of the unexplained variation between samples and the author is unaware of there being DSF-specific primers. An additional benefit of DNA-

fingerprinting a narrower selection of fungi is the greater likelihood of capturing all pertinent species. For instance, a fungal community of 70 species is unlikely to yield a total-fungal ARISA profile with even 30 OTUs if a few fungi dominate the community and a 1% relative abundance cutoff were used, yet all eight Basidiomycetes present could be readily detected using a more-specific primer set.

Future studies should better-integrate dark-septate endophytic fungi into research of mycorrhizal-dependency relationships between plants. The current study details a scenario where two mycorrhizal-dependent plants, one native and one an invasive exotic, had equally-high levels of AMF hyphal colonization but nearly 100% differences in the amount of DSF and the presence of AM vesicles, opening the possibility that DSF may have influenced mycorrhizal activity and/or competitive outcomes between the plants. It is possible that the differences in endophyte levels turned a situation that theoretically promoted coexistence between plant species via shared AMF (Van der Heijden, 2002) into a relationship that favored one plant through possibly through alteration of mycorrhizal activity (Scervino et al., 2009). Thus, our limited understanding of AMF-DSF interactions by host plant, habitat, and composing fungi may require a reworking of the mycorrhizal-dependency model once more-comprehensive data has been collected and analyzed. More growth-promotion and competition studies that focus on simple (controlled pot and greenhouse) and complex (wild) multi-species assemblages of endophytes are needed to evaluate plant responses, as are more cultivation and sequencing-based surveys of DSF to characterize the fungi involved and their activities.

The ultimate payback of such research would not only be a greater understanding of mechanisms of plant-invasiveness and factors regulating plant community structure, but the likely discovery of beneficial fungal consortia that may prove superior to the commercial mycorrhizal inocula now available to agriculture.

Appendix 1. Root Clearing-and-Staining Protocol

1. Place at least 0.2 g (wet weight) of well-cleaned, fine roots cut to approximately 1-cm lengths into tissue cassettes labeled by sample in permanent marker, being sure to keep the roots moist.

2. In a beaker, cover cassettes with 10% potassium hydroxide (KOH) and autoclave at 15 PSI for 10 minutes. 100ml of 10% KOH will cover several tissue cassettes.

3. Remove beakers containing solution and cassettes from autoclave and let cool on bench to allow handling.

4. In a clean beaker, wash cassettes with five changes of cool tap water for about 30 seconds per soaking to neutralize pH.

5. Cover cassettes with commercial 3% hydrogen peroxide solution mixed with four sprays of commercial glass cleaner with ammonia (to approx. 3% cleaner by volume) and let soak for 15 minutes or until roots are suitably pale.

6. Repeat Step 4.

7. Cover cassettes with 0.1 N hydrochloric acid (HCl) in a beaker or Pyrex petri dish for 3-4 minutes and pour-off acid (DO NOT RINSE ROOTS!).

8. Cover cassettes with 0.05% w/v Trypan blue stain in lacto-glycerin* and autoclave at 15 PSI for 12 minutes.

9. Repeat Step 3.

10. Destain roots by covering cassettes in simple lacto-glycerin (w/o stain) and swirl to remove excess blue coloration.

11. Remove roots from destaining solution within several days and store in 50% glycerin thereafter.

*Trypan blue powder dissolved in equal-volume ratios of lactic acid, glycerin, and deoinized water.

Appendix 2. DNA Extraction Protocol for Root Fungi

1. Add extra ceramic bead to each Lysing Matrix A tube (two beads total).
2. Add up to 200 mg finely ground roots to tubes.
3. Add 800 µl CLS-VF and 200 µl 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 600 µl of supernatant to 2 ml centrifuge vial.
7. Resuspend Binding Matrix and add 600 µl 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 100 µl of 65º C-warmed DES water and incubate at RT for 2 minutes.
16. Centrifuge for 1 minute at 14000 rpm to collect DNA extract in catch tube.

Appendix 3. Fungal Sequence Designations

Fungal Sequence Designation	Accession Number	Sequence Lengths (bp)[1]	Query Coverage (%)	Max Identity (%)	Number Samples N = 17
Alternaria alternata strain GrS8	FJ904919	266^, 281^	100	100	7
Ascomycete sp. 5/97-81	AJ279443	255^, 256	100	98	12
Ascomycete sp. A1	AJ279460	265	100	83	3
Aspergillus flavipes strain BH	GU566209	289	100	99	4
Aureobasidium pullulans	AF013229	295, 296^, 297	100	100	4
Bionectria ochroleuca strain G11	GU566253	272	100	98	7
Cladophialophora chaetospira strain CBS 514.63	EU035406	277	100	98	1
Cladosporium sp. F40	FJ755823	271, 272^	100	100	17
Dactylaria sp. P7	AY265338	285	86	88	1
Devriesia strelitziicola strain X1045	GU214635	259	98	92	1
Epicoccum nigrum strain GrS7	FJ904918	257^, 258	100	99	11
Exophiala sp. NH1238	AB488490	292	100	98	1
Fungal endophyte sp. ECD-2008 isolate 226	EU686118	255	99	96	1
Fungal sp. G17	GU566255	270	100	99	2
Fusarium oxysporum strain BD	GU566205	263	98	100	13
Fusarium oxysporum strain F-X.1.7	EU364863	264^, 267	98	100	10
Fusarium sp. 5/97-45	AJ279478	266	100	100	4
Glomus intraradices	AM980841	225	98	98	1

clone C14.1d_3					
Glomus luteum strain SA101, clone pMK020-6	FM876812	234	100	95	2
Glomus mycorrhizal symbiont of *Marchantia foliacea* Otira Valley sample, clone c3	AJ716311	219	99	94	1
Glomus proliferum specimen voucher MUCL41827, clone pHS117-IID14	FM992402	225	98	96	1
Kappamyces sp. ARG063	EF585664	261, 262^	94	83	3
Leptosphaeria sp. MH-2001	AJ317958	270	85	100	3
Cf. *Leptosphaerulina* sp. BOER-OM-1C clone JERPCR567	HQ026491	289	100	99	2
Lophiostoma sp. ICMP 18081	HM116744	261	100	96	1
Magnaporthe grisea 70-15 clone 61J04	DQ493955	250	98	89	3
Marasmius apatelius voucher NW437	EU935562	279	94	82	1
Myrmecridium sp. TMS-2011 voucher SC17d100p18-10	HQ631062	265	100	99	2
Nectria haematococca isolate N35	FJ903374	266	100	100	3
Unmatched Fungus[2] A – “Amylostereum areolatum”	NA	267	NA	NA	1
Unmatched Fungus B – “Articulospora tetracladia”	NA	284	NA	NA	1
Unmatched Fungus C – “Chytriomyces poculatus”	NA	293	NA	NA	1
Unmatched Fungus D – “Rhizopogon sp.”	NA	258	NA	NA	1
Unmatched Fungus E – “Tomentella sublilacina”	NA	290	NA	NA	2
Tremellales sp. LM630 (originally Unmatched	EF060914	225	100	85	1

Fungus F)					
Unmatched Fungus H – "Uncultured Glomus"	NA	274	NA	NA	6
Olpidium brassicae isolate GBR1	AY373011	249	100	99	1
Olpidium brassicae isolate GBR7	EU726300	219	100	95	2
Paecilomyces cateniannulatus strain: BCMU IF05	AB263742	282	98	99	1
Penicillium citrinum strain G53	GU566273	255	100	99	3
Penicillium sp. H17	GU566281	278, 285^	99	98	3
Periconia macrospinosa isolate 00023	AJ246159	265	98	99	1
Phaeosphaeriaceae sp. LM38	EF060422	271	98	98	4
Pleosporales sp. MH179	FM995624	285	74	98	1
Preussia sp. L34	FJ903358	257	100	98	2
Rhodotorula sp. JJP-2009a isolate 56853-10-123-UL-b	AJ317958	253	97	97	1
Sebacina vermifera isolate K251	EU626002	248	100	97	2
Stachybotrys elegans	AF081481	291	100	98	1
Uncultured ascomycete clone BF-OTU304	AM901918	274	98	97	2
Uncultured ascomycete clone C31_C12	EU490053	289	100	92	1
Uncultured ascomycete clone Mesq_A12	EU490121	269	98	100	2
Uncultured ascomycete isolate 1	DQ182434	297	99	96	1
Uncultured ascomycete isolate dfmo0726_131	AY970031	303	79	100	1
Uncultured Ascomycota clone 374	HM239958	292, 293, 296^	100	100	16
Uncultured Ascomycota clone 722	HM162159	242	100	97	1
Uncultured Ascomycota clone 985	HM162309	267	100	100	3

Uncultured Ascomycota clone 995	HM162316	255	98	100	1
Uncultured Agaricales clone G5c823H	GQ923993	262	84	97	2
Uncultured Basidiomycota isolate unk624	GU246996	272^, 273^	100	96	6
Uncultured *Ceratobasidium* clone B8-7	FM866376	252	100	94	1
Uncultured *Ceratobasidium*, clone B9-7	FM866384	261^, 263	99	99	1
Uncultured *Cryptococcus* clone LTSP_EUKA_P6H21	FJ554344	267	100	100	1
Uncultured ectomycorrhiza (Ceratobasidiaceae) 4099	AY634129	257	93	88	1
Uncultured endophytic fungus clone R2-51	FJ524314	294	100	94	1
Uncultured fungus (349-1936_2605)	FJ782358	266	90	99	2
Uncultured fungus clone 15	FJ386912	252	100	99	1
Uncultured fungus clone 3	AY702072	284	100	100	1
Uncultured fungus clone C9 37C	GU366746	235	100	89	2
Uncultured fungus clone Contig1-05-2815_1306	FJ756950	255	100	100	4
Uncultured fungus clone Contig125-128-1205_1844	FJ776142	254	100	100	2
Uncultured fungus clone Contig1369-45-2971_1605	FJ758318	258	100	96	1
Uncultured fungus clone Contig245-157-1205_0763	FJ776262	275	100	99	1
Uncultured fungus clone Contig476-107-1136_1549	FJ776493	267	100	98	1

Uncultured fungus clone Contig640-118-1183_0450	FJ776657	254	100	99	1
Uncultured fungus clone Contig782-37-2694_2147	FJ757731	255	100	99	2
Uncultured fungus clone Contig815-127-1145_1623	FJ776832	219	100	96	2
Uncultured fungus clone fTHITc29	GU721702	257	100	100	1
Uncultured fungus clone iG03_P_1_G5	FN397249	271	100	99	1
Uncultured fungus clone LMRF_73	GU078639	283	100	99	1
Uncultured fungus clone L042885-122-065-F12	GU054206	214	92	92	1
Uncultured fungus clone OTU#2868-44-3267_1024	GQ511117	256, 258^	100	98	2
Uncultured fungus clone OTU#3532-56-3623_1269	GQ511780	255	99	99	1
Uncultured fungus clone S288	FJ820775	291	94	93	1
Uncultured fungus clone Singleton_(128-1096_0772)	FJ779050	243	100	79	4
Uncultured fungus clone Singleton_(159-1139_0933)	FJ778470	267	100	92	2
Uncultured fungus clone Singleton_(159-1142_2884)	FJ779498	256	64	98	2
Uncultured fungus clone Singleton_(201-1699_0621)	FJ780083	262	96	100	1
Uncultured fungus clone Singleton_(214-1617_2974)	FJ780566	271	98	100	3
Uncultured fungus clone Singleton_(303-1838_2662)	FJ783246	266	96	99	1
Uncultured fungus clone	FJ759393	288	100	98	2

Singleton_37-2678_2491					
Uncultured fungus clone Singleton_38-2755_1230	FJ758739	213	100	99	3
Uncultured fungus clone Singleton_62-2994_2652	FJ761594	269	100	100	2
Uncultured fungus clone Unisequence#37-3587_2101	GQ519196	261	100	99	1
Uncultured fungus clone Unisequence#43-3211_1806	GQ525082	256	100	99	1
Uncultured fungus clone: IU-FSC Fun19_FuB186	AB520309	255	85	97	1
Uncultured fungus isolate BC063531-19	FM999594	278	100	90	1
Uncultured fungus isolate DGGE gel band F1	GU372937	253	98	98	3
Uncultured *Fusarium* sp.	FN689685	267	100	100	6
Uncultured Herpotrichiellaceae clone LTSP_EUKA_P6P13	FJ554453	292^, 297	100	98	1
Uncultured *Lachnum* isolate B35A_1	FJ378855	258	100	97	1
Uncultured *Mortierella* clone LTSP_EUKA_P2O07	FJ553286	262	100	100	8
Uncultured root-associated fungus clone R5M5c43P	EU144705	268	97	89	2
Uncultured soil fungus (clone 9a16)	DQ421267	270	100	98	2
Uncultured soil fungus clone 115-22	DQ421237	265, 274, 275^	100	99	2
Uncultured soil fungus clone 115-64	DQ420799	249	100	98	1
Uncultured soil fungus clone 130-31	DQ421155	258	100	98	1
Uncultured soil fungus clone 137-18	DQ421226	272	100	100	1
Uncultured soil fungus clone 137-19	DQ421058	299	100	99	2
Uncultured soil fungus	DQ421190	297	100	98	1

clone 137-31					
Uncultured soil fungus clone 138-33	DQ421246	251	100	93	2
Uncultured soil fungus clone 138-35	DQ420981	256	100	91	3
Uncultured soil fungus clone 2_L_C08	EU807366	259	92	97	2
Uncultured soil fungus clone 30-5	DQ421072	289, 297^	100	96	2
Uncultured soil fungus clone 53-31	DQ421238	264	100	94	3
Uncultured soil fungus clone 9a25	DQ421296	241	100	95	1
Uncultured soil fungus clone RS4M5c8P	EU480016	243	98	92	1
Uncultured soil fungus clone RS5M5c34P	EU479985	250	98	99	1
Volutella ciliata	AJ301967	273	98	88	1

[1]Sequence lengths in basepairs from NCBI BLAST-reevaluation of pyrosequencing results.
[2]"Unmatched Fungus" indicates that no sequence match at >70% similarity was found on NCBI nucleotide database.
^Sequence length(s) corresponding to similarity data where a fungus is matched to multiple sequences of different lengths.

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Curriculum Vitae

Robert Scott Andrews is a Northern Virginia native who graduated from Oakton High School, Vienna, VA in 2000 before graduating *Summa cum Laude* in Environmental Science from the Virginia Polytechnic Institute and State University (VA Tech) as part of the Class of 2004. He enrolled in the George Mason University Masters Program in Environmental Science and Policy in Spring 2005 after completing several non-degree courses the previous Fall. Robert currently lives in South Riding, VA with his wife Holly and son Joseph Scott. He plans to eventually obtain a Doctorate in Molecular Biology, Microbiology, or Ecology to further his research on the relationships between invasive plant communities and their associated microbial flora.