Influence of plant diversity and perennial plant identity on *Fusarium* communities in soil

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Chapter 1

Introduction

1.1 Fungi in soil

The biological diversity of soil is integral to the productivity of native and agricultural ecosystems. Among the different diverse taxonomic groups found in soil (p. 49 Wall et al., 2001), organisms in the Kingdom Fungi are unique in light of their evolutionary history. Based on a variety of data it has been hypothesized that the colonization of land by plants was dependent on a fungal partner, likely a close relative of the extant group of arbuscular mycorrhizal fungi (AMF) found in the phylum Glomeromycota. [1] While AMF in the Glomeromycota continue to play important and complex (Hoeksema et al., 2010) roles in soil, they represent a relatively well defined lifestyle limited to a narrow phylogenetic group of early diverging fungi. Ultimately, these obligate biotrophic fungi represent a small fraction of the diversity of lifestyles and fungal taxa found in soil. This is highlighted in a recent global sequence-based survey of fungal taxa in soil

[1] This hypothesis (Pirozynski and Malloch, 1975) is supported by: recovery of fossils resembling extant AMF in the fungal lineage Glomeromycota from an extinct plant dated from the Devonian period (Remy et al., 1994) as well as dolomite rock dated from the Ordovician period (Redecker et al., 2000), as well as more recent empirical evidence that extant glomeromycotan fungi can increase fitness of basal land plants (Humphreys et al., 2010). There is also evidence that this ancient symbiosis was not limited to the Glomeromycota, but was more widely distributed among basal phylogenetic lineages within the kingdom Fungi (Strullu-Derrien et al., 2014; Bidartondo et al., 2011).

(Tedersoo et al., 2014) which found that sequences from the Glomeromycota (i.e. AMF) represented only 0.2 percent of the data.

In contrast to the narrow phylogenetic distribution of the obligate biotrophic AMF, saprotrophic fungi can be found in most major phylogenetic lineages, even those dominated by different lifestyles (e.g. the lichenized Lecanaromycetes) (James et al., 2006). Fungi with saprotrophic lifestyles also predominate in soil. This was shown by Tedersoo et al. (2014) who found that globally 44 percent of the fungal taxa in soil were likely saprotrophs; note that 28 percent of fungal taxa had unknown lifestyles. This highlights the role of fungi in decomposition and soil nutrient cycling, but broadly labelling a fungus as a saprotroph overlooks the many other important functional roles these fungi may play.

1.2 *Fusarium* in soil

Of the different saprotrophic fungi commonly found in soil, few have recieved the amount of basic and applied research as those in the genus *Fusarium* (Summerell, 2001; Nelson et al., 1982). The most recent comprehensive genus-wide phylogeny suggests there are at least 17 to 20 species-complexes, with each species-complex comprised of 2 to 60 phylogenetically distinct species within *Fusarium* (Ma et al., 2013; O'Donnell et al., 2013). As a genus, *Fusarium* is ubiquitous in the environment, even extreme environments such as areas exposed to high levels of radiation contamination (Zhdanova et al., 2000) or low levels of oxygen (Cathrine and Raghukumar, 2009). Ultimately, soil serves as a major reservior for these fungi (Burgess, 1982; Balmas et al., 2010), where they may play both well characterized and poorly understood functional roles.

1.2.1 *Fusarium* as soilborne plant pathogens

Fungi in the genus *Fusarium* have been well studied as soilborne plant pathogens in agricultural ecosystems. Individuals in multiple different species-complexes within the

genus can cause disease in cultivated monocotyledonous and dicotyledonous plants. Diseases caused by these soilborne plant pathogenic fungi include root-rots, crown-rots, and vascular-wilts (e.g. Burgess et al., 2001; Elmer, 2001; Moore et al., 2001). Examples of specific diseases caused by these soilborne plant pathogenic fungi that are threatening major crops on multiple continents are Fusarium-wilt of banana (i.e. Panama Disease) caused by fungi in the *F. oxysporum* species-complex (Blomme et al., 2013) and sudden death syndrome (SDS) of soybean caused by multiple different phylogenetic species in the *F. solani* species-complex (Hartman et al., 2015).

Fungi in the genus *Fusarium* can also cause disease in native plants in native ecosystems. For example, Liu et al. (2012) found that seedling survival of the woody legume *Ormosia glaberrima* increased with greater distance from a full grown *O. glaberrima* plant. The most common fungus isolated from rotten seeds and seedlings of *O. glaberrima* found in the soil around the full grown tree was identified as belonging to the *F. oxysporum* species-complex. Pathogenicity assays using the *F. oxysporum* isolate showed it was a pathogen of *O. glaberrima* seedlings, but not other native plant species growing in the same plant community.

1.2.2 *Fusarium* as suppressors of plant disease

Though fungi in the genus *Fusarium* are important plant pathogens, many if not most of these fungi in soil are not pathogenic on plants they are associated with (i.e. closest to in soil or in the rhizosphere) (Demers et al., 2015; Edel et al., 1997; Nel et al., 2006). These nonpathogenic fungi may play many different functional roles in soil. One characterized role is the ability to reduce plant disease symptoms of vascular-wilt pathogens in the *F. oxysporum* species-complex. Soils displaying this naturally occuring phenomenon have been observed on multiple continents and are generally referred as Fusarium-wilt suppressive soils (Alabouvette, 1986; Larkin et al., 1993).

To understand the mechanisms of disease suppression in Fusarium-wilt suppressive soils, Larkin et al. (1996) characterized isolates of *F. oxysporum* cultured from soil

suppressive to Fusarium-wilt of melon caused by the soilborne pathogen *F. oxysporum* f. sp. *niveum* in Florida, USA. About one third of the cultured *F. oxysporum* isolates showed an ability to reduce plant disease caused by the pathogen. Physical separation (using a split root assay) of the pathogen and antagonistic isolates still resulted in disease suppression, even though the pathogen colonized the plant. This suggests disease suppression can resulted from induction of plant immune responses by the antagonistic isolates that were physically separated from the pathogen. Induction of plant immune responses is just is one of multiple mechanisms underlying Fusarium-wilt suppressive soils.

Competition between nonpathogenic and plant pathogenic individuals for growth limiting resources (e.g. carbon) in soil is another mechanism underlying Fusarium-wilt suppressive soils (Fravel et al., 2003). Couteaudier and Alabouvette (1990) measured growth and competitive ability for a set of *F. oxysporum* isolates in sterile soil microcosms (i.e. in the absence of any plants) to see if carbon use phenotypes explained an isolate's competitive ability. In glucose amended soil they measured an index of yield (fungal biomass/unit glucose consumed) for each isolate. They also measured an index of competition for each isolate, based on inhibition of an isolate with a selectable phenotype. With one slight exception, greater yield corresponded to a greater competition index. This highlights the importance of carbon use in determining competitive outcomes between these fungi in soil and its role in disease suppression.

1.2.3 Additional roles of *Fusarium* in soil

Fungi in the genus *Fusarium* can also influence decomposition and soil nutrient cycling. The high lignolytic capacity of these fungi is shown in a recent comparison of over 100 sequenced fungal genomes. Sequenced isolates of *F. oxysporum* and *F. solani* show the highest number of CAZymes, genes producing enzymes potentially involved in the breakdown of lignocellulose (Zhao et al., 2013). The ability of these fungi to breakdown and use lignocellulose has also been demonstrated based on in-vitro (King et al., 2011;

Li et al., 2008) measurement.

In addition to the expansion of genes associatied with lignocellulose breakdown, genomes of sequenced *Fusarium* isolates show a high number of genes predicted to produce secondary metabolites (Hansen et al., 2015). Many of these characterized metabolites have primarily been studied as mycotoxins. However, their demonstrated biological activities suggest they may play an important ecological function for the producing fungi. For example fusaric acid, which is produced by many different *Fusarium* taxa (Bacon et al., 1996; Brown et al., 2014), can suppress the production of phenazine-1-carboxamide (van Rij et al., 2005) and other antifungal compounds (Duffy and Défago, 1997) produced by common soil bacteria in the genus *Pseudomonas*. Fungi in the genus *Fusarium* can also produce secondary metabolites that are known plant hormones such as gibberellic acid and indole-acetic acid (Tsavkelova et al., 2012; Wiemann et al., 2013). The demonstrated biological activities of *Fusarium* produced secondary metabolites suggests these fungi may influence plant growth as well as the activity of other soil microorganisms.

From these poorly understood functional roles as secondary metabolite producers and decomposers, to their aforementioned well-studied roles as suppressors and promoters of plant disease, it is clear that fungi in the genus *Fusarium* are important components of the biological diversity found in soil. Despite this, very little is known about the ecology of these fungi in soil.

1.3 Research background and thesis structure

This research focuses on the influence of plant diversity and perennial plant identity on *Fusarium* communities in soil. The soil studied was sampled from the rhizosphere of perennial prairie plants maintained as monocultures or growing within diverse plant communities (i.e. polyculture). These perennial plant species and differences in plant

diversity are part of the Cedar Creek Long Term Ecological Research (LTER) experiment, described in Tilman et al. (1997), located in Minnesota, USA. Native perennial plants and changes in plant diversity of the surrounding plant community has implications for understanding and managing native prairie grassland ecosystems as well as agricultural ecosystems.

The current native North American prairie ecosystem is a small fraction compared to its historical predominance (Samson and Knopf, 1994; DeLuca and Zabinski, 2011). This prairie and other grassland ecosystems are important reservoirs of biological diversity (Werling et al., 2014; Glover et al., 2010*a*) that warrent conservation in light of the alarming rate of species extinction (Monastersky, 2014; Dirzo and Raven, 2003; Barnosky et al., 2011). Grasslands also serve as important carbon sinks in the context of global climate change (Beniston et al., 2014; DeLuca and Zabinski, 2011). Additionally, the well characterized relationship between grassland plant diversity and productivity (i.e. biomass production) (Tilman et al., 1997) has been suggested as a strategy to produce plant biomass on marginal lands while maintaining soil carbon due to the perennial nature of many prairie plant species (Tilman et al., 2006).

Focusing on perennial plants growing in contrasting levels of plant diversity has further implications for understanding and managing agricultural ecosystems. Many important cultivated plants are perennial and perennial cropping systems have been proposed as long-term, low-input alternatives to current annual cereal cropping systems (Glover et al., 2010*a*,*b*). Plant diversity is also an important component of agricultural ecosystems. Increased plant diversity is reflected in the use of polyculture cropping systems and the use of crop rotations. Increased plant diversity in agricultural ecosystems of one mangement strategy to reduce plant disease pressure (Abawi and Widmer, 2000) from soilborne plant pathogens and can also increase the concentration of carbon in soil (Tiemann et al., 2015).

Changes in *Fusarium* communities in soil may have many different effects, considering the diverse functional roles these fungi can play (discussed above). Accumulation

of host specific pathogens is known to limit crop yield in agricultural ecosystems, but may also act to maintain plant diversity in native ecosystems (Bever et al., 2012; Liu et al., 2012). The saprotrophic nature of these fungi also implicates them in decomposition and nutrient cycling in soil. As saprotrophic competitors for carbon or inducers of plant immune responses, these fungi may also suppress disease caused by soilborne plant pathogens. Furthermore, the ability to produce small molecules with a wide range of biological activities, suggest these fungi may have additional effects on plant growth and the acitivity of other soil microorganisms.

This thesis is structured as five chapters, this being the first. The four remaining chapters describe original research. Chapters Two and Three describe the development and use of targeted-metagenomic methods to characterize *Fusarium* communities in soil. Specifically, it was hypothesized that *Fusarium* diversity would be greater in soil of more diverse plant communities and *Fusarium* community structure would be influenced by both plant identity and plant diversity. Chapter Four focuses on carbon use of cultured *Fusarium* isolates from soil. Similarly, it was hypothesized that carbon use of these fungi would be influenced by both plant identity and plant diversity. Through further phylogenetic characterization of the isolates, this chapter also tested the relationship between phylogenetic distance and carbon use distance (i.e. testing if isolates within a phylogenetic clade show more similar carbon use phenotypes than isolates between clades). Chapter Five discusses the development and use of a secondary metabolism genotyping assay to screen *Fusarium* isolates for the genetic potential to produce different secondary metabolites. This assay was then used to to measure variation in the genetic potential to produce these biologically active compounds in cultured *Fusarium* isolates. Altogether this work provides a novel look at a diverse group of fungi, for which much is known, yet little regarding its ecology in soil.

Chapter 2

Soil fungal communities respond to grassland plant community richness and soil edaphics characteristics

2.1 Introduction

With 1.5 million extant species representing the lower estimate for total fungal diversity, much of the fungal Kingdom remains to be characterized (Blackwell, 2011; Hawksworth, 2001). Recent and ongoing coordinated efforts have delimited major phylogenetic lineages, yet the ecology of most fungi remains poorly understood (Schoch et al., 2009; Blackwell et al., 2006; James et al., 2006; Lutzoni et al., 2004). Soil is often cited as the environment harboring the greatest fungal diversity and analysis of grassland soils has revealed extraordinarily diverse communities with taxa spanning the Kingdom Fungi (Blackwell, 2011; Jumpponen, 2011; Jumpponen et al., 2010). Despite this diversity and the significance of fungi to terrestrial ecosystem processes (Horwath, 2007), factors

influencing the diversity, presence, and abundance of most soil-dwelling fungi are largely unknown. This can be attributed to the complex growth requirements of many fungi, making it difficult to census all fungi present in a soil sample.

The limitations to studying fungi in the environment by culturing have been partially overcome by sequencing amplicon libraries derived from environmental nucleic acids (i.e. targeted metagenomics). Metagenomic analyses have been used to uncover patterns in global fungal diversity (Amend et al., 2010*b*) and describe temporal dynamics in plant-associated fungal communities (Dumbrell et al., 2011; Jumpponen and Jones, 2010). Use of targeted metagenomics also has revealed large groups of novel fungi (Porter et al., 2008; Schadt et al., 2003) that were subsequently verified through culture-based studies and phylogenetics (Rosling et al., 2011). Despite these advances, there are major computational and biological limitations to sequence-based approaches to be overcome. With no morphological characters to observe, taxonomic assignment of sequence data is entirely dependent on the quality of available reference sequences. The poor quality of annotation (Nilsson et al., 2006; Buée et al., 2009) and sometimes-incorrect annotation of publicly available sequence data (Bridge et al., 2003) are often cited as major caveats to metagenomic studies.

This study had two primary objectives. The first was to test the effect of plant diversity and identity on the diversity and structure of fungal communities in soil. Specifically, it was hypothesized that fungal diversity would be greater in soil of more diverse plant communities and have a positive relationship with soil edaphic characteristics. It was also hypothesized that both plant diversity and plant identity would influence fungal community structure. The second objective was to test the use of a single copy protein-coding locus (*RPB2*) to characterize *Fusarium* communities in soil using sequence-dependent methods. Fungi in the genus *Fusarium* are well studied as plant pathogens in agricultural ecosystems (Goswami and Kistler, 2004; Michielse and Rep, 2009), but these fungi may also play important roles in decomposition of plant lignocellulose and carbon sequestration in soil (Murase et al., 2012; Rodriguez et al.,

1996).

To test these hypotheses and the ability to characterize *Fusarium* communities, this work focused on soil associated with the roots of two perennial legumes (*Lespedeza capitata* and *Lupinus perennis*) and two perennial C4 grasses (*Andropogon gerardii* and *Schizachyrium scoparium*) growing in monoculture or within diverse (i.e. polyculture) plant communities. Fungal and *Fusarium* communities were characterized by targeting both the multiple-copy rDNA locus *LSU* and the single-copy locus *RPB2*, respectively. Altogether, this work extends our understanding of fungal communities in soil and the link between plant and fungal communities in grassland ecosystems.

2.2 Methods

2.2.1 Field sampling

The Cedar Creek Ecosystem Science Center, an NSF Long Term Ecological Research site, is located in East-Central Minnesota. Soil cores were collected in 2009 from experiment E120 (`http://www.cedarcreek.umn.edu/research/experiments/e120.php`), which was established in 1994 (Tilman et al., 1997). Four different plant species were sampled growing in two levels of plant community richness, monoculture (stands of a single plant species) and polyculture (stands of 16 randomly selected native plant species from a pool of 18 (Tilman et al., 1997)). Soil was sampled from the base of two perennial C4 grasses, *A. gerardii* and *S. scoparium*, and two perennial legumes *L. capitata* and *L. perennis*. Each sample consisted of four bulked soil cores (5 cm diameter, 30 cm depth) taken from the base of the individual plants sampled. Soil cores were bulked and homogenized as recommended in Lindahl et al. (2013). To remove plant debris the soil was passed through a 2 mm mesh, prior to subsampling. The subsampled soil was used to measure soil edaphic characteristics and extract DNAs. The soil was also used to characterize *Streptomyces* communities, published elsewhere (Bakker et al., 2013).

The PowerSoil Kit (MO BIO) was used to extract DNAs by adding an initial

sonication step (10 min) and increased bead-beating (2 rounds) to the standard extraction protocol (Schlatter et al., 2010; Bakker et al., 2012). Soil organic matter (OM), extractable nitrogen (N), extractable potassium (K), and pH were measured at The Research Analytical Laboratory at the University of Minnesota (`http://soiltest.cfans.umn.edu/`). Organic matter was measured by loss of mass after drying at 360 °C for two hours. Nitrogen was measured using a LECO FP-528 Nitrogen Analyzer. Potassium was measured using a Perkin Elmer Analyst 100 spectrometer. Soil pH was measured in 1:1 soil/water mixture, with a Mettler Toledo Seven-Multi pH meter.

2.2.2 Amplicon library preparation

Amplicon libraries were created from environmental DNAs using two rounds of PCR. The initial PCR was used to create an amplicon library. The second PCR was used to tag the amplicons with 454 GS FLX+ specific adaptors and sample-specific barcodes. To create an amplicon library targeting the *LSU* locus, LROr (5'-ACCCGCTGAACTTAAGC-3') LR3 (5'-CCGTGTTTCAAGACGGG-3') (`http://www.biology.duke.edu/fungi/mycolab/primers.htm`) primers were used in both rounds of PCR.

To target the *RPB2* locus, the two rounds of PCR differed in the primers used. The first round used the previously published RPB2-5F2 and RPB2-7cR primers (O'Donnell et al., 2007). Due to the large size of the amplicons produced (1.2 kb), a second reverse primer was designed nested within the first amplicon for compatability with 454-sequencing technology. To design this reverse primer an alignment *RPB2* sequences from 73 different *Fusarium* spp. was used to predict the reverse multiplex primer RPB2-RP1 (5'-GGNGTCATGCARATCATNGC-3'). This reverse primer was used with RPB2-5F2 in the second round of PCR to produce amplicons of 800 bp.

All reactions were performed in an Eppendorf Mastercycler. For the *LSU* locus the first round of PCR used 20 µL reactions with 2 µL Ex-Taq buffer, 1.6 µL dNTPs (2.5

µM of each), 2 µL of each forward and reverse primers (10 µM), 11.3 µL water, 0.1 µL Ex-Taq polymerase (5 units μL^{-1}), and 1 µL template (10 ng DNA μL^{-1}). The reactions were performed with an initial denaturing step at 95 °C (5 min) followed by 24 cycles of 95 °C (1 min), 57 °C (1 min), 72 °C (1 min), and ended with an extension of 72 °C (10 min). Three reactions were performed per individual DNA sample and pooled to account for potential bias in PCR amplification (Lindahl et al., 2013). The pooled PCR reactions were cleaned using a QIAquick PCR purification kit (Qiagen) and used in the second round of PCR. The second round of PCR was performed using the same reaction ingredients except 0.4 µL of forward and reverse primers, 7.5 µL water, and 8 µL template (1 ng DNA μL^{-1}). The reactions were performed similarly, but with an annealing temperature of 69 °C and only twelve cycles (to reduce non-specific priming). Three PCR reactions per sample were pooled and gel extracted at 600 bp using a QIAquick Gel Extraction Kit (Qiagen).

Amplification of the *RPB2* locus followed similar methods with the following changes. The annealing temperature for the initial round of PCR was 60 °C, and 25 cycles were used for three replicate reactions. For the second round of PCR the annealing temperature was 65 °C and 20 cycles were performed for three replicate reactions per individual DNAs sample.

Sample DNAs were quantified using a DyNA Quant 200 fluorometer (Hoefer) and pooled in equal concentrations. Libraries were sequenced using the Roche 454 GS FLX+ platform at the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center. Demultiplexed raw sequence data can be found at the NCBI Sequence Read Archive accession number SRS600205.

For the *LSU* locus 17 samples were analyzed. Originally, 24 samples (3 replications for each plant species, diversity combination) were used, however seven samples failed to produce greater than 50 sequences. These low abundance samples were excluded from subsequent analyses. For the *RPB2* locus only 9 monoculture samples were analyzed with three plant replicates each from *S. scoparium*, *L. capitata*, and *L. perennis*.

2.2.3 Operational taxonomic unit construction from *LSU* sequence data

Generation of OTU data for the *LSU* locus was performed using mothur v.1.27.0 (Schloss et al., 2009) and followed steps outlined in Schloss et al. (2011) to minimize PCR and sequencing artifacts. The mothur implementation of the PyroNoise (Quince et al., 2009) algorithm was used to screen the pyrosequence flowgram files. This algorithm helps correct for sequence error associated with pyrosequencing technology, such as insertion/deletions. Following use of the PyroNoise algorithm, sequences were screened for the following criteria: presence of sample barcode sequence, presence of primer sequence, and a minimum length of 250 nucleotides (following removal of barcode and primer). Sequences failing to meet these criteria were culled and the sequences meeting these criteria were truncated to keep the first (5') 250 nucleotides. A set of unique sequences was generated and from it a random group (n=1000) was selected and used to create a template alignment using the program MUSCLE (Edgar, 2004). The template alignment was then used to align the unique sequence set in mothur. The preclustering and UCHIME algorithms (Edgar et al., 2011) were used to further reduce the effect of sequence and PCR error and sequences were then clustered into OTUs (0.97 identity) using the average neighbor algorithm.

To account for differences in sequence depth per sample of the *LSU* data, a random sub-sampling/rarefaction was performed based on the sample with the fewest sequences (n=400). These standardized *LSU* data were used to calculate fungal OTU richness as the number of non-singleton OTUs in a given sample. These data also were also used to analyze differences in fungal community structure between individual soil samples.

2.2.4 Taxonomic classification of pyrosequence data

A separate sequence processing pipeline was developed to assign a taxonomy to sequence data for both the *LSU* and *RPB2* loci. Raw sequences were initially

processed using mothur v.1.27.0 with the following criteria: a minimum length of 200 nucleotides, presence of sample barcode sequence, and an average quality score of 20. A set of 4030 *LSU* and 3319 *RPB2* reference sequences were compiled from data generated in the Assembling the Fungal Tree of Life project (http://aftol.org/), review of phylogenetic research, and mining sequenced fungal genomes available through the Broad Institute (`http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative`) and Joint Genome Institute (`http://genome.jgi.doe.gov/programs/fungi/index.jsf`). Taxonomic assignment to reference sequences was based on the NCBI Taxonomy derived using BioPerl modules (Stajich et al., 2002). Higher taxonomic classifications were derived from NCBI using the R package CHNOSZ (Dick, 2008). The BLAST+ programs (Altschul et al., 1997) were downloaded from the NCBI FTP site (`ftp://ftp.ncbi.nih.gov/blast/executables/blast+/2.2.25/`) and used to align sequences generated by pyrosequencing to the *LSU* and *RPB2* reference sequences. A cutoff of $\geq$400 bit score was used to guard against alignment of non-fungal sequences from the *LSU* locus to basal lineages within the Kingdom Fungi.

2.2.5 Statistics

All statistics were performed in R utilizing the base packages (R Development Core Team, 2012) plus the ggplot2 (Wickham, 2009), and vegan (Oksanen et al., 2013) packages.

Two-sided t-tests were used to test if individual soil edaphic characteristics were significantly different between soil of monoculture or polyculture plant communities. Two-sided t-tests were also used to test if fungal OTU richness (number of non-singleton OTUs) differed between soil of monoculture or polyculture plant communities. Pearson's correlation coefficient was calculated and used to test for a significant relationship between fungal OTU richness and individual soil edaphic characteristics.

To visualize differences in fungal community structure, Nonmmetric Multidimensional Scaling (NMDS) was performed with presence/absence OTU data (Jaccard distance) and OTU abundance data (Bray-Curtis distance). Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) (Anderson, 2001) was used to test for significant differences between fungal community structure in monoculture and polyculture soils as well as between grasses and legumes. Distance matrices were calculated from OTU presence/absence (Jaccard distance) as well as OTU abundance (Bray-Curtis distance) and the test was performed using the adonis function in the vegan package.

2.3 Results

2.3.1 Sequence output and taxonomic classification

Sequence output varied between the individual samples and for both sequenced loci. For OTU construction using the *LSU* locus sequence data, the total number of sequences after processing (see Methods: Operational taxonomic unit construction from *LSU* sequence data) was 90338. Per sample the average number of sequences was 5314 (σ=1519). The smallest sample had 400 sequences and the largest had 34406 sequences. Sequences from both loci were were assigned a taxonomy based on alignment to reference databases. For the *LSU* locus there was a total of 106844 sequences that fulfilled our criteria and passed our alignment threshold; sixty nine percent of the sequences that passed initial sequence processing aligned to reference sequences (see Section 2.2.4). Per sample the average number of sequences was 6285 (σ=9466) the smallest sample had 337 sequences and the largest had 34268 sequences. For alignment based taxonomic assignment for *RPB2* sequences a total of 23122 sequences fulfilled our criteria and passed our alignment threshold; eighty seven percent of the sequences that passed initial sequence processing aligned to reference sequences. Per sample the average number of sequences was 2569 (σ=1519) the smallest sample had 322 sequences and the largest

had 4586 sequences.

The reference databases compiled for taxonomic assignment of unknown sequence data covered two loci. The *LSU* locus database had broad taxonomic coverage within Fungi, with 4030 sequences representing 1446 genera within 129 orders. The database for the *RPB2* locus, known to have greater divergence among closely related fungi in the genus *Fusarium* than the *LSU* locus (O'Donnell et al., 2008), had 3318 sequences representing 805 genera within 103 orders.

Since the amplicon libraries for the *RPB2* locus were generated from the same extracted DNAs as the larger set of amplicon libraries for the *LSU* locus, it was possible to compare more generally how the taxonomic distribution differed between the two loci. As summarized in Table 2.1 it is clear that the *LSU* locus covered a much greater taxonomic breadth of Fungi than the *RPB2* locus, whereas the *RPB2* locus enriched for taxa in the Sordariomycetes. It should be noted that although the underlying reference databases differ between the loci, all the classes represented in Table 2.1 are in each reference database.

2.3.2 Soil edaphic characteristics

Soil organic matter, nitrogen, and potassium were significantly higher in polyculture than monoculture plant communities based on a two-sided t-test(Table 2.2).

2.3.3 Fungal OTU richness and community structure

Fungal OTU richness (number of non-singleton OTUs from *LSU* data) was greater in soil of polyculture plant communities compared to soil of monoculture plant communities based on a two-sided t-test ($t = -3.8295, P < 0.01$) (Fig. 2.1). Fungal OTU richness was positively correlated (Pearson) with soil potassium ($R^2 = 0.30, P < 0.05$). No other soil edaphic characteristic was significantly correlated with fungal OTU richness.

Fungal community structure was influenced by both plant diversity and plant identity. Fungal communities in soil of monoculture plant communities were differentiated

from fungal communities in soil of polyculture plant communities (Fig. 2.2). Fungal communities in soil associated with different plant families (grasses or legumes) grown in monoculture were differentiated from each other. However, fungal communities in soil associated with different plant families were not distinct when plants were grown in polyculture plant communities (Fig. 2.2). The ordination plots for OTU abundance and OTU presence/absence data showed similar patterns in ordination space (Figs. 2.2 A-B).

The clustering seen in ordination was used as a basis to test for differences between fungal communities associated with monocultures and polycultures as well as grasses and legumes. Based on OTU presence/absence data fungal communities were significantly different between soil of monoculture and polyculture plant communities (PERMANOVA, $P < 0.001$) as well as grasses and legumes (PERMANOVA, $P < 0.05$). Similar statistically significant results were obtained from OTU abundance data (data not shown).

2.3.4 *Fusarium* taxa present in soil samples

Fungi in the genus *Fusarium* were of particular interest, due to their documented diversity in soil (Balmas et al., 2010). Sequences aligning to references from this genus were found in every sample from both the *LSU* and *RPB2* locus. Across the *RPB2* samples 13 percent of the sequences aligned to *Fusarium* references. This compares with 4 percent of the sequences from the *LSU* locus that aligned to *Fusarium* references.

As shown in Fig. 2.3, sequences from the *RPB2* locus aligned to reference sequences from five different species-complexes *sensu* O'Donnell et al. (2013) in the genus *Fusarium*. Summed across the nine samples that were sequenced, 53 percent of the sequences aligned to references in the *F. tricinctum* species-complex followed by 45 percent which aligned to references in the *F. oxysporum* species-complex.

2.4 Discussion

This work characterized fungal communities in soil associated with the roots of perennial grasses and legumes growing as monocultures or within polyculture plant communities. Overall, the results support our initial hypotheses. Fungal diversity (i.e. OTU richness) was greater in soil of polyculture plant communities and had a positive relationship with soil potassium. Additionally, fungal community structure was influenced by both plant diversity and plant identity. However, the effect of plant identity on fungal community structure was only found when the different types of plants (legumes or grasses) were growing in monoculture, not in polyculture plant communities.

Previous work has often failed to find an effect of plant diversity on fungal diversity in soil (Waldrop et al., 2006; McGuire et al., 2012). However, the results reported here are in agreement with Peay et al. (2013) and Lange et al. (2015) who both found increasing plant diversity had a positive relationship with fungal diversity in soil. Interestingly, the relationship between plant and fungal diversity in soil is often restricted to certain taxonomic groups of fungi (Pellissier et al., 2014; Peay et al., 2013). This necessitates study of fungal diversity at other taxonomic hierarchies, such as common fungal genera, to determine how often plant diversity has a positive relationship with the diversity of fungi in soil.

The effect of plant family identity and diversity on fungal community structure in these grassland soils is similar to results from a global comparison of plant community structure (i.e. beta diversity) with fungal community structure in grassland soils. Prober et al. (2015) found that across globally distributed grassland soils plant community structure was predictive of fungal community structure in soil. Similar relationships between plant community structure and fungal community structure have been observed in both Alpine grassland soils and Amazonian forest soils (Pellissier et al., 2014; Peay et al., 2013). Altogether, these studies highlight the consistent link between plant community structure and the structure of fungal communities in soil.

The second objective of this work was to test the use of the *RPB2* locus to characterize *Fusarium* communities in soil. Based on taxonomic classification of the *RPB2* sequence data, our methods did not exclusively target the genus *Fusarium*. However, through exclusion of many fungal taxa (i.e. fungal taxa outside the class Sordariomycetes), it was possible to increase the percentage of *Fusarium* sequences. Additionally, the presence of sequences aligning to *Fusarium* references in all of the sequenced samples, regardless of the locus, highlights the ubiquity of these fungi in these grassland soils. Within the genus *Fusarium*, sequence data from the *RPB2* locus aligned to five different species-complexes. Future work will focus on characterizing the diversity within these different species-complexes as well as the effect of plant diversity and identity on the diversity and structure of *Fusarium* communities in soil.

There are multiple caveats to this work that are inherent to any sequence-based study. Two ongoing issues are the question of correlation of sequence abundance with a biologically relevant measurement (e.g. biomass) (Amend et al., 2010*a*) and the effect of sequencing error on estimates of diversity (Kunin et al., 2010). To address these issues we have used multiple methods. To address concerns of sequence error we used an algorithm that accounts and corrects for common errors associated with pyrosequencing. This method has been shown to give more accurate estimates of OTU richness in mock communities (Quince et al., 2009). To address the issue of sequence abundance we used abundance as well as presence/absence data with respect to fungal community structure. Results using the abundance and presence/absence data were in agreement. Additionally, abundance data from a single copy locus (e.g. *RPB2*) should be more representative of underlying taxon abundance than a locus that exhibits inter- and intraspecific variation in copy number such as rRNA genes (Herrera et al., 2009)

In summary, through sequence-dependent methods we have characterized grassland fungal communities in soil associated with different grassland plants growing within monoculture and polyculture plant communities. Fungal diversity was greater in soil of polyculture plant communities and increased with the concentration of soil potassium.

Fungal community structure was effected by differences in plant diversity and plant identity within monoculture plant communities. Furthermore, use of the *RPB2* locus will aid in future work focused on studying *Fusarium* communities in these same soils. Altogether, this work shows that fungal communities in grassland soil are tightly linked to plant community dynamics and as such need to be more explicitely integrated into our current understanding of these grassland ecosystems.

Table 2.1: Relative abundance of sequences (summed across individual sequenced samples) from the *LSU* and *RPB2* loci assigned to fungal classes. Taxonomic classification is based on alignment (BLASTN) of sequences from the two loci to custom reference databases (see Section 2.2.4).

Class	***LSU***	***RPB2***
Dothideomycetes	0.393	<0.001
Sordariomycetes	0.195	0.999
Leotiomycetes	0.160	<0.001
Eurotiomycetes	0.138	<0.001
Lecanoromycetes	0.047	0
Agaricomycetes	0.046	0
Chytridiomycetes	0.008	0
Orbiliomycetes	0.003	0
Tremellomycetes	0.002	0
Pezizomycetes	0.002	0
Blastocladiomycetes	0.001	0
Archaeorhizomycetes	0.001	0
Ustilaginomycetes	0.001	0
Geoglossomycetes	<0.001	0
Glomeromycetes	<0.001	0
Saccharomycetes	<0.001	0
Agaricostilbomycetes	<0.001	0
Paraglomeromycetes	<0.001	0
Exobasidiomycetes	<0.001	0
Taphrinomycetes	<0.001	0
Monoblepharidomycetes	<0.001	0

Table 2.2: Comparison of soil edaphic characteristics in soil of monoculture and polyculture plant communities using two-sided t-tests.

Edaphic characteristic	**Monoculture** [1]	**Polyculture** [2]	**P-value** [3]
pH	5.96 ± 0.213	6.11 ± 0.181	0.125
OM (%)	0.87 ± 0.071	1.31 ± 0.53	< 0.05
N (%)	0.03 ± 0.006	0.05 ± 0.031	< 0.05
K (ppm)	33.78 ± 5.869	59.13 ± 11.679	< 0.001

[1] Mean +/- standard deviation of soil edaphic characteristic in monoculture soil samples.

[2] Mean +/- standard deviation of soil edaphic characteristic in polyculture soil samples.

[3] P-value from two-sided t-test comparing soil edaphic characteristic in soil of monoculture and polyculture plant communities.

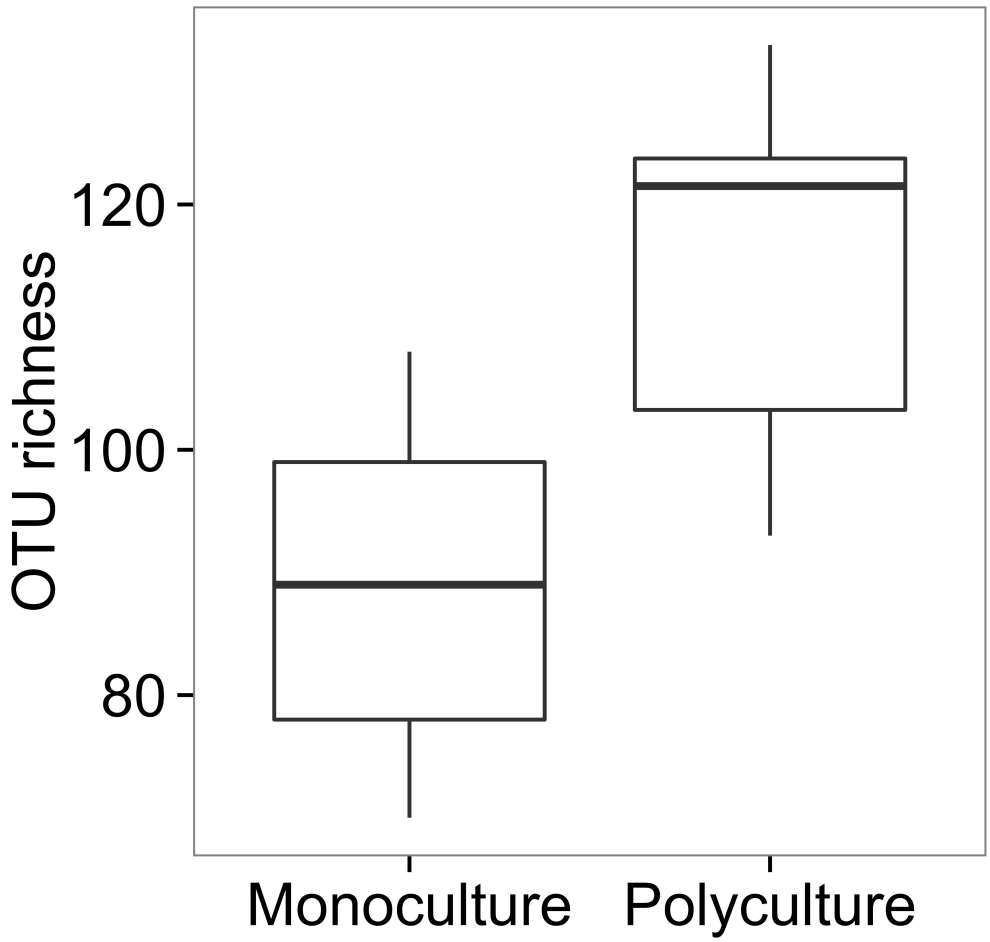

Figure 2.1: Box plot of fungal OTU (0.97 identity) richness in soil from monoculture and polyculture plant communities. Fungal OTU richness (the number of OTUs from *LSU* sequence data in a sample) is significantly greater in polyculture soil compared to monoculture soil based on a two-sided t-test ($t = -3.82, P < 0.01$).

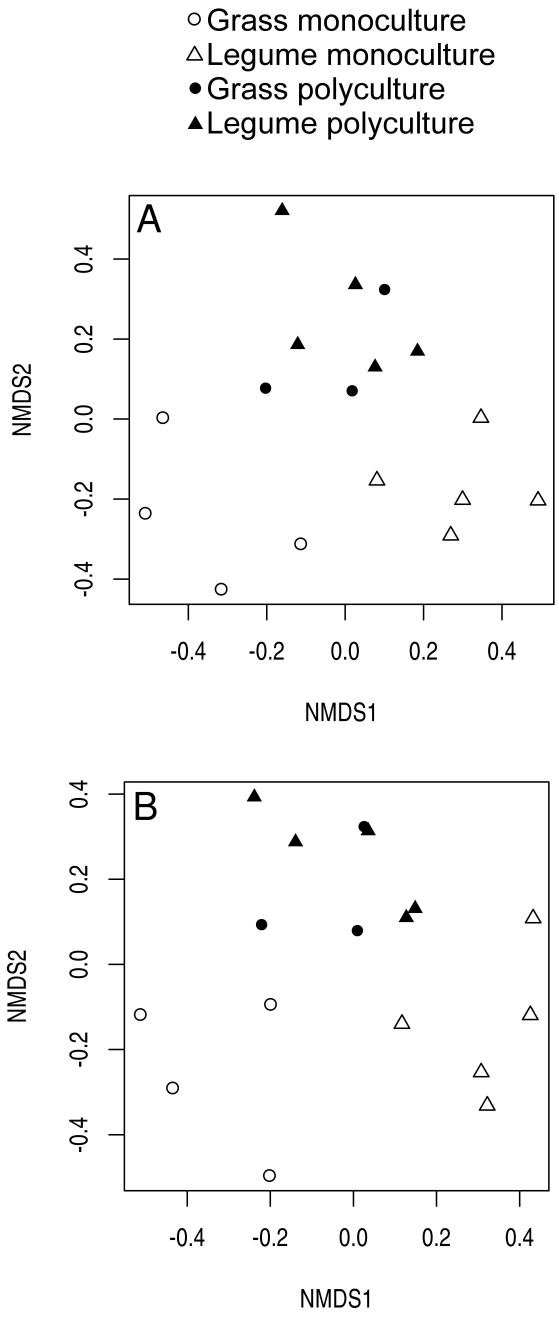

Figure 2.2: Nonmetric Multidimensional Scaling plots showing differences in fungal community structure between soil from monoculture and polyculture plant communities and differences between different plant families (grasses and legumes) in monoculture. The two plots represent results for OTU abundance (A) and OTU presence/absence (B) data.

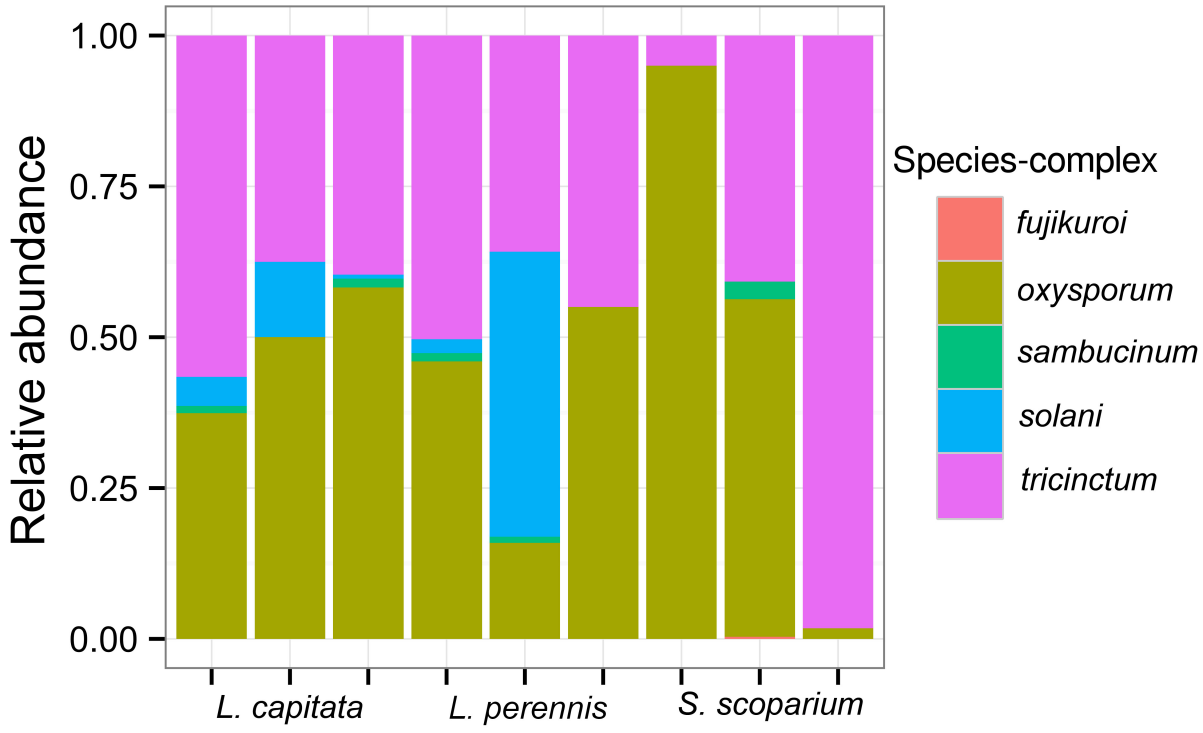

Figure 2.3: Relative abundance of sequences from the *RPB2* locus that aligned (BLASTN) to *Fusarium* references. Each column represents the classification of sequence data from individual soil samples. Samples represent soil associated with the roots of *L. capitata*, *L. perennis*, and *S. scoparium* growing in monoculture.

Chapter 3

Plant diversity and identity influence *Fusarium* community structure in soil

3.1 Introduction

Fungi in the genus *Fusarium* show high species and genotypic diversity in soil (Balmas et al., 2010; Gordon et al., 1992) where they play many important functional roles. Plant pathogenic fungi in this genus have been linked to both the maintenance (Liu et al., 2012) and decline of plant diversity (Mangla and Callaway, 2008) in native ecosystems. Through competition for limiting resources in soil, nonpathogenic individuals can suppress plant disease caused by plant pathogenic individuals (Alabouvette et al., 2009). The ability of these fungi to breakdown and use lignocellulose also suggests their growth will influence decomposition and soil nutrient cycling (King et al., 2011; Murase et al., 2012). Despite the diversity and functional importance of these fungi, the factors that influence the structure or diversity of *Fusarium* communities in soil are largely unknown.

Changes in the diversity or community structure of soil microbial communities in soil

may in turn influence the growth of individual plants and consequently plant community dynamics (Bever et al., 2012). For example, the displacement of native plant diversity by invasive plant species can occur as a result of microorganisms in soil. This displacement occurs with the accumulation of microorganisms in the rhizosphere of the invasive plant that suppress the growth of the native plants (Mangla and Callaway, 2008). Changes in the diversity or community structure of microorganisms in soil may also influence decomposition and soil nutrient dynamics. For example, the positive effect of plant diversity on soil carbon accumulation over time has been linked to the activity and diversity of soil microbial communities (Lange et al., 2015). Hence, understanding the effect of plant diversity and identity on the diversity and structure of soil microbial communities, such as communities of fungi in the genus *Fusarium*, may help to predict plant community and soil nutrient dynamics.

The barriers to studying the ecology of *Fusarium* communities in soil do not come from complex in-vitro growth requirements of these fungi, as is common with many microorganisms in soil. In fact there is a long history of culturing and characterization of *Fusarium* taxa from soil, which has been important for the description of novel diversity and biogeographical distributions within the genus (Balmas et al., 2010; Laurence et al., 2011; Backhouse et al., 2001). Culture-based surveys of *Fusarium* suggest that some taxa are cosmopolitan with a global distribution, whereas others are endemic to particular continents or climatic regions (Backhouse et al., 2001; Sangalang et al., 1995).

Beyond the recognition that fungi in the genus *Fusarium* can be recovered from a wide variety of soils, studies to understand what influences the diversity and structure of *Fusarium* communities have been inconclusive. For example, Yergeau et al. (2010) characterized *Fusarium* communities in soil 1 meter from asparagus plants with or without symptoms of Fusarium crown-rot. Using denaturing gradient gel electrophoresis targeting the genus *Fusarium*, it was found that there was no effect of plant disease on *Fusarium* community structure. Other culture-based surveys using morphological species definitions found that the isolation frequency of particular *Fusarium* species

correlated with soil edaphic characteristics such as the concentration of organic matter or potassium (Bissett and Parkinson, 1979; Windels and Kommedahl, 1974). Though these latter studies did not explicitly measure *Fusarium* community structure they provide some evidence that changes in soil edaphic characteristics can influence the composition of *Fusarium* communities in soil.

Sequencing amplicon libraries generated from environmental DNAs can be an efficient method to characterize fungal communities in complex environments like soil. Though these methods usually characterize diversity at the Kingdom level by targeting multiple-copy rDNA loci (e.g. internal transcribed spacers or 28S rRNA), they can also be applied to study lower taxonomic hierarchies (e.g. Davey et al., 2013). Previously, it was shown that it is possible to amplify and sequence a portion of the single-copy *RPB2* locus (second largest subunit of the RNA polymerase) from soil DNAs (Ch. 2). Additionally, through primer specific, PCR-based exclusion of a large portion of the fungal kingdom it was possible to enrich for sequences within the Sordariomycetes and consequently the genus *Fusarium*. The *RPB2* locus has been used to study evolution within *Fusarium* (O'Donnell et al., 2013) and for genotyping clinical isolates (O'Donnell et al., 2007) making it a good candidate to characterize communities of these fungi in soil.

The purpose of this research was to characterize *Fusarium* communities in soil to address three specific hypotheses. First, based on previous sequence-dependent characterization of the *Fusarium* taxa in soil (Ch. 2), it was expected that the most common taxa would be the *F. tricinctum* and *F. oxysporum* species-complexes. Second, as increased plant diversity can have a positive effect on fungal diversity in soil (Lange et al., 2015; Peay et al., 2013, Ch. 2), it was hypothesized that *Fusarium* diversity would be greater in soil of more diverse plant communities. Finally, based on the previously observed effect of plant diversity and plant identity on fungal community structure (Ch. 2), it was hypothesized that plant diversity and plant identity would influence *Fusarium* community structure. To address these hypotheses, this work characterized *Fusarium*

communities in soil associated with the roots of a native perennial grass and a native perennial legume. By characterizing *Fusarium* communities associated with these two plant species maintained as monocultures or growing within diverse (i.e. polyculture) grassland plant communities, this work provides insight into the ecology of a diverse group of fungi found in soil.

3.2 Materials and methods

3.2.1 Field sampling and soil processing

Samples were collected from the Cedar Creek Long Term Ecological Research site in Minnesota, USA in late July, 2012. The samples represented soil associated with the roots of two different perennial grassland plant species grown within two different plant diversity treatments. The plant species were the C4 grass *Andropogon gerardii* (common name: big bluestem, turkey's foot) and the legume *Lespedeza capitata* (common name: round headed bush-clover). Plant community richness treatments were monoculture of each target plant species and polyculture plant communities representing 16 grassland plant species, including the target plant species. Twelve individual plants were sampled, six from each plant species, three from monoculture and three from polyculture plant communities.

To sample rhizosphere soil, 2-2.5 $\times$ 10 cm soil cores, separated by a ninety-degree angle around the base of the plant, were sampled directly from the base of each plant. The two soil cores per individual plant were pooled in the field and kept on ice until storage at -20 °C. The soil samples were passed through a 2 mm sieve to remove large plant debris. Extractable phosphorus, extractable potassium, extractable nitrate, pH, and percent organic matter of the soil were measured at the University of Minnesota Research Analytical Laboratory (`ral.cfans.umn.edu`).

Soil DNA extraction order was randomized. Extractions were optimized for the final adjustments to the PowerSoil (MoBio) protocol: 0.5 g of soil was used, the soil was

disrupted using 2, 1- minute pulses in a Mini-BeadBeater 8 (BioSpec) on the highest setting, and 50 µL Tris was used for the final spin step. Extracted DNAs were diluted to 5 ng DNA/µL and stored at -20 °C.

3.2.2 Amplicon library preparation

Amplicon libraries were created from extracted DNAs using nested PCR. All reactions were performed in an Eppendorf Mastercycler. The initial PCR was used to create an amplicon library from the soil DNAs using the RPB2-5F2 (5'-GGGGWGAYC AGAAGAAGGC-3') and RPB2-7cR (5'-CCCATRGCTTGYTTRCCCAT-3') primers (O'Donnell et al., 2007). The second PCR was used to tag the amplicons with 454 GS FLX+ specific adaptors and sample-specific barcodes as well as to reduce the size of the amplicons from 1.2 kb to 0.8 kb for compatibility with 454 sequencing technology. For the second round of PCR the RPB2-5F2 primer was paired with the reverse primer RPB2-RP1 (5'-GGNGTCATGCARATCATNGC-3') which is nested within the 1.2 kb amplicon from the initial PCR.

The first round of PCR used 20 µL reactions with 2 µL Takara Ex-Taq buffer, 1.6 µL dNTPs (2.5 µM of each), 2 µL of each forward and reverse primers (10 µM), 11.3 µL water, 0.1 µL Takara Ex-Taq polymerase (5 units/µL), and 1 µL template (5 ng DNA/µL). The reactions were performed with an initial denaturing step at 95 °C (5 min) followed by 24 cycles of 95 °C (1 min), 60 °C (1 min), 72 °C (1 min), and ended with an extension of 72 °C (10 min). Three reactions were performed per sample and gel extracted at 1.2 kb using a Promega Wizard kit (Promega). The second round of PCR was performed using the same reaction ingredients except 0.4 µL of forward and reverse primers, 7.5 µL water, and 8 µL template (1 ng DNA/µL). The reactions were performed similarly, but with an annealing temperature of 65 °C. Three reactions per sample were pooled and gel extracted at 0.8 kb using a Promega Wizard kit (Promega). The amplicon DNAs were quantified using a DyNA Quant 200 fluorometer (Hoefer) and pooled in equal sample concentrations. The amplicon libraries were sequenced using the

Roche 454 GS FLX+ platform through the Roy J. Carver Biotechnology Center at the University of Illinois (`http://www.biotech.uiuc.edu/`). Demultiplexed raw sequence data is archived in the NCBI Sequence Read Archive accession SRX894060.

3.2.3 Sequence processing

Sequences were processed in mothur v.1.27.0 (Schloss et al., 2009). The mothur implementation of the PyroNoise algorithm (Quince et al., 2009) was used to screen for and correct sequencing errors common to the pyrosequencing technology, such as insertion/deletions. Following use of the PyroNoise algorithm, sequences were then clustered into operational taxonomic units (OTUs) following the recommendations highlighted in Schloss et al. (2011). Sequences were screened for the presence of sample barcodes, sequencing primer, and a minimum length of 400 nucleotides; sequences not fulfilling these criteria were culled. Following barcode and primer removal the sequences were truncated to the first 400 (5') nucleotides. From this complete sequence set a random subset (n=1000) was aligned in MUSCLE (Edgar, 2004), which was then used as a template alignment to align the complete sequence set in mothur. To further reduce the effect of sequencing and PCR error, sequences were preclustered and screened for chimeras using the UCHIME algorithm in mothur (Edgar et al., 2011). Following removal of chimeras, sequences were clustered into OTUs (0.97) using the average neighbor algorithm. To account for differences in sequencing depth per sample the final OTU table was subsampled/rarefied based on the sample with the fewest sequences (n=1082). Operational taxonomic units represented by a single sequence prior to subsampling were not considered in downstream analysis.

3.2.4 Taxonomic classification of *Fusarium* OTUs

Representative sequences from each OTU were used for phylogenetic classification. First the sequences were aligned to a custom database of reference *RPB2* sequences (Ch.

2) using the default parameters of the BLASTN algorithm (Altschul et al., 1997). Sequences that showed a minimum of 0.90 identity with reference sequences from *Fusarium sensu* O'Donnell et al. (2013) were further classified using the Evolutionary Placement Algorithm (EPA) (Berger et al., 2011).

The EPA enables classification of short sequences (e.g. sequences representing OTUs) within a reference phylogeny. Use of the EPA requires both a reference phylogeny and the underlying sequence alignment used to create the phylogeny. This work used data from a recent *Fusarium* genus-wide reference phylogeny constructed from *RPB1* and *RPB2* sequence data (O'Donnell et al., 2013). The *Fusarium* OTU sequences were aligned to each other and then the reference alignment from O'Donnell et al. (2013) using MUSCLE (Edgar, 2004). The EPA was used through a web server at `http://epa.h-its.org/raxml`.

Sequences classified as *F. beomiforme* using the EPA were further classified using *RPB2* reference sequences from closely related *Fusarium* taxa. The OTU sequences were aligned with the following accessions from the NCBI nucleotide database: HQ646389, HQ646391-HQ646397, and HQ662683-HQ662689 (Laurence et al., 2011). A neighbor joining tree based on Jukes-Cantor distance with 100 bootstraps was constructed in MEGA6 (Tamura et al., 2013).

Phylogenies were plotted using Archaeopteryx (Han and Zmasek, 2009).

3.2.5 Statistics

All statistical analyses were performed in R (R Development Core Team, 2012) using the vegan (Oksanen et al., 2013), ggplot2 (Wickham, 2009), and base packages.

Pearson's correlation coefficient was calculated and used to test for a relationship between *Fusarium* OTU (number of non-singleton OTUs) and species (number of unique classifications within a reference phylogeny using the EPA) richness with individual soil edaphic characteristics.

Two-sided t-tests were used to test for an effect of plant diversity (i.e. monoculture

versus polyculture) on individual soil edaphic characteristics as well as *Fusarium* OTU and species richness. One-way Analysis of Variance (ANOVA) was used to test for an effect of the four plant treatments (each plant species growing in monoculture or polyculture plant communities) on individual soil edaphic characteristics as well as *Fusarium* OTU and species richness. Where the one-sided ANOVA gave a significant result ($\alpha < 0.05$), Tukey Honest Significant Differences (TukeyHSD) post-hoc test was used to determine differences between the four plant treatments.

Using the R package vegan, Nonmetric Multidimensional Scaling (NMDS) was used to visualize differences in *Fusarium* community structure between individual soil samples. Both *Fusarium* OTU abundance data (Bray-Curtis distance) and OTU presence/absence data (Jaccard distance) were used in NMDS analyses.

3.3 Results

3.3.1 Soil edaphic characteristics

Across plant species, soil in polyculture plant communities had greater phosphorus ($t = -3.0225, P < 0.05$) and organic matter ($t = -3.3499, P < 0.01$) than soil from monoculture plant communities. Across plant richness treatments, soil from the legume *L. capitata* had greater nitrogen than soil from the grass *A. gerardii* ($t = -2.756, P < 0.05$).

Soil edaphic characteristics also varied between the four different plant treatments (each plant species growing in monoculture or polyculture plant communities). Based on one-way ANOVA there was a significant effect of the different plant treatments on organic matter (Table 3.2; $F = 5.125, P < 0.05$) and phosphorus (Table 3.3; $F = 4.471, P < 0.05$). Post-hoc (TukeyHSD) comparisons of the four plant treatments showed organic matter and phosphorus were greater in soil from *A. gerardii* growing in polyculture than soil from *A. gerardii* growing in monoculture (TukeyHSD, $P < 0.05$).

3.3.2 Sequence output and processing

After sequence processing, sequence numbers per sample (total of 12 samples, 3 from each plant species growing in monoculture or polyculture plant communities) ranged from 1082 to 2350, with a mean of 1927 ($\sigma = 414$). The processed *RPB2* sequences clustered into 497 OTUs (0.97 identity). Sequences representing 89 percent of the OTUs aligned to reference *RPB2* sequences in the class Sordariomycetes. Within the Sordariomycetes, sequence data representing 72 OTUs aligned to reference sequences in the genus *Fusarium*. Only the *Fusarium* OTUs were included in subsequent analyses.

3.3.3 Phylogenetic characterization of *Fusarium* soil communities

Using the EPA (Berger et al., 2011), the 72 *Fusarium* OTUs were classified within a genus-wide reference phylogeny generated with data from O'Donnell et al. (2013). As shown in Table 3.1 and Fig. 3.1, the OTUs were classified as 19 unique positions within the reference phylogeny (these unique classifications served as a measurement of "species" richness below). The OTUs were classified in the following species or species-complexes *sensu* (O'Donnell et al., 2013): *F. sambucinum*, *F. incarnatum-equisiti*, *F. tricinctum*, *F. fujikuroi*, *F. oxysporum*, *F. beomiforme*, and *F. solani* (Table 3.1 and Fig. 3.1). Thirty five of the OTUs were classified to the single reference species *F. avenaceum* 13995; this species was also represented by the greatest number of sequences (summed across the OTUs classified as such) (Table 3.1). Operational taxonomic units assigned to a single position within the *F. oxysporum* complex were the next richest unique classification represented by 6 OTUs. Since *F. beomiforme* had not been reported in the Northern Hemisphere (Laurence et al., 2011), classification of OTUs within this phylogenetic species warranted further analysis. As shown in Fig. 3.2 the OTUs originally classified as *F. beomiforme* fall within the closely related species-complex *F. burgessii*, specifically within the morphotype B clade as described in Laurence et al. (2011).

3.3.4 *Fusarium* community structure

Fusarium community structure was influenced by plant diversity as shown by separation in ordination space of *Fusarium* communities in soil of monoculture and polyculture plant communities (Fig. 3.3). *Fusarium* communities in soil of monoculture plant communities were also separated between the two different plant species. This separation by plant species was not observed for *Fusarium* communities in soil of polyculture plant communities (Fig. 3.3). Similar differences in *Fusarium* community structure were observed regardless of the use of OTU abundance or OTU presence/absence data (Figs. 3.3 A-B).

3.3.5 *Fusarium* OTU and species richness

Fusarium OTU richness (the number of non-singleton OTUs in a sample) and *Fusarium* species richness (the number of unique EPA classifications in a sample (Table 3.1 and Fig. 3.1)) were not effected by plant diversity. Based on a two-sided t-test there was no significant difference in *Fusarium* OTU richness ($t = 1.564, P = 0.149$) and species richness ($t = 0.528, P = 0.609$) between soil of monoculture and polyculture plant communities. Based on a one-way ANOVA there was no effect of the four different plant treatments (each plant species growing in monoculture or polyculture plant communities) on *Fusarium* OTU richness ($F = 2.219, P = 0.163$) or species richness ($F = 0.236, P = 0.869$). *Fusarium* OTU and species richness were also not significantly correlated (Pearson) with any of the individual soil edaphic characteristics (data not shown).

3.3.6 Subgenus OTU and species richness

To measure subgenus richness within *Fusarium*, two primary lineages were identified within the genus. These represent two major groups that have evolved within the "Gibberella" section of *Fusarium*, where the majority of the OTUs were classified using the

EPA (see Fig. 3.1). These two main lineages were, *F. oxysporum* and relatives (hereafter referred to as the "oxysporum" lineage) and *F. tricinctum* and relatives (hereafter referred to as the "tricinctum" lineage) (Fig. 3.1). Both OTU and species richness within the two lineages are defined as described above.

Within the "oxysporum" lineage both OTU and species richness were positively correlated (Pearson) with pH ($R^2_{otu} = 0.57, P < 0.01$; $R^2_{species} = 0.62, P < 0.01$) and potassium ($R^2_{otu} = 0.39, P < 0.05$; $R^2_{species} = 0.52, P < 0.01$) (Fig. 3.4). Two-sided t-tests showed there was no significant difference in OTU or species richness between soil of monoculture and polyculture plant communities. One-way ANOVA showed there was no significant effect of the four plant treatments on species or OTU richness within the "oxysporum" lineage (data note shown).

Within the "tricinctum" lineage, OTU richness was negatively correlated (Pearson) with organic matter ($R^2_{otu} = 0.41, P < 0.05$). Two-sided t-tests showed OTU richness within the "tricinctum" lineage was significantly greater in soil of monoculture plant communities compared to polyculture plant communities ($t = 2.866, P < 0.05$). One-way ANOVA showed there was a significant effect of the four different plant treatments (each plant species growing in monoculture or polyculture plant communities) on OTU richness within the "tricinctum" lineage (3.4; $F = 5.063, P < 0.05$). Post-hoc (TukeyHSD) comparisons of the four plant treatments showed that OTU richness within the "tricinctum" lineage was significantly greater in soil from *L. capitata* growing in monoculture than soil from *L. capitata* or *A. gerardii* growing in polyculture (TukeyHSD, $P < 0.05$) (Fig. 3.6).

3.4 Discussion

Using a combination of amplicon sequencing and phylogenetic classification, this research focused on the ecology of *Fusarium* communities in soil. Overall, we found evidence for 72 different OTUs falling within the genus *Fusarium*, tentatively assigned

to 19 phylogenetic species, within 5 species-complexes. As hypothesized, the most common (found in the most soil samples and with the greatest number of sequences classified as such) species-complexes were *F. tricinctum* and *F. oxysporum*. These two species-complexes were also the most diverse, having the greatest number of OTUs. Of the remaining species-complexes found in these soils, classification of OTUs within the *F. beomiforme* species-complex was unexpected. Through further phylogenetic characterization of the OTUs classified as *F. beomiforme*, we found these OTUs fall within the *F. burgessii* species-complex (Laurence et al., 2011). As *F. burgessii* has only been found in a region of Australia (Laurence et al., 2011) the presence of this fungus in North America suggests a more cosmopolitan distribution. Interestingly, based on this and previous aforementioned work (Laurence et al., 2011), these fungi have only been reported in non-agricultural soils, suggesting they may play important, uncharacterized roles in native ecosystems.

Our second hypothesis was that *Fusarium* diversity (i.e. number of species or OTUs) would be greater in soil of more diverse plant communities (i.e. polyculture). To test this we compared *Fusarium* OTU and species richness in soil of monoculture and polyculture plant communities, as well as between the four different plant treatments. In all cases there was no statistical support for our initial hypothesis, that *Fusarium* diversity in soil is effected by plant diversity. Additionally, there was no relationship between *Fusarium* diversity and any of the individual soil edaphic characteristics. In part, the inability to find significant predictors of *Fusarium* diversity can be explained by the different patterns of diversity within different subgenus lineages.

To further differentiate *Fusarium* diversity within subgenus lineages, we defined the "oxysporum" and "tricinctum" lineages. It is important to note that these lineages differ from the formal *F. tricinctum* and *F. oxysporum* species-complexes discussed above and defined by O'Donnell et al. (2013). Species and OTU richness within the "oxysporum" lineage showed a positive relationship with soil pH and potassium. However, OTU richness in the "tricinctum" lineage showed a negative relationship with soil organic

matter as well as greater OTU richness in monoculture, particularly in soil associated with the legume *L. capitata*. While the different plant and soil edaphic characteristic effects are limited to specific subgenus lineages, this may not reflect *lineage-specific* (e.g. Pellissier et al., 2014; Peay et al., 2013) responses *per se*. Instead, as the two lineages differed in the ratio of OTUs to species (much greater in the "tricinctum" lineage), the different responses may reflect different scales of diversity (i.e. intra- versus interspecific richness) within the lineages (e.g. Silvertown et al., 2009).

By characterizing *Fusarium* communities in soil associated with the roots of two different grassland plant species growing in monoculture and polyculture plant communities we were able to test the effect of plant diversity and plant identity on *Fusarium* community structure. Based on previous work (Ch. 2), we initially hypothesized that both plant identity and diversity would influence *Fusarium* community structure in soil. The differences in *Fusarium* community structure found here are in agreement with previous observations and support our hypothesis. Specifically, *Fusarium* communities were differentiated between soil of monoculture and polyculture plant communities as well as by plant species in monoculture, but not in polyculture plant communities. If differences in *Fusarium* community structure influence the functional roles of these fungi in soil, these results have broader implications. Functional differences of the *Fusarium* communities associated with the different plant species as they grow in monoculture will be changed by increased diversity of the surrounding plant community. Ultimately, understanding the relationship between *Fusarium* community structure and function as well as the importance of these fungi in plant soil feedbacks will require a better understanding of the phylogenetic distribution of functionlly relevant traits within this genus of diverse fungi.

Table 3.1: Classification of *Fusarium* OTUs in a genus-wide reference phylogeny using the EPA (Berger et al., 2011).

EPA classification[1]	**Species-complex**[2]	**Sequences**[3]	**OTUs**[4]	**Samples**[5]
F. avenaceum 13995	*tricinctum*	1810	35	12
I61	*oxysporum*	610	6	11
F. brachygibbosum 31008	*sambucinum*	530	2	5
F. flocciferum 25473	*tricinctum*	104	3	9
F. verticillioides 20956	*fujikuroi*	30	1	5
F. beomiforme 25174	*beomiforme*	18	2	2
F. scirpi 13402	*incarnatum-equisiti*	13	1	3
F. proliferatum 22944	*fujikuroi*	13	1	2
F. neocosmosporiella 22436	*solani*	10	2	4
F. oxysporum 34936	*oxysporum*	9	5	4
F. torulosum 22748	*tricinctum*	7	2	2
F. tricinctum 25481	*tricinctum*	5	1	1
F. ambrosium 20438	*solani*	5	2	3

[1]Classification to a specific branch within the reference phylogeny for which at least one OTU was assigned. Alphanumeric codes begining with "I" indicate classification of an OTU deeper within the phylogeny (see Fig. 3.1). These individual classifications were used as a proxy for "species".

[2]Species-complex for a given classification according to O'Donnell et al. (2013).

[3]Total number (summed across OTUs) of sequences assigned to the classification in column 1.

[4]Number of OTUs assigned to the classification in column 1.

[5]Number of individual soil samples (out of 12) in which the classification in column 1 was found.

I59	*oxysporum/foetens*	4	3	4
I110	*tricinctum*	2	2	2
F. brachygibbosum 13829	*sambucinum*	2	1	1
I24	*solani*	2	1	1
F. subglutinans 22016	*fujikuroi*	2	1	1
I107	*tricinctum/heterosporum*	1	1	1

Table 3.2: Table representing one-way ANOVA testing the effect of the four plant treatments on soil organic matter.

Source	**Degrees of freedom**	**SS**	**MS**	**F-statistic**	**P-value**
Treatment	3	6.97	2.32	5.125	0.029
Error	8	3.627	0.453		

Table 3.3: Table representing one-way ANOVA testing the effect of the four plant treatments on soil phosphorus.

Source	**Degrees of freedom**	**SS**	**MS**	**F-statistic**	**P-value**
Treatment	3	648.3	216.08	4.471	0.04
Error	8	386.7	48.33		

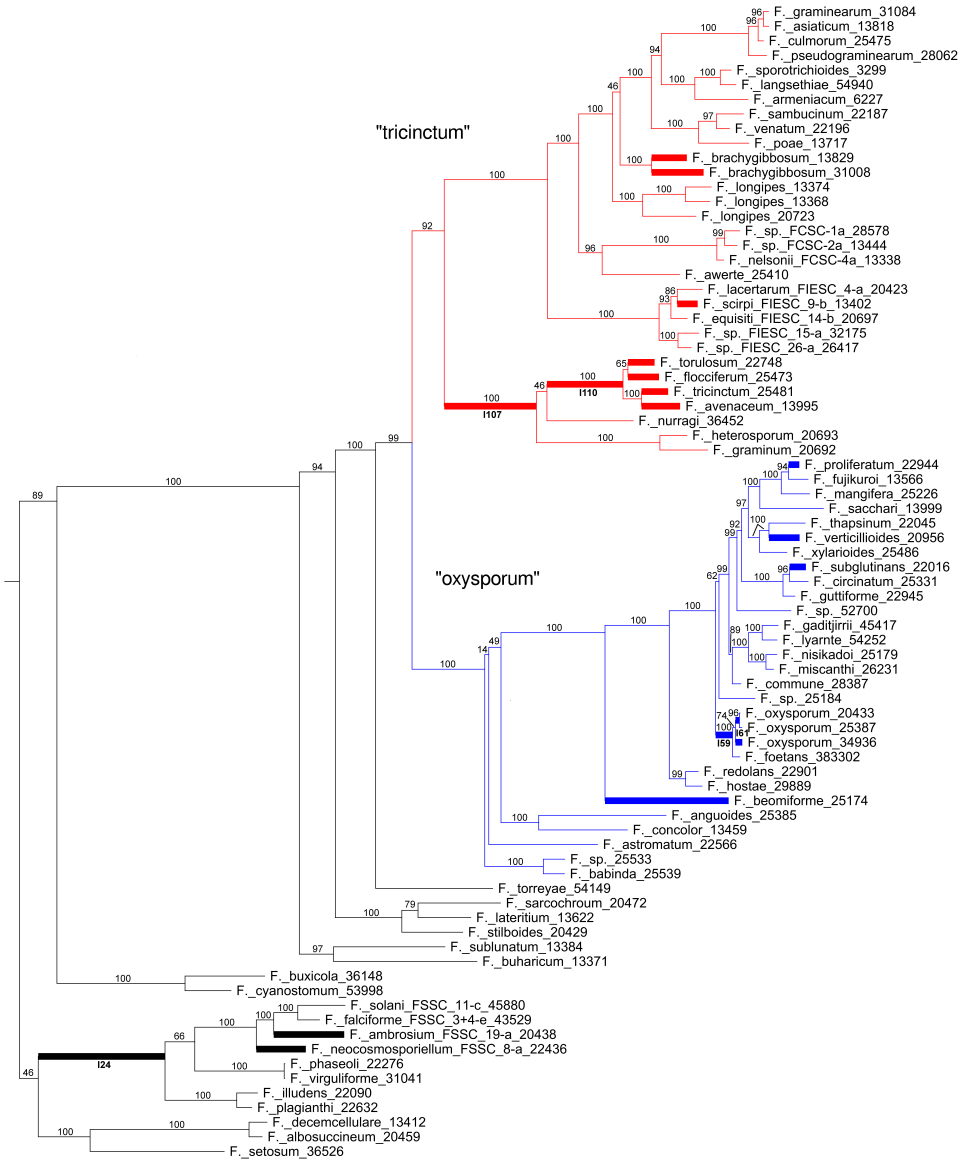

Figure 3.1: Classification of *Fusarium* OTUs within a genus-wide reference phylogeny. The reference phylogeny was constructed from the two locus (*RPB1* and *RPB2*) alignment from O'Donnell et al. (2013) and sequences representing OTUs were classified using the EPA (Berger et al., 2011). The phylogeny shown represents a subset of the complete reference phylogeny, showing the portion relevant to the classified OTUs. Thick branches indicate classification of an OTU to that branch in the reference phylogeny. The blue subtree represents the "oxysporum" and the red subtree represents the "tricinctum" lineage. Numbers above branches are bootstrap values for the reference phylogeny and bold text below thick branches correspond to the EPA classification shown in column 1 of Table 3.1.

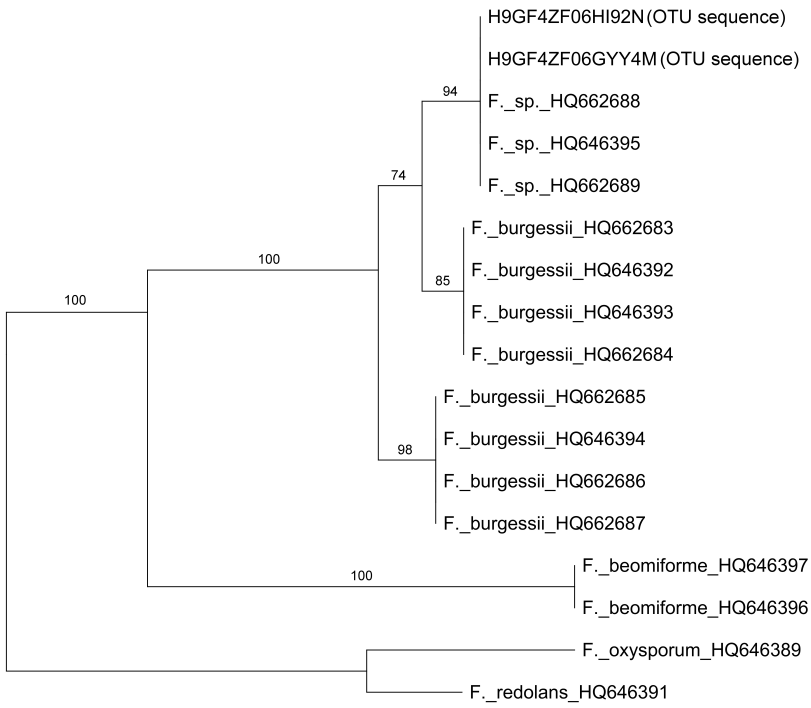

Figure 3.2: A neighbor joining tree constructed from *RPB2* reference sequences representing isolates from the *F. beomiforme* and *F. burgessii* clades as well as sequences from OTUs classified as *F. beomiforme* using the EPA (see Table 3.1 and Fig. 3.1). Numbers above branches are bootstrap values, the tip labels beginning with H9GF4ZF06 are sequences from the OTUs and the last string of digits in the remaining tip labels are NCBI accession numbers. The OTUs fall within the the same clade as *Fusarium* sp. morphotype B as described in Laurence et al. (2011).

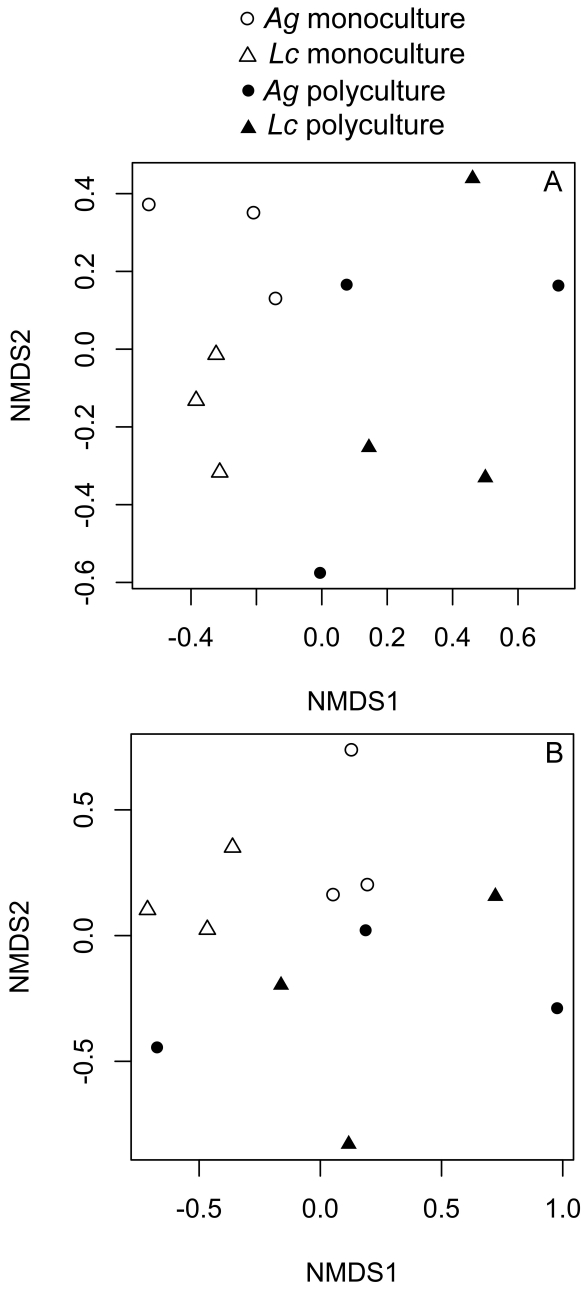

Figure 3.3: Nonmetric Multidimensional Scaling plots showing differences in *Fusarium* community structure between soil from monoculture and polyculture plant communities and differences between the two plant species in monoculture. The two plots represent results for OTU abundance (A) and OTU presence/absence (B) results.

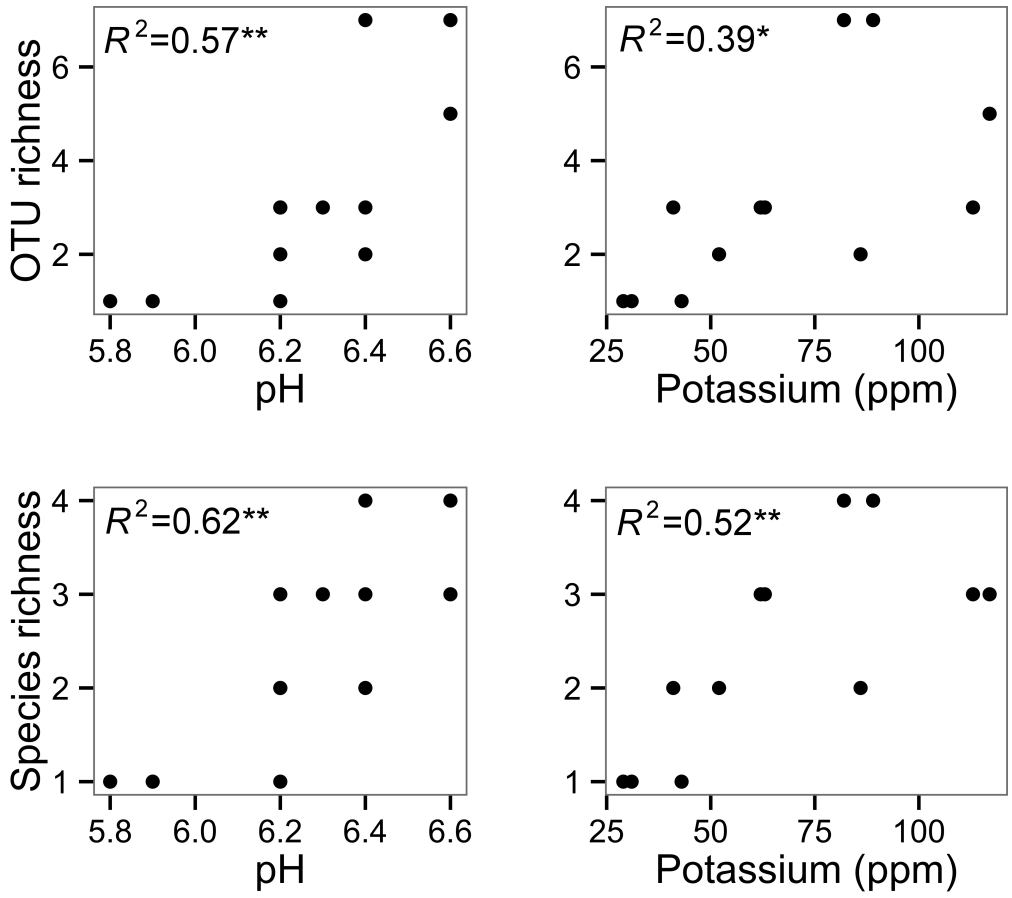

Figure 3.4: Scatter plots showing the relationship of OTU and species richness (i.e. number of OTUs or species in a sample) within the "oxysporum" lineage (see Fig. 3.1) with soil edaphic characteristics. Pearson correlation coefficients were used to calculate R^2 and p-values. P-values indicated as: $P < 0.01 = **, P < 0.05 = *$.

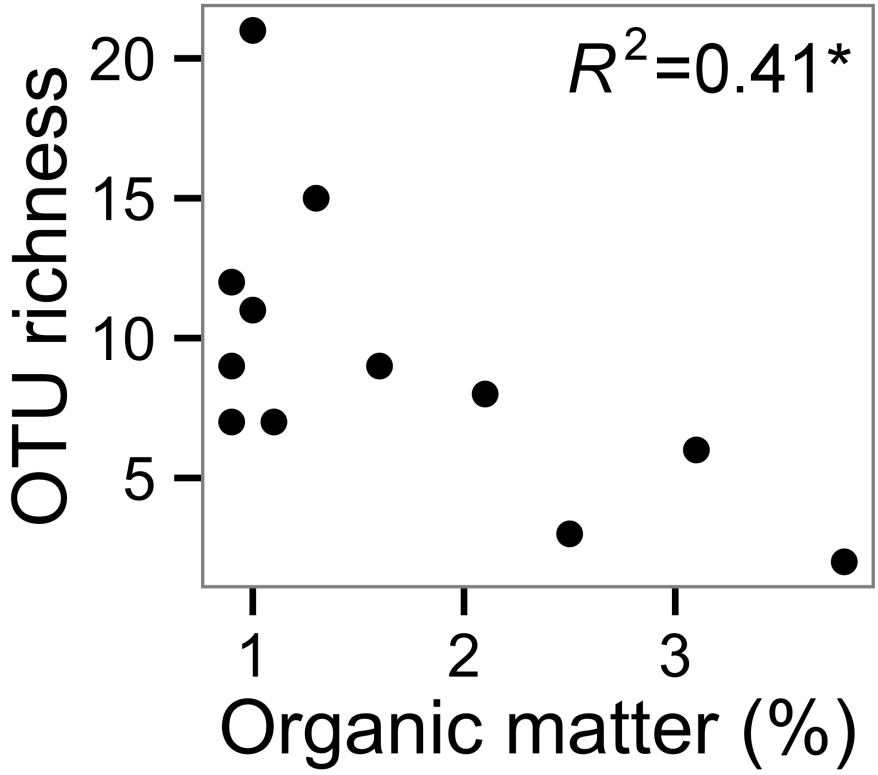

Figure 3.5: Scatter plot showing the relationship of OTU richness (number of OTUs in a sample) within the "tricinctum" lineage (see Fig. 3.1) with soil organic matter. Pearson correlation coefficients were used to calculate R^2 and p-values. P-values indicated as: $P < 0.05 = *$.

Table 3.4: Table representing one-way ANOVA testing the effect of the four plant treatments on OTU richness within the "tricinctum" lineage.

Source	**Degrees of freedom**	**SS**	**MS**	**F-statistic**	**P-value**
Treatment	3	193.7	64.56	5.063	0.02
Error	8	102	12.75		

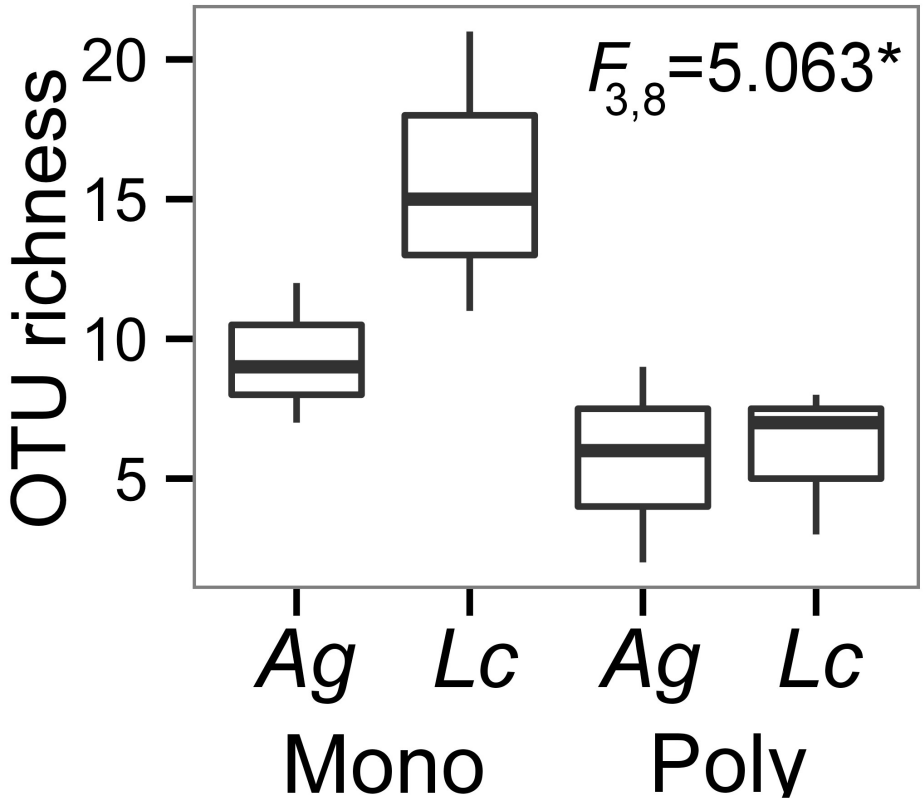

Figure 3.6: Box-plot showing OTU richness (number of OTUs in a sample) within the "tricinctum" lineage (see Fig. 3.1) in the four plant treatments. F-statistic and p-value are results from one-way ANOVA testing the effect of the four plant treatments on OTU richness. Post-hoc (TukeyHSD) comparisons of the four plant treatments showed OTU richness within the"tricinctum" lineage was significantly greater in soil from *L. capitata* growing in monoculture than soil from *L. capitata* or *A. gerardii* growing in polyculture ($P < 0.05$). P-values indicated as: $P < 0.05 = *$.

Chapter 4

Carbon use of *Fusarium* soil isolates is influenced by isolate phylogeny and plant identity

4.1 Introduction

Fungi in the genus *Fusarium* are well-known soilborne plant pathogens of agronomic plants (Michielse and Rep, 2009; Hartman et al., 2015). However, there is strong evidence that the growth of these fungi in soil play significant functional roles in agricultural and native ecosystems beyond causing plant disease.

Comparison of over 100 sequenced fungal genomes has shown genes predicted to produce enzymes involved in the breakdown of plant lignocellulose (i.e. CAZymes) are enriched within *Fusarium* genomes (Zhao et al., 2013). The high lignolytic capacity of fungi in the genus *Fusarium* is also supported by in-vitro measurement of plant cell-wall and lignocellulose decomposition (King et al., 2011; Li et al., 2008). In addition to the breakdown of plant biomass, the use of carbon for heterotrophic growth by these filamentous fungi is linked to their ability to suppress disease caused by soilborne plant

pathogens in the same genus (Couteaudier and Alabouvette, 1990; Alabouvette et al., 2009).

Multiple phylogenetic lineages of *Fusarium* are commonly found in soil (Ch. 3 McMullen and Stack, 1983; Balmas et al., 2010). Despite this diversity, there is little information on how carbon use varies between different lineages of these fungi found in soil. If the use of distinct carbon sources is restricted to certain lineages, then the presence or absence of *Fusarium* lineages may influence carbon turnover in the soil or the suppression of disease caused by soilborne plant pathogens.

Both plant identity and diversity may influence the type and quantity of carbon substrates in soil and carbon use of soil microorganisms such fungi in the genus *Fusarium*. High concentrations of a particular carbon substrate in the rhizosphere of specific plant species are known to select for *Fusarium* individuals able to use that substrate as a resource for growth (Rodriguez-Carres et al., 2008). Because small genotypic differences between closely related plants can change the concentration and type of carbon substrates entering the soil from plant roots (Badri et al., 2009), it is likely that different plant species will influence carbon use of microorganisms in soil. Changes in plant diversity may also influence carbon use of soil microorganisms. Increased plant diversity often results in greater soil carbon concentrations (Lange et al., 2015; Tiemann et al., 2015). Additionally, though untested, greater plant diversity could potentially increase the number of different types of carbon substrates entering the soil.

The objectives of this research were to characterize carbon use of cultured *Fusarium* isolates from soil. The first objective was to study how carbon use varies across the genus. Specifically, it was hypothesized that more phylogenetically similar isolates (e.g. within a clade) would display greater similarity in carbon use than more phylogenetically dissimilar isolates (e.g. between clades). The second objective was to test the effect of plant diversity and identity on carbon use of these fungi. With respect to plant diversity, it was hypothesized that *Fusarium* isolates from soil of polyculture (high diversity) plant communities would use a greater number of carbon substrates and display greater

growth than isolates from soil of monoculture (low diversity) plant communities. It was further hypothesized that *Fusarium* isolates from soil associated with the roots of different plants species would differ in the number of carbon substrates they use or display differences in growth.

4.2 Materials and methods

Soil was sampled from the Cedar Creek Ecosystem Science Reserve LTER site in Minnesota, USA in July, 2012. Two soil cores, separated by a ninety-degree angle around the base of the sampled plant were taken from the base of individual plants growing in monoculture or 16 species (i.e. polyculture) plant communities as part of experimental plots established and maintained since 1994 (Tilman et al., 1997). The individual plants sampled were the perennial legume *Lespedeza capitata* and the perennial grass *Andropogon gerardii*. The two soil cores from individual plants were pooled in the field and kept on ice until storage at -20 °C. Extractable phosphorus, extractable potassium, extractable nitrate, pH, and organic matter of the soil were measured at the University of Minnesota Research Analytical Laboratory (`ral.cfans.umn.edu`).

4.2.1 Culturing *Fusarium* isolates from soil

To culture *Fusarium* isolates from the soil, *Fusarium* selective Komada and Nash-Snyder media (Leslie and Summerell, 2006) were inoculated with soil suspensions. Soil suspensions were created by diluting 1 gram of air-dried soil in sterile water for a final concentration of 1×10^{-3} grams soil/mL water. For each individual soil sample, 1 mL of the diluted soil suspension was inoculated onto two plates of each type of media. The inoculated plates were incubated at 28 °C for five days. Individual colonies for subculturing were selected randomly using a grid and randomized coordinates. Randomized colonies were subcultured onto half-strength Potato Dextrose Agar (1/2-PDA: 12 g Potato Dextrose Broth (Difco) + 15 g agar / L water) and grown under a 12 hour photoperiod at

25 °C for 5-7 days. A culture was classified as within the genus *Fusarium* based on the production of fusiform (banana or canoe) shaped spores. For soil suspensions failing to produce more than five *Fusarium* isolates after the initial plating, repeated culturing efforts were performed. Single spore isolates were generated from the cultured isolates and stored in 50 percent glycerol at -70 °C.

4.2.2 Carbon use phenotyping

Carbon use phenotypes were measured based on the ability of the cultured *Fusarium* isolates to grow on 95 different carbon substrates using Biolog SF-P2 plates (Biolog). The methods used to standardize fungal material and inoculate the Biolog SF-P2 plates followed the protocol outlined in Schlatter et al. (2013). Individual isolates were grown for 5 days on 1/2-PDA at 25 °C under a 12 hour photoperiod. Spores from each isolate were harvested from the media using sterile water and suspensions were adjusted to an optical density (OD) of 0.22 at 590 nm. One and a half mL of the adjusted spore suspensions were dispensed into 13.5 mL of sterile water. The spore suspensions for each isolate were then inoculated into individual Biolog SF-P2 plates (i.e. one isolate per plate). Optical densities were measured every 12 hours for 4 days using a Synergy H1 Reader (BioTek Instruments) at 590 nm.

Processing of the Biolog data and calculating indices of carbon use followed a similar rationale outlined in Schlatter et al. (2013). Optical densities in the water control in each plate at every time point were subtracted from all the remaining 95 wells, after which individual wells with OD $\leq$ 0.005 were set to zero. Two indices of carbon use were calculated from the processed OD values on the 95 carbon substrates. Growth was calculated as the mean OD across the 95 substrates for a given isolate at a given timepoint and the number of substrates used was calculated based on the number of substrates yielding positive OD values greater than 0.005 by a given *Fusarium* isolate at a given timepoint.

4.2.3 PCR methods

A portion of the *RPB2* gene was sequenced from each *Fusarium* isolate. This was done to determine the phylogenetic relatedness among and genetic distance between isolates as well as to assign a taxonomic classification to the isolates. Deoxyribonucleic acids were extracted as described in Gale et al. (2011) and diluted to 10 ng/µL. A portion of the *RPB2* (RNA polymerase 2nd largest subunit) gene was amplified from the extracted DNAs using the RPB2-5F2 (5'-GGGGWGAYCAGAAGAAGGC-3') and RPB2-7cR (5'-CCCATRGCTTGYTTRCCCAT-3') primers (O'Donnell et al., 2007). Reactions were performed in an Eppendorf Mastercycler using 20 µL reactions with 2 µL Ex-Taq buffer, 1.6 µL dNTPs (2.5 µM of each), 2 µL of each forward and reverse primers (10 µM), 11.3 µL water, 0.1 µL Takara Ex-Taq polymerase (5 units/µL), and 1 µL template (10 ng DNA/µL). The PCR reactions were performed with an initial denaturing step at 95 °C (5 min) followed by 24 cycles of 95 °C (1 min), 57 °C (1 min), 72 °C (1 min), and ended with an extension of 72 °C (10 min). Amplicons were purified using a Promega Wizard kit (Promega, USA) and sequenced with the 5F2 primer using Sanger technology at the Biomedical Genomics Center at the University of Minnesota (`http://www.bmgc.umn.edu/`). Low quality sequence data were trimmed using 4Peaks (`http://nucleobytes.com/index.php/4peaks`) and the trimmed sequences were aligned using MUSCLE (Edgar, 2004).

4.2.4 Phylogenetics and taxonomy

To determine the phylogenetic relationships between the cultured *Fusarium* isolates, the aligned *RPB2* sequences from the isolates were used to construct a neighbor joining phylogeny using MEGA6 (Tamura et al., 2013). Phylogenetic clade support was assessed based on nonparametric bootstrap values, with 100 replications. For taxonomic identification of the isolates, the same sequence data were aligned to culture-backed *RPB2* reference sequences in the Fusarium ID database (Geiser et al., 2004) using BLASTN

(Altschul et al., 1997).

4.2.5 Statistics

All statistics were performed in the R environment (R Development Core Team, 2012) using the corrplot (Wei, 2013), ggplot2 (Wickham, 2009), seqinr (Charif and Lobry, 2007), vegan (Oksanen et al., 2013), and base packages.

A Mantel-test was used to test for a relationship between genetic and phenotypic (i.e. carbon use) distance. To perform the Mantel-test a genetic distance (square root of the pairwise sequence identity) matrix was calculated from the aligned *RPB2* sequence data using the seqinr package and a carbon use distance (Euclidean) matrix was calculated from OD values on the 95 carbon substrates at 96 hours for each *Fusarium* isolate. The Mantel test was performed with the vegan package using the Pearson correlation method with 999 permutations.

To visualize pairwise correlation of growth among *Fusarium* isolates across the 95 carbon substrates, a heatmap was generated with the corrplot package. Pearson correlation coefficients were calculated from OD values on the 95 carbon substrates at 96 hours for a given comparison between pairwise isolates. The isolates represented in the heatmap were reordered to match the order of the isolates represented in the phylogeny constructed from the *RPB2* sequence data (see above). This was done to compare correlation coefficients within and between phylogenetic clades.

Two-sided t-tests used to test for differences of individual soil edaphic characteristics between the two levels of plant diversity or the two plant species. Based on Q-Q plots and a Shapiro-Wilk test for normality, both growth and the number of substrates used of the isolates were not normally distributed. Consequently, nonparametric Mann-Whitney tests were used to test for differences in growth and the number of substrates used of the isolates between the two levels of plant diversity or the two plant species. These Mann-Whitney tests were performed with data from the termination of the timecourse (i.e. 96 hours).

Spearman rank correlation coefficients (ρ) were used to test for relationships between growth or number of substrates used and individual soil edaphic characteristics, including organic matter, potassium, pH, phosphorus, and nitrogen.

4.3 Results

4.3.1 Variation in isolate number and carbon use

A total of 84 *Fusarium* isolates were cultured from the 12 individual soil samples. As shown in Table 4.1, there was variation in the number of isolates cultured from individual soil samples. Within the plant species treatments, a mean of 4.333 (σ=2.944) isolates were cultured from *A. gerardii* and a mean of 9.667 (σ=0.816) isolates were cultured from *L. capitata*. Within the plant diversity treatments, a mean of 6.333 (σ=3.445) isolates were cultured from monoculture and a mean of 7.667 (σ=3.67) isolates were cultured from polyculture.

All of the 95 carbon substrates were used by at least one *Fusarium* isolate. The fewest substrates used was 74 with an average of 91.31 (σ = 3.818) substrates being used by the isolates. Among the 84 *Fusarium* isolates there were 56 unique carbon use patterns (i.e. combinations of substrates used by individual isolates). Fifty of these patterns were singletons, while the most common carbon use pattern, use of all 95 substrates, was found in 14 isolates. The five substrates supporting the greatest growth (i.e. highest mean OD of the isolates) were turanose, N-acetyl-D-glucosamine, polyoxyethylene (20) sorbitan monopalmitate (Tween 40), D-trehalose, and polyoxyethylene (20) sorbitan monooleate (Tween 80). The five substrates supporting the least growth (among isolates exhibiting growth on them) were 3'-methyl-D-glucose, adenosine, adenosine-5'-monophosphate, D-fructose-6-phosphate, and D-L-α-glycerol phosphate.

4.3.2 Taxonomy of *Fusarium* isolates

Taxonomy of the isolates was assigned based on alignment and similarity of *RPB2* sequence data from the isolates with *RPB2* reference sequences in the Fusarium ID database (Geiser et al., 2004). Sequences from all of the isolates showed similarity to reference sequences in the database with a minimum alignment of 453 bases. Based on the alignments, the *RPB2* sequences from the isolates were similar to seven different culture-backed reference sequences, representing six different species-complexes *sensu* O'Donnell et al. (2013): *F. oxysporum*, *F. foetens*, *F. nisikadoi*, *F. sambucinum*, *F. incarnatum-equisiti*, and *F. solani*, (Table 4.2).

4.3.3 Phylogeny and carbon use of *Fusarium* isolates

A neighbor joining tree was constructed using *RPB2* sequence data to assess the phylogenetic relationships among the *Fusarium* isolates. The *Fusarium* isolates formed four different phylogenetic clades supported by bootstrap values of at least 97 percent based on the *RPB2* sequence data (Fig. 4.1). The previous taxonomic classification of the isolates showed that the largest clade represents isolates showing high sequence similarity to references in the *F. oxysporum* and *F. foetens* species-complexes (Table 4.2, Fig. 4.1). As shown in the heatmap, Pearson correlation coefficients (calculated from growth across the 95 carbon substrates for pairwise isolates) were higher for isolate pairs within phylogenetic clades than isolate pairs between phylogenetic clades (Fig. 4.1). Using a Mantel test there was also a significant relationship between genetic distance (calculated from *RPB2* sequence data) and carbon use distance (calculated from growth on the 95 carbon substrates) (Mantel correlation $= 0.294, P < 0.01$).

4.3.4 Effect of plant diversity and identity on carbon use

There was no effect of plant diversity on carbon use of the *Fusarium* isolates. Based on the nonparametric Mann-Whitney test, the number of substrates used by the isolates from soil of monoculture or polyculture plant communities were not significantly different (Mann-Whitney, $P = 0.306$). Growth also was not significantly different between *Fusarium* isolates from soil of monoculture or polyculture plant communities (Mann-Whitney, $P = 0.218$).

Plant species influenced carbon use of the cultured *Fusarium* isolates. The number of substrates used was significantly greater for *Fusarium* isolates from soil associated with the roots of the plant species *L. capitata* than the plant species *A. gerardii* (Mann-Whitney, $P < 0.01$; Fig. 4.2). There was no significant difference in growth of isolates from soil associated with the two different plant species (Mann-Whitney, $P = 0.187$).

4.3.5 Soil edaphic characteristics and carbon use

Soil phosphorus and organic matter were greater in soil of polyculture plant communities compared to soil of monoculture plant communities ($t = -3.003, P < 0.05; t = -2.61, P < 0.05$, respectively). Growth of the *Fusarium* isolates was significantly correlated (Spearman) with soil potassium ($\rho = -0.243, P < 0.05$). The number of substrates used was significantly correlated with organic matter ($\rho = -0.34, P < 0.01$), pH ($\rho = -0.485, P < 0.001$), potassium ($\rho = -0.452, P < 0.001$), and nitrogen ($\rho = 0.226, P < 0.05$).

4.4 Discussion

The goal of this research was to characterize carbon use of *Fusarium* isolates cultured from soil to test three primary hypotheses. Our first hypothesis was that isolates within a phylogenetic clade would be display greater similarity in carbon use then isolates between phylogenetic clades. In other words, with increasing genetic distance between

isolates there would be a corresponding dissimilarity in carbon use. There was some evidence to support our hypothesis. Visualization of the similarity in carbon use among isolates, showed that isolates within a given phylogenetic clade displayed greater similarity in carbon use (i.e. higher correlation in growth across the carbon substrates) than comparison of isolates between phylogenetic clades. In addition, a Mantel-test provided statistical evidence for a positive relationship between genetic distance and carbon use distance. As suggested by McGuire et al. (2010), a positive relationship between genetic and carbon use distance, may have implications for predicting the functional consequences of changes in the diversity or community structure of fungi in soil. For example, with the presence or absence of different *Fusarium* clades in soil there may be quantitative differences in carbon use, potentially influencing decomposition and soil nutrient dynamics.

In addition to a potential relationship between phylogeny and carbon use, we hypothesized that plant diversity would influence carbon use of the *Fusarium* isolates. This reasoning was based on observations that increased plant diversity often has a positive effect on the concentration of carbon in soil (Lange et al., 2015; Tiemann et al., 2015). While this research found higher organic matter in soil of polyculture plant communities compared to monoculture plant communities, there was no statistical evidence for an effect of plant diversity on carbon use of the *Fusarium* isolates.

Our third hypothesis was that isolates from soil associated with different plant species would differ in carbon use. This was motivated by empirical research that has shown even closely related plants produce different concentrations and types of carbon substrates in their rhizosphere (Badri et al., 2009). Additionally, the high concentration of particular carbon substrates can select for individuals able to use those substrates in the rhizosphere (Rodriguez-Carres et al., 2008). Considering isolates from soil associated with the roots of *L. capitata* used more carbon substrates than isolates from *A. gerardii*, we found support for our hypothesis. This suggests soil nutrient dynamics, as influenced by these fungi, may differ within the rhizosphere of these different plant

species which could influence the growth of the focal or neighboring plant species. This plant species effect on carbon use of *Fusarium* fungi in soil may have further effects on soil nutrient cycling in the rhizosphere of these different plant species. This could potentially alter the availability of soil nutrients and growth of the focal or neighboring plant species, ultimately influencing the diversity or structure of plant communities.

We also found a primarily negative relationship between individual soil edaphic characteristics and carbon use. These results are similar to observations made by Schlatter et al. (2013) who characterized carbon use of bacteria in the genus *Streptomyces* from soil. Ultimately, greater insight into the mechanisms underlying the variation in carbon use of these *Fusarium* isolates will require a better understanding of how these fungi interact with other microorganisms in soil and how these interactions are influenced by different plant species or variation in plant diversity.

In summary, there was support for two of our three hypotheses. *Fusarium* isolates that were more phylogenetically closely related displayed more similar carbon use phenotypes and there was an effect of plant species on carbon use of these fungi. However, there was no support for an effect of plant diversity on carbon use of the *Fusarium* isolates. Altogether this work shows that both the evolutionary history as well as the local environment influence carbon use of these ubiquitous soil fungi and that changes in *Fusarium* communities in soil are likely to have functional consequences.

Table 4.1: The number of cultured *Fusarium* isolates from 12 individual soil samples (rows) associated with the roots of the plant species *A. gerardii* or *L. capitata* growing in monoculture or polyculture plant communities.

Plant diversity	**Plant species**	**Number of isolates**
Monoculture	*A. gerardii*	4
Monoculture	*A. gerardii*	2
Monoculture	*A. gerardii*	4
Monoculture	*L. capitata*	10
Monoculture	*L. capitata*	10
Monoculture	*L. capitata*	8
Polyculture	*A. gerardii*	4
Polyculture	*A. gerardii*	2
Polyculture	*A. gerardii*	10
Polyculture	*L. capitata*	10
Polyculture	*L. capitata*	10
Polyculture	*L. capitata*	10

Table 4.2: Taxonomic classification of *Fusarium* isolates by alignment of isolate *RPB2* sequence data to the Fusarium-ID database (Geiser et al., 2004).

Reference[1]	**Species-complex**[2]	**Isolates**[3]	**Identity**[4]
F. oxysporum NRRL34936	*oxysporum*	35	0.995
F. oxysporum NRRL38302	*foetens*	24	0.99
F. commune NRRL28387	*nisikadoi*	6	0.979
F. brachygibbosum NRRL31008	*sambucinum*	1	1
F. incarnatum-equiseti 14-b NRRL20697	*incarnatum-equisiti*	1	0.985
F. solani 5-d NRRL43681	*solani*	14	1
F. solani 5-l NRRL32791	*solani*	3	1

[1]Identity of the references in Fusarium ID that were most similar to the *RPB2* sequences from the *Fusarium* isolates.

[2]Species-complex identity of references shown in column 1.

[3]Total number of *Fusarium* isolates for which *RPB2* sequence data aligned to references shown in column 1.

[4]Mean identity between *RPB2* sequences of the isolates and *RPB2* sequences of the references shown in column 1.

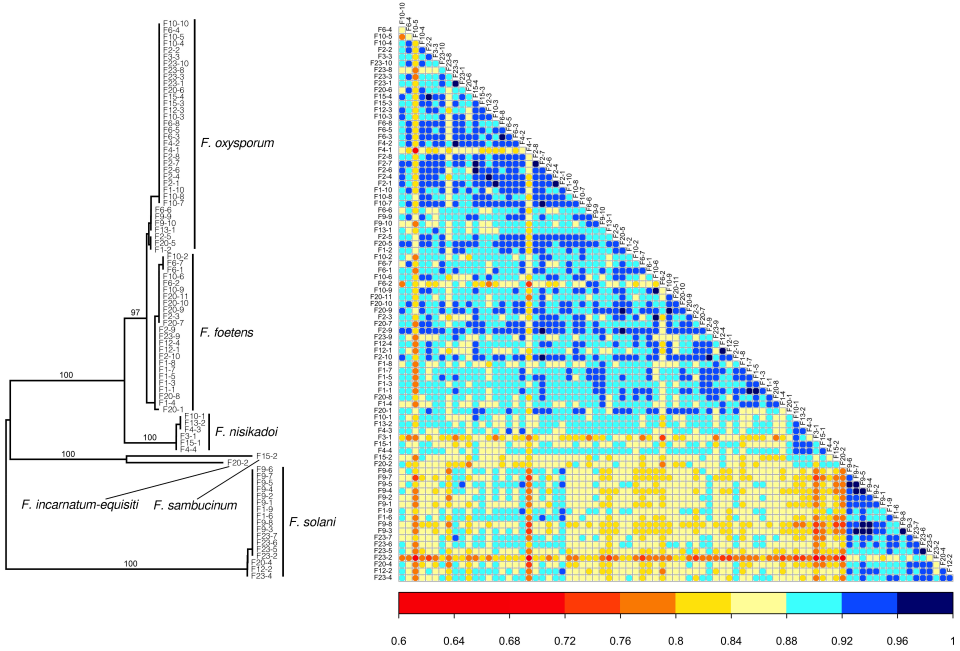

Figure 4.1: A neighbor joining phylogeny representing phylogenetic relationships between *Fusarium* isolates and a heatmap representing similarity in carbon use among isolates. The phylogeny was constructed from *RPB2* sequence data from the isolates. Numbers above branches represent nonparametric bootstrap values and taxonomy is based on alignment of *RPB2* sequence data to Fusarium ID (Geiser et al., 2004) (Table 4.2). The heatmap represents Pearson correlation coefficients calculated from OD values (growth) on the 95 carbon substrates at 96 hours for a given comparison between pairwise isolates. The isolates represented in the heatmap were reordered to match the order of the isolates represented in the phylogeny.

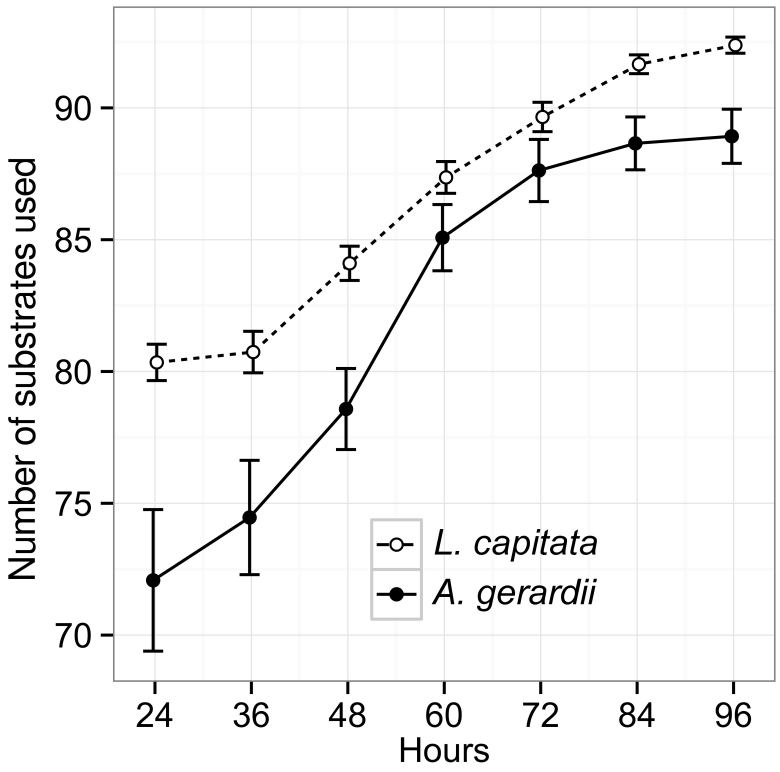

Figure 4.2: The number of substrates used over time by *Fusarium* isolates from soil associated with the roots of the plant species *L. capitata* or *A. gerardii*. Based on the nonparametric Mann-Whitney test, the isolates from *L. capitata* use significantly more substrates than isolates from *A. gerardii* at the termination of the timecourse (i.e. 96 hours) (Mann-Whitney, $P < 0.01$).

Chapter 5

Fusarium soil isolates show variation in genetic potential to produce secondary metabolites

5.1 Introduction

The ecological relevance of fungal secondary metabolites is poorly understood, yet there is evidence that these small molecules mediate the functional roles of fungi as they interact with plants and other microorganisms. For example, the ability to produce the plant hormone gibberellic acid is associated with increased colonization of rice plants by the fungus *F. fujikuroi* (Wiemann et al., 2013). Fungi in the genus *Fusarium* are also able to produce other plant hormones such as indole-acetic acid (Tsavkelova et al., 2012) or alter plant hormone regulation through the secretion of small peptides (Bailey, 1995). In addition to plant hormones or plant hormone elicitors, fungi can produce secondary metabolites with demonstrated effects on other microorganisms. Fusaric acid, which is produced by many different species of *Fusarium* (Bacon et al., 1996), can reduce the production of antifungal compounds produced by Gram negative bacteria in the genus

Pseudomonas (van Rij et al., 2005). This provides a mechanism of defence for the producing fungi against antifungal compounds produced by the bacteria.

The production of specific secondary metabolites may be advantageous for the producing fungus in certain situations (e.g. interacting with other microorganisms or colonizing plant tissue), however there is in-vitro evidence that the production of secondary metabolites is metabollically costly (Malz et al., 2005; Gaffoor et al., 2005). This suggests a potential tradeoff between secondary metabolism and growth.

The objective of this work was to develop a PCR based assay to screen for the genetic potential to produce five secondary metabolites in a collection of *Fusarium* isolates. With the expectation that the isolates would display variation in the genetic potential to produce different metabolites, it was hypothesized that isolates lacking the genetic potental to produce a given secondary metabolite would display increased growth compared to isolates with the genetic potential to produce that metabolite. Additionally, as many fungal secondary metabolites are biologically active toward plants (e.g. the plant hormone gibberellic acid), it was hypothesized that isolates with genetic potential to produce particular secondary metabolites would influence plant growth compared to isolates without the genetic potential to produce that metabolite.

5.2 Materials and methods

5.2.1 *Fusarium* isolates

Fusarium isolates were cultured from rhizosphere soil collected from the plant species *Lespedeza capitata* and *Andropogon gerardii* growing in monoculture and polyculture plant communities as described in Section 4.2. The isolates characterized in this work are a subset (n=65) of the previously described 84 isolates. The subset represents isolates that show *RPB2* sequence similarity to *Fusarium* taxa in the *F. oxysporum*, *F. foetens*, and *F. nisikadoi* species-complexes *sensu* O'Donnell et al. (2013). This subset of isolates was used because the taxa are closely related to the members of the

F. fujikuroi and *F. oxysporum* species-complexes used to develop the PCR assay.

5.2.2 Development of PCR assay

Secondary metabolism genotypes were determined based on the presence of genes responsible for the production of individual secondary metabolites in the *Fusarium* isolates. A PCR assay was developed to screen for genes that through deletion were previously shown to be required for the production of indole-acetic acid (IAA), gibberellic acid (GA), fusaric acid (FA), bikaverin (BIK), and the necrosis and ethylene inducing peptide (NEP) (see Table 5.1 and references within). Reference nucleotide sequences for genes of interest were downloaded from the Broad Institute (`http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html`) or the National Center for Biotechnology Information (`http://www.ncbi.nlm.nih.gov/nucleotide/`) as required. These reference sequences were aligned to the genomic sequence data of the reference *Fusarium* genomes at the Broad Institute to identify homologous sequences in additional *Fusarium* genomes. Homologous sequences were downloaded from the reference genomes and aligned using MUSCLE (Edgar, 2004). Primers were designed and selected based on a 100 % consensus sequence from the alignment using Primer3 (Untergasser et al., 2012) (`http://www-genome.wi.mit.edu/genome_software/other/primer3.html`).

5.2.3 PCR methods

To screen for the genes required for the production of the five different secondary metabolites (Table 5.1) DNAs were extracted from the fungal isolates as described in Gale et al. (2011). For PCR amplification, 10 µL reactions were used with 1 µL Ex-Taq buffer, 0.8 µL dNTPs (2.5 µM of each), 1 µL of each forward and reverse primers (10 µM), 5.15 µL water, 0.05 µL Takara Ex-Taq polymerase (5 units/µL), and 1 µL template (10 ng DNA/µL). The PCR reactions were performed in an Eppendorf Mastercycler with an initial denaturing step at 95 °C (5 min) followed by 24 cycles of 95 °C (1 min),

57 °C (1 min), 72 °C (1 min), and ended with an extension of 72 °C (10 min). Amplicons were resolved on an agarose gel and scored for presence or absence of the genes in each individual isolate. Each isolate was screened twice, with a positive control as well as water and DNAs without the gene of interest as negative controls.

Growth of the individual isolates was measured based on average optical densities across 95 carbon substrates as described in 4.2.2.

5.2.4 Plant infection assay

A growth chamber experiment was used to test if the *Fusarium* isolates influenced the growth or caused plant disease symptoms in the plant species *A. gerardii* and *L. capitata*. Plant seed was purchased from Prairie Restoration (`http://www.prairieresto.com/`). Seed coats were removed and the seeds were surface-sterilized by rinsing in 95 % ethanol, two minutes in 10 % bleach (8.25 % sodium hypochlorite), and two minutes in 70 % ethanol. Prior to planting, medium-grain vermiculite was autoclaved for one hour and cone-tainers were surface sterilized with 10 % bleach. Five seeds of each plant species were planted in each cone-tainer and thinned to one plant per cone-tainer following seedling emergence. The plants were grown under a 16 hour photoperiod, alternating between 22 °C (light) and 20 °C (dark).

The 65 isolates were divided into two randomized inoculation sets. For each plant species, the two randomized inoculation sets were used to inoculate the plants in separate experiments. Within each inoculation set, individual treatments and water negative controls were randomized and replicated nine times.

A standardized concentration of spores was prepared from each of the 65 isolates as described in Menke et al. (2012) and adjusted to 1×10^6 spores/mL water. Fourteen days post planting, 1 mL of the isolate spore suspension was inoculated at the base of individual seedlings.

Fourteen days post inoculation the plants were harvested and phenotyped. Symptoms of necrosis and wilting were visually inspected compared to the water controls.

Plant biomass as well as root and shoot length were also measured.

5.2.5 Recovery of inoculated fungal isolates

A subsequent growth chamber experiment was used to test if the inoculated fungal isolates could be recovered from surface-sterilized plants. Using the same sterilization and inoculation methods described above, two individual plants per *Fusarium* isolate were inoculated. Fourteen days post inoculation the plants were harvested and surface-sterilized. The surface-sterilized plants were placed on 1/2-PDA (see 4.2.1) amended with antibiotics (2 g streptomycin sulfate + 0.24 g neomycin sulfate/L media). Fungi emerging from the surface-sterilized plants were subcultured and identified to genus based on spore morphology.

5.2.6 Statistics

One-way Analysis of Variance (ANOVA) was used to test for an effect of isolate inoculation on the individual plant growth phenotypes. Separate one-way ANOVAs were used to test the effect of isolate inoculation on each plant phenotype measured (root length, shoot length, and biomass). Two-sided t-tests were used to determine if *Fusarium* isolates with or without the genetic potential to produce a given secondary metabolite differed in growth at individual timepoints.

5.3 Results

5.3.1 Variation in secondary metabolism genotypes

The 65 *Fusarium* isolates were evaluated for the presence or absence of genes previously shown to be essential for the production of five different secondary metabolites (Table 5.1). Ninety one percent of the isolates had the genetic potential to produce BIK and FA, 89 % NEP, 78 % IAA, and 6 % GA. Based on presence or absence of genes for the ability to produce the five different secondary metabolites, there were 10 unique secondary

metabolism profiles (combinations of presence/absence of the five screened genes). A total of 42 isolates (65 %) represented the most common secondary metabolism profile, having the genetic potential to produce all the secondary metabolites except GA. Of the remaining secondary metabolism profiles, 5 were singletons including one isolate having the assayed genes for all five metabolites and one isolate lacking genes for all five (Fig. 5.1).

5.3.2 Genetic potential to produce secondary metabolites and growth

Isolates with or without the genetic potential to produce individual secondary metabolites differed in growth. Where statistically significant, isolates with the genetic potential to produce a metabolite showed reduced growth compared to isolates without the genetic potential to produce that metabolite. Based on a two-sided t-test, growth of isolates without the genetic potential for GA grew more than isolates with the genetic potential for GA at two timepoints. This phenomenon was also observed for isolates with or without the genetic potential for IAA production at six timepoints (Fig. 5.2).

5.3.3 Effects of *Fusarium* isolates on plant growth

To test if isolates with the genetic potential to produce individual secondary metabolites influence plant growth, the 65 individual *Fusarium* isolates were used to inoculate the two plant species *A. gerardii* and *L. capitata* in a growth chamber experiment. The isolates were inoculated at the base of the two plant species growing as seedlings. Two weeks post inoculation the plants were harvested and phenotyped. Relative to the water controls there were no symptoms of wilting or necrosis observed in any inoculated plant of either plant species. Additionally, based on a one-way ANOVA there was no effect of inoculating the fungal isolates on plant root length, shoot length, or biomass. The absence of an effect was found for both randomized inoculation sets for each plant species (Table 5.2).

5.3.4 Recovery of inoculated fungi

Thirteen of the 65 inoculated *Fusarium* isolates were recovered from surface-sterilized *L. capitata* plants and 6 of the 65 inoculated *Fusarium* isolates were recovered from surface-sterilized *A. gerardii* plants. No fungi with fusiform *Fusarium* conidia were recovered from nine replicate water controls from either plant species. There were no symptoms of wilting or necrosis observed on any plants in this experiment either.

5.4 Discussion

This work characterized secondary metabolism genotypes of *Fusarium* isolates from soil associated with the roots of two perennial plant species. Using a PCR assay developed here, the isolates were genotyped for their potential to produce five different secondary metabolites. As expected, there was variation in the genetic potential to produce all of the individual secondary metabolites. Though most isolates tested positive for amplicons associated with most of the metabolites, the genetic potential to produce gibberellic acid was relatively rare among the isolates.

By measuring growth of the isolates we tested the hypothesis that isolates with the genetic potential to produce a given secondary metabolite would show a reduction in growth. For three of the five screened secondary metabolite genes there was no difference in growth of isolates with or without the genetic potential to produce the metabolite. However, isolates with the genetic potential to produce either indole-acetic acid or gibberellic acid had reduced growth compared to isolates without the genetic potential to produce these two secondary metabolites. In general, this agrees with in-vitro comparisons of isogenic fungal strains differing in only the presence or absence of genes linked to the production of secondary metabolites (Malz et al., 2005; Gaffoor et al., 2005). This raises the question as to why the genetic potential to produce these metabolites is maintained in naturally ocurring isolates. One potential explanation is that the production of these metabolites may be advantageous during in-planta growth

of the producing fungi. More generally, this tradeoff between growth and the potential to produce these secondary metabolites provides a potential mechanism underlying the maintenance of the diversity of these fungi in soil (Kassen and Rainey, 2004).

The inoculated *Fusarium* isolates did not have a discernible effect on the growth of either plant species under the conditions tested. As there was no effect on plant growth, we found no support for our hypothesis that isolates with the genetic potential to produce specific metabolites would influence plant growth. The absence of an effect of these fungi on plant growth was surprising, but does not mean these fungi do not influence plant growth under other conditions. For example, fungi in the genus *Fusarium* often influence plant phenotypes when the plant is under an abiotic stress (Redman et al., 2011) or challenged with a plant pathogenic fungus (Jaroszuk-Ściseła et al., 2008; Larkin et al., 1996).

In addition to the lack of an effect of these fungi on plant growth, even with some isolates able to infect the plant roots, there was no evidence that these isolates cause plant disease symptoms on either plant species. The argument could be made that under other environments or interacting with other plant species these fungal isolates may induce plant disease symptoms. That being said, there is growing evidence that nonpathogenic *Fusarium* isolates may predominate in the rhizosphere of both annual and perennial plants (Demers et al., 2015; Edel et al., 1997; Nel et al., 2006; Jaroszuk-Ściseła et al., 2008). Additionally, fungi in the genus *Fusarium* display high levels of diversity as endophytes in many different plant species (Demers et al., 2015; Kuldau and Yates, 2000). This suggests that – though fungi in the genus *Fusarium* are well known plant pathogens – the broader relevance of these fungi to the productivity of agricultural and native ecosystems may be answered through their study as nonpathogenic members of diverse soil communities.

Table 5.1: Secondary metabolism PCR genotyping assay developed to screen for the presence or absence of genes previously validated as essential for the production of five different secondary metabolites.

Product[1]	**Acession (Locus)**[2]	**Primers**[3]	**Amplicon size**[4]	**References**[5]
BIK	FOXG04757 (BIK1)	ACCAGCCAAGTCCAGTACG CAGTAACCGTCTGCACCATC	500	(Wiemann et al., 2013)
FA	FOXG15248 (FUB1)	CTCGACTCCTCCAAGACAGG GCCGCAGAGTGAATCAAGAC	500	(Brown et al., 2012)
GA	Y15280 (GGS2)	GATGTCCCGGAAGACAAGAC TTCAGAGTTCAGCGCCAGTA	488	(Tsavkelova et al., 2008)
IAA	FOXG02586 (IAAH)	AGGCCTTCTCAGGGAATGAT GATCGACAGGGGATGCAG	688	(Tsavkelova et al., 2012)
IAA	FOXG02584 (IAAM)	GAGCCGAGCCATGAACTTT TGACGTGCAGTCACTGTTGA	559	(Tsavkelova et al., 2012)
NEP	AF036580 (NEP1)	GCTCCAGGACAGCGGTAGTA AGGCCTTGTTGAGGTTGTTG	506	(Bailey et al., 2002)

[1]Secondary metabolite associated with the given gene: bikaverin (BIK), fusaric acid (FA), gibberellic acid (GA), indole-acetic acid (IAA), and the necrosis and ethylene inducing peptide (NEP).

[2]The nucleotide sequence and locus name in parenthisis used to identify homologous sequences in sequenced *Fusarium* genomes.

[3]Primer sequences (5' to 3') for amplification of the gene associated with the given secondary metabolite.

[4]Predicted size of the amplicon.

[5]Literature reporting deletion of the given gene and its effect on the production of the given secondary metabolite.

Table 5.2: One-way ANOVAs testing the effect of *Fusarium* isolate inoculation on plant growth phenotypes in a growth chamber environment. Individual plant seedlings (14 days post planting) were inoculated with 1 mL (1×10^6 spores/mL) of spores from individual isolates. Each *Fusarium* isolate inoculation treatment had nine replications. Plants were harvested and phenotyped 14 days post inoculation.

Plant[1]	**Phenotype**[2]	**F-statistic**[3]	**P-value**[4]	**Set**[5]
L. capitata	Plant biomass	0.529	0.981	1
	Root length	0.964	0.524	
	Shoot length	0.619	0.942	
A. gerardii	Plant biomass	0.792	0.775	1
	Root length	1.12	0.313	
	Shoot length	1.202	0.224	
L. capitata	Plant biomass	1.406	0.071	2
	Root length	0.765	0.83	
	Shoot length	0.918	0.606	
A. gerardii	Plant biomass	0.767	0.816	2
	Root length	0.755	0.829	
	Shoot length	1.309	0.14	

[1]Plant species inoculated.

[2]Plant phenotype measured.

[3]F-statistic from one-way ANOVA testing the effect of inoculating the isolates on the given plant phenotype.

[4]P-value from one-way ANOVA testing the effect of inoculating the isolates on the given plant phenotype.

[5]Inoculation set consisting of a randomized subset of the total 65 isolates.

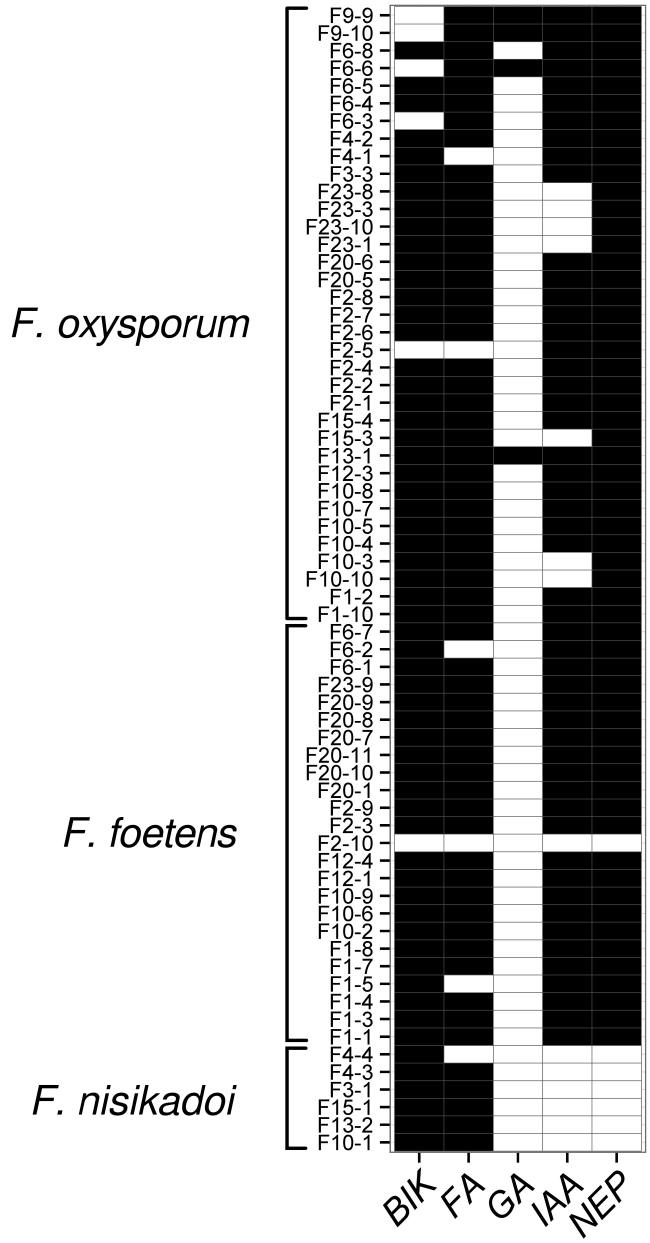

Figure 5.1: Secondary metabolism genotypes of the screened *Fusarium* isolates. Black indicates presence and white absence of the screened genes underlying the production of the secondary metabolites within the screened isolates. The metabolites represented are bikaverin (BIK), fusaric acid (FA), gibberellic acid (GA), indole-acetic acid (IAA), and the necrosis and ethylene inducing peptide (NEP). Taxonomy of the isolates is based on alignment of isolate *RPB2* sequence data to the Fusarium ID database (Geiser et al., 2004) (see Section 4.2.4).

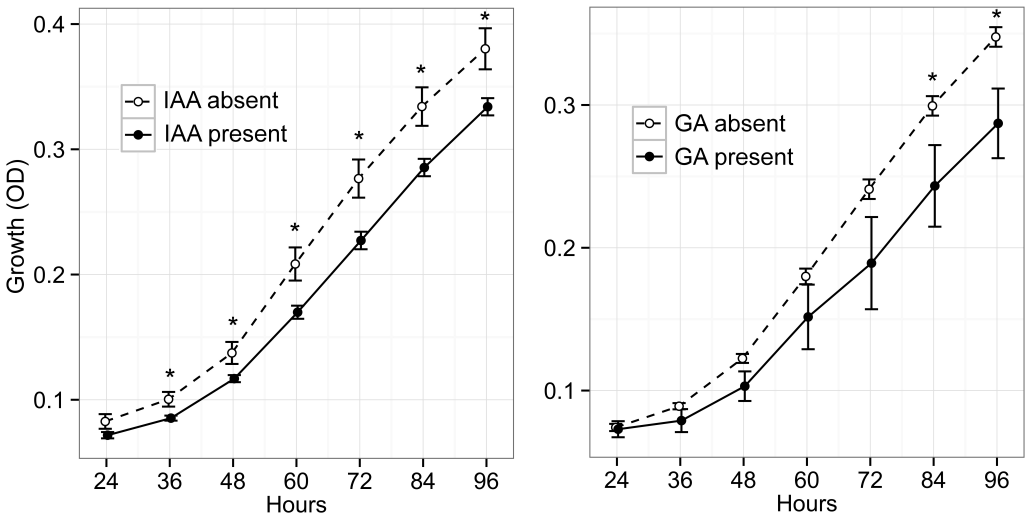

Figure 5.2: Growth of isolates with and without the genetic potential to produce indole-acetic (IAA) and gibberellic acid (GA). Stars indicate a significant ($P < 0.05$) difference between isolates with or without the genetic potential to produce the metabolites at the given timepoint, based on a two-sided t-test.

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