INVASIVE *LESPEDEZA CUNEATA* AND ITS RELATIONSHIP TO SOIL MICROBES AND PLANT-SOIL FEEDBACK

BY

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DISSERTATION

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ABSTRACT

Globalization has lead to increased frequencies of exotic plant invasions, which can reduce biodiversity and lead to extinction of native species. Physical traits that increase competitive ability and reproductive output encourage the invasiveness of exotic plants. Interactions with the soil microbial community can also increase invasiveness through plant-soil feedback, which occurs through shifts in the abundance of beneficial and deleterious organisms in soil that influence the growth of conspecific and heterospecific progeny.

Lespedeza cuneata is an Asian legume that has become a problematic invader in grasslands throughout the United States. While *L. cuneata* has numerous traits that facilitate its success, it may also benefit from interactions with soil microbes. Previous studies have shown that *L. cuneata* can alter bacterial and fungal community composition, benefit more from preferential nitrogen-fixing symbionts than its native congener, *L. virginica*, and disrupt beneficial fungal communities associated with the native grass *Panicum virgatum*. *L. cuneata* litter and root exudates also have high condensed tannin contents, which may make them difficult to decompose and allow them to uniquely influence microbial communities in ways that benefit conspecific but not heterospecific plants. Plant-mediated shifts in the relative abundance of beneficial and deleterious microbes that influence future plant growth are known as plant-soil feedbacks.

The objective of this dissertation is to examine plant-soil feedback in the *L. cuneata* system. In Chapter 2, I conduct a greenhouse experiment that identifies

plant-soil feedbacks between *L. cuneata* and native potential competitors and use whole-community DNA sequencing to identify potential microbial drivers of observed feedbacks. Results showed that *L. cuneata* limits the growth of itself and native plants, but it benefits from microbes that were enriched in native-conditioned soil. This negative plant-soil feedback may facilitate early stages of *L. cuneata* invasion. In Chapter 3, I conduct a greenhouse experiment that examines the role that multiple plant chemical inputs may play in facilitating plant-soil feedback. Results suggest that *in situ* plant-soil feedback is mediated by complex sums of multiple plant inputs, but that root exudates may be especially important for structuring symbiont and pathogen populations. Metabolites from *L. cuneata* chemical inputs may also directly harm native plant growth. In Chapter 4, I conduct a follow up study to determine whether observations from my greenhouse study are applicable in the field. I found that *L. cuneata* invasion has lasting affects on soil microbial community composition in the field, which may be sufficient to drive plant-soil feedback *in situ*. Similar symbiont and decomposer OTUs were identified as being enriched by *L. cuneata* in both the greenhouse and the field.

The results from this dissertation provide a novel examination of the influence of *L. cuneata* on plant-soil feedback patterns. As plant-soil feedbacks influence the outcome of plant competitive dynamics, they may have large implications for effects of exotic plants on invaded ecosystems.

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CHAPTER 1: INTRODUCTION

BACKGROUND

Globalization has greatly increased the distribution and abundances of exotic species around the world (Hulme 2009). Approximately ten percent of these introduced species will spread into surrounding areas, with the potential to establish long-term populations that alter ecosystem processes (Raizada et al. 2008), alter community composition (Hejda et al. 2009), and decrease the abundance of native species (Gaertner et al. 2009).

Once transported, introduced exotic plants can become invasive by overcoming abiotic filters to colonize the new habitat, establishing large population sizes at the introduction site, and dispersing to new sites across the landscape (Theoharides and Dukes 2007, Vermeij 1996). Exotic plants are more likely to become invasive if they have traits that increase their ability to compete with native plants for resources, such as fast growth rates and large leaf areas, and traits that increase their reproductive output, such as high seed number and germination rate (Van Kleunen et al. 2010). Invaders typically produce more above and belowground biomass than their native counterparts (Leishman et al. 2007, Liao et al. 2008). Site characteristics can also influence the invasive success of an exotic plant. Plant communities with low biodiversity may leave light, nutrient, and water resource niches available for exotic species, and thus they may be more susceptible to invasion than sites with high species diversity (Tilman 1997). Additionally, the history of disturbance at a site can create niche

space for exotics by decreasing populations of native species or by directly increasing resource availability (Davis et al. 2000).

Once successfully established, invasive plants often influence the composition and function of soil microbial communities in introduced ranges (Hawkes et al. 2005, Wolfe and Klironomos 2005). Plants interact with and alter the abundance of organisms that they directly associate with, such as mutualistic or pathogenic bacteria and fungi, and they also influence the abundance of soil microbes that mediate nutrient cycling and availability. These influences can have large implications for the success of the invader (Callaway et al. 2004) and the health of the invaded ecosystem (Van Der Heijden et al. 2008). Because distinct plant-microbe interactions can have varying influences on plant growth and invasive success, it is important to understand which particular interactions are at play within a system to understand how microbes may be facilitating or hindering plant invasion.

Plant-microbe relationships that help plants acquire nutrients can have particularly strong effects on invasion success and on invader's impacts on the invaded system. Many plants form influential symbioses with bacteria that fix atmospheric nitrogen into ammonia inside specialized nodules on plant roots in exchange for carbon (Limpens et al. 2005). These symbioses have important implications for plant productivity because they provide an essential nutrient to plants that would otherwise be unavailable (Chapin III 1980).
Relationships between leguminous plants and rhizobia bacteria are the most well-studied examples of nitrogen-fixing symbioses (Van Rhijn and Van der

Leyden 1995), and they have been shown to supply significant portions of nitrogen to plant communities (Van der Heijden et al. 2006a).

Most terrestrial plants also form symbioses with mycorrhizal fungi, which increase the supply of nutrients, most importantly phosphorus, to plants by extending the range of roots, increasing the rate at which nutrients can be taken up, and solubilizing nutrients that are bound to clay minerals in soil (Bolan 1991). These symbioses have been shown to influence plant productivity and diversity (Van der Heijden et al. 1998) and the distribution of nutrients throughout plant communities (Van der Heijden et al. 2006b). Simultaneous plant mutualisms with nitrogen fixing bacteria and mycorrhizal fungi, termed tripartite symbioses, are common and have been shown to increase legume performance and nitrogen fixation (Chalk et al. 2006). Distinct types of mycorrhizal fungi differ in their ability to acquire nutrients from soil and uniquely influence decomposition. Arbuscular mycorrhizal fungi are obligate symbionts that are unable to break down organic matter in soil, and thus rely on taking up inorganic forms of nutrients that have been made available by other microbes in soil. Conversely, ectomycorrhizal fungi produce extracellular enzymes that enable them to increase nutrient availability by decomposing organic matter in soil (Smith and Read 2010). These differences in nutrient acquisition may influence nutrient constraints on plant productivity (Phillips et al. 2013).

Microbes can also harm plant productivity directly as pathogens. Pathogen populations in soil are influenced by plant community composition and soil characteristics (LeBlanc et al. 2017). Microbial pathogens can function as

generalists, which infect a large range of hosts, or specialists, which only infect specific hosts (Woolhouse et al. 2001). Pathogen accumulation under parent plants can limit the germination and growth of nearby offspring despite high seed numbers (Augspurger and Kelly 1984). Thus, specialized pathogens can maintain the diversity of a community by limiting the growth of host species and facilitating coexistence (Chesson 2000). Pathogens can facilitate plant community succession by reducing the growth of sensitive plant species and creating space for the growth of less sensitive species (Van der Putten et al. 1993).

Microbes can also influence plant productivity without participating in specific symbioses. Free-living soil microbes can increase nitrogen availability to plants through the secretion of extracellular enzymes that mineralize organic material in soil, producing inorganic forms of nitrogen that are available for plant uptake (Schimel and Bennett 2004). Free-living nitrogen-fixing bacteria can also contribute significant portions of nitrogen into soil (Cleveland et al. 1999). Microbes can also increase nutrient availability to plants by weathering primary materials in soil (Landeweert et al. 2001) and solubilizing phosphorus (Kucey 1983). Conversely, microbes can limit nutrient availability to plants by competing for nutrient resources and immobilizing nutrients in microbial biomass (Bardgett et al. 2003). Free-living microbes can also increase plant yields by producing plant hormones that directly stimulate plant growth and development (Steenhoudt and Van der Leyden 2000), and suppressing pathogens (Bais et al. 2004).

Plant-microbe interactions can play a role in facilitating or hindering invasion by exotic plant species. Because specialized host-pathogen pairs have co-evolved together over time (Combes and Théron 2000), exotic plants may experience a release from specialist pathogens in introduced ranges (Keane and Crawley 2002). This release from pathogen pressure may enable exotic plants to allocate more resources to growth instead of defense mechanisms, thus facilitating invasion (Callaway and Ridenour 2004). Additionally, the availability of compatible symbionts in introduced ranges may determine the invasive success of an exotic plant that relies on specific mutualisms for nutrient supply (Parker et al. 2006).

Plant-mediated shifts in the relative abundance of beneficial and deleterious organisms in soil that impact future con- and heterospecific plant growth are known as plant-soil feedback (Bever 2002, Mills and Bever 1998). Plant-soil feedback functions as a density-dependent force that influences the outcome of plant competition (Bever 2003). Positive plant-soil feedback works in concert with competition, and increases the likelihood of competitive exclusion and the dominance of the stronger competitor. Negative plant-soil feedback works in opposition to competition, and increases the likelihood of stable coexistence of competitors. Plant-soil feedback can have dramatic influences on plant community structure (Mangan et al. 2010), and may influence important processes such as succession (Van de Voorde et al. 2011), community development after land use change (Kardol and Wardle 2010), and soil nutrient cycling (Lee et al. 2012).

Plant-soil feedback has been shown to both facilitate and hinder invasion by exotic plant species (Bever et al. 2010, Klironomos 2002, Suding et al. 2013, Van der Putten 2010). Previous work suggests that invaders may experience more positive plant-soil feedback in introduced ranges than native ranges due to pathogen release (Mitchell and Power 2003), but studies have also observed negative plant-soil feedback for successful invaders (Nijjer et al. 2007, Suding et al. 2013). Negative plant-soil feedback can contribute to invasive success by creating heterogeneous soil environments and patchy distributions of plant species that can lead to mutual invasibility (Burns and Brandt 2014), allowing the invader to establish populations at newly introduced sites. Although shifts in the abundance of beneficial and deleterious microbes are the underlying cause of plant-soil feedback, there has been very little work investigating which specific microbes are responsible for plant-soil feedback to native and exotic plants.

Plant litter inputs from living or dead plants can facilitate plant-soil feedback. Litter mediated plant-soil feedback operates through fluctuations in primary productivity or the nutrient content of litter inputs, which drive nutrient cycling and resource availability for subsequent generations of plants (Aerts 1997, Aerts et al. 2003). Because plants compete for nutrient resources in soil, litter decomposition dynamics have the capacity to influence plant competition (Berendse 1998). Nitrogen-rich litter tends to decompose quickly and stimulate nutrient mineralization, while nitrogen-poor litter tends to decompose more slowly (Lavorel and Garnier 2002). Additionally, leachates from leaf tissue often contain

high concentrations of phenolic compounds that can slow decomposition and nutrient release in soil (Kalburtji et al. 1999, Sariyildiz and Anderson 2003).

Plants also facilitate plant-foil feedback through the release of carbon and metabolites into soil in the form of root exudates, which alter the abundance of organisms in the zone of influence of the root (Ehrenfeld et al. 2005). The amount and composition of root exudates secreted into soil varies by plant species and characteristics such as plant age, soil type, plant stress and nutrient availability (Badri and Vivanco 2009). Root exudates contain large quantities of ions, free oxygen, water, enzymes, mucilage, and carbon-based primary and secondary metabolites (Uren 2007) into soil. Labile carbon compounds in root exudates are readily available for consumption by soil microbes, which leads to abundant microbial populations in plant rhizospheres (Bais et al. 2006). The rapid utilization of these labile carbon compounds can stimulate decomposition and nutrient cycling in soil (Meier et al. 2017, Phillips et al. 2011). Secondary metabolites in root exudates can function as chemoattractants that attract potential symbionts and pathogens towards roots (Bacilio-Jiménez et al. 2003, Zheng and Sinclair 1996). Flavonoid and strigolactone components of root exudates are especially important for the establishment of mutualisms with nitrogen-fixing bacteria and mycorrhizal fungi, respectively (Akiyama et al. 2005, Besserer et al. 2006, Peters et al. 1986, Phillips 2000). The individual contribution of plant chemical inputs from litter and roots to plant-soil feedback is currently unknown.

OVERVIEW OF DISSERTATION

Lespedeza cuneata is a perennial legume, introduced from Asia in the late 1800's, that has become a problematic invader throughout the United States (Cummings et al. 2007, Eddy and Moore 1998). *L. cuneata* has a variety of physical traits, such as large aboveground biomass (Allred et al. 2010), high reproductive output (Woods et al. 2009), and high stress tolerance (Allred et al. 2010), that contribute to its invasive success. In addition to these physical traits, chemical traits of *L. cuneata* may also facilitate invasive success. Both litter residues and root exudates from *L. cuneata* have been shown to decrease the germination or growth of native grasses (Kalburtji and Mosjidis 1992, Kalburtji and Mosjidis 1993a, Kalburtji and Mosjidis 1993b), and phenolic compounds isolated from *L. cuneata* litter residues have been shown to be phytotoxic (Langdale and Giddens 1967). Additionally, there is preliminary evidence that *L. cuneata* root exudates are chemically distinct from those of native congeners and grasses (Ringelberg et al. 2017, *in press*).

L. cuneata invasion has also been shown to alter bacterial and fungal communities at invasion sites (Yannarell et al. 2011). *L. cuneata* has been shown to disrupt mycorrhizal fungal communities associated with the native grass, *Panicum virgatum* (Andrews 2011). *L. cuneata* may also benefit more from nitrogen-fixing symbionts (Hu et al. 2014) and may have a stronger preference for interacting with Rhizobiales nitrogen fixers than its native counterpart, *L. virginica* (Busby et al. 2016). Additionally, there is evidence that arbuscular

mycorrhizal fungal associations can increase *L. cuneata* yields in phosphorus-limiting environments (Wilson 1988).

The degree to which chemically mediated plant-microbe interactions influence interactions between invasive *L. cuneata* and native plants is currently unknown. *L. cuneata*'s unique chemistry and interactions with microbes suggest that plant-soil feedback may play a role in facilitating *L. cuneata* dominance. Therefore, the purpose of this dissertation is to examine microbially and chemically mediated plant-soil feedback between *L. cuneata* and the native plants *L. virginica* and *P. virgatum*. In **Chapter 2**, I conduct a greenhouse experiment to characterize pairwise plant-soil feedback between these plants, and use whole-community DNA sequencing to identify potential microbial drivers of observed feedbacks. In **Chapter 3**, I conduct a second greenhouse experiment to examine plant-soil feedback generated by *L. cuneata* root exudates, root leachates, and litter leachates, and identify unique chemical components of these solutions that may drive observed plant-soil feedbacks. In **Chapter 4**, I conduct a follow-up observational study to show that *L. cuneata* invasion has lasting influences on soil microbial communities that could be driving plant-soil feedback in the field. Taken together, these chapters offer a novel examination of the plant-microbe interactions that may facilitate dominance by *L. cuneata*.

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CHAPTER 2: DISRUPTION OF NATIVE-ASSOCIATED MICROBIAL COMMUNITIES DRIVES PLANT-SOIL FEEDBACK BETWEEN NATIVE PLANTS AND *LESPEDEZA CUNEATA*[1]

ABSTRACT

Aims: Invasive plants, such as *Lespedeza cuneata*, may benefit from interactions with soil communities, and the resulting plant-soil feedback can influence coexistence with native plants. Our objective for this study is to characterize plant-soil feedback between invasive *Lespedeza cuneata* and its potential native competitors, and to document potential microbial drivers of observed feedback.

Methods: We conducted a greenhouse experiment to compare the effect of con- and heterospecific-conditioned microbes on *L. cuneata* and native plant biomass, and we used DNA sequencing to compare the effect of plant conditioning treatments on soil bacterial, fungal, and nitrogen-fixing communities. We extracted constrained correspondence analysis axis scores to identify microbes that were enriched under each conditioning treatment.

Results: We found negative feedback between *L. cuneata* and its native congener *L. virginica*, and neutral feedback between *L. cuneata* and the native grass *Panicum virgatum*. *L. cuneata* experienced increased growth in the presence of native-conditioned microbes.

Conclusions: Overall, these findings suggest that increased *L. cuneata* growth in the presence of native-selected microbes may be important for the *L. cuneata* invasion process.

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INTRODUCTION

Plant-mediated changes to the soil microbial community can impact the growth of both con- and heterospecific plants, and these changes may play a role in facilitating or hindering invasion by non-native plant species. Changes to soil microbial communities can have serious impacts on plant community composition and productivity (Van der Heijden et al. 2008, Schnitzer et al. 2011). Plant growth benefits from interactions with both specific mutualistic partners and general soil microbial communities, which mediate nutrient availability and increase plant yields in numerous other ways (Gray and Smith 2005). Conversely, microbes can be deleterious for plant health and productivity (Dodds and Rathjen 2010). Plants communicate with and attract specific microbes toward their roots via root exudation (Bais et al. 2004). By mediating shifts in the abundance of beneficial and deleterious microbes in the soil (Mills and Bever 1998, Bever 2002), plants can impact the growth of their progeny and their competitors through plant-soil feedback (Bever et al. 1997). If the growth of a plant species alters soil communities in ways that confer a net positive effect on future conspecifics, either by benefitting itself or by harming competing species, then it will have a positive feedback on future conspecific plant growth. Conversely, if the growth of a plant species alters soil communities in ways that confer a net negative effect on future conspecifics, either by harming itself or benefitting competing species, then it will have a negative feedback on future conspecific plant growth.

Plant-soil feedback functions as a frequency-dependent force that can work in concert with or against interspecific competition between plants (Bever 2003). Positive feedback works in concert with competition and increases the likelihood of competitive exclusion. Conversely, negative feedback works in opposition to competition and increases the likelihood of coexistence between two plant species. Thus, plant-soil feedback can have large-scale influences on the structure of *in situ* plant communities (Mangan et al. 2010), plant community succession (Van de Voorde et al. 2011), plant community development after land use change (Kardol and Wardle 2010), and soil nutrient cycling (Lee et al. 2012).

Both positive and negative plant-soil feedbacks may play a role in facilitating successful plant invasion (Klironomos 2002, Bever et al. 2010, Van der Putten 2010, Suding et al. 2013). Invasive plants have been shown to be both positive and negatively influenced by growth in invaded soil (Suding et al. 2013). Positive feedback on the growth of the invader in introduced systems can be carried out through a variety of mechanisms. Invaders can experience a release from natural enemies in introduced ranges (Mitchell and Power 2003), and those that do accumulate local pathogens may be less harmed by them than their native neighbors (Eppinga et al. 2006). In addition to this release from pathogen pressure, invaders may benefit from the presence of soil mutualists (Klironomos 2002), and invaders have been shown to limit native plant growth by disrupting native soil mutualist communities (Stinson et al. 2006). Thus, a combination of reduced pathogen pressure and increased mutualist benefit compared to native plants may lead to positive plant-soil feedback that facilitates

dominance by the invader. However, negative plant-soil feedback can also support plant invasions. In negative plant-soil feedback, a rare plant species has a growth advantage at low frequencies that can help its population to grow (Bever et al. 1997, Bever 2002). In this way, negative plant-soil feedback may confer a growth advantage to an invader that occurs at low frequency, and this can allow the invasive plant to gain a "foothold" in newly invaded ecosystems. Negative plant-soil feedback increases the heterogeneity of the soil environment and the ability of both plants to maintain individuals within competitor populations (Burns and Brandt 2014). This maintenance of invader populations within native communities poises the invader to take advantage of any niche opportunities that arise from a reduction in native plant populations or an alteration in resource availability (Shea and Chesson 2002). Thus, negative feedback may contribute to the colonization of low-density invader populations that may increase in size under the appropriate circumstances.

In addition to mediating changes to soil microbial communities and downstream plant-soil feedbacks, plants can directly affect future plant growth by altering soil nutrient availability. Plants take up multiple forms of nitrogen from soil (Weigelt et al. 2005) and can decrease nitrogen in soil throughout the growing season (Koottatep and Polprasert 1997). Plants can also increase nitrogen availability in soil through high quality litter inputs and symbiotic nitrogen fixation (Knops and Tilman 2000). These alterations to nitrogen availability can directly influence plant competition (Wilson and Tilman 1991) and therefore affect the outcome of plant-soil feedback.

Lespedeza cuneata is a widespread grassland invader that was introduced into the United States from Asia during the late 1800's (Eddy and Moore 1998). In addition to its ability to alter plant community composition (Dudley and Fick 2003), previous work suggests that *L. cuneata* may have distinctive interactions with members of the soil microbial community. *L. cuneata* has been shown to alter bacterial and fungal community composition (Yannarell et al. 2011), and to disrupt mycorrhizal fungal communities associated with the native grass, *Panicum virgatum* (Andrews 2011). Additionally, *L. cuneata* participates in symbioses with nitrogen-fixing bacteria, and may benefit more from this symbiosis than its native counterpart, *L. virginica* (Hu et al. 2014). *L. cuneata* may also have a stronger preference for associating with Rhizobiales nitrogen-fixers than native *Lespedeza* species (Busby et al. 2016). *L. cuneata* has been shown to increase nitrogen concentrations at nutrient depleted sites over time (Lynd and Ansman 1993). This ability to influence soil resources and plant and microbial communities suggests that plant-soil feedback may be involved in structuring *L. cuneata*-dominated plant communities.

In this study, we used a greenhouse plant-soil feedback experiment and microbial community analysis to examine the role that plant-soil feedbacks may play in *L. cuneata* invasion. We address the following research goals: 1) characterize plant-soil feedback patterns that exist between *L. cuneata* and the native plants *L. virginica* and *P. virgatum*, and 2) examine the effects that conditioning treatments have on soil nitrogen availability and the composition of soil microbial communities that may be driving observed feedbacks.

METHODS

Greenhouse Experiment

We performed a two-phase "home and away" experiment (Bever et al. 1997) to determine whether *Lespedeza cuneata* and two native plants experience positive or negative plant-soil feedback (i.e. "home effects") and whether plant-soil feedback between pairs of plant species would reinforce or oppose competitive exclusion (i.e. "away effects"). Our experiment consisted of a *conditioning phase*, in which plants from each of our targeted species influenced the assembly of soil microbial communities in sterile soil containing a starter inoculum of prairie microbes, and a *feedback phase*, in which subsequent con- and heterospecific plant growth was quantified (Van der Putten et al. 2007). The effect that each conditioning treatment had on plant growth was determined by comparing plant growth in the conditioned soil to plant growth in sterile soil with no microbial community.

Live prairie soil was collected from John English Memorial Prairie, Comlara Park, Mclean County, IL, USA in spring 2015. This live soil was stored at 4°C for 24 hours before use as a 3% by volume inoculum into autoclave-sterilized potting soil made up of equal parts soil, calcinated clay and torpedo sand. Concurrently, we produced a sterile soil control by autoclave sterilizing the soil from the prairie and using this as a "killed" inoculum (3% by volume) into sterilized potting soil. To begin the conditioning phase of the experiment, we placed approximately 450 cubic centimeters of the live-inoculated soil mixture into 45 pots and 450 cubic centimeters of the killed inoculum soil mixture into 45

pots. Five *L. cuneata*, *L. virginica*, or *P. virgatum* seeds were placed in each conditioning pot (n=30) and were allowed to germinate. After two weeks, pots were thinned to contain one seedling each. Each plant was allowed to influence microbial community assembly over a total period of three months. Plants were watered weekly with sterile water and no nutrient solution was added.

After this conditioning phase, plant tissue was removed and soil was homogenized within each pot. A subsample of soil was taken for subsequent analysis of the soil microbial community that developed during conditioning by each plant species. In order to examine the effect of each plant's selection of soil microbes on the subsequent growth of each plant species, the remaining soil from each pot was divided into thirds and placed into new, sterile pots. These 270 feedback pots were filled with sterile soil to a total volume of approximately 450 cubic centimeters and homogenized. For the feedback phase of the experiment, we again placed five *L. cuneata*, *L. virginica*, or *P. virgatum* seeds in each pot (n=45) and thinned to one seedling per pot after two weeks of growth. Plants were watered weekly and no nutrient solution was added. After three months of growth, aboveground plant tissue was collected and air-dried to a constant weight, and the mass was recorded.

Soil Nitrogen and Microbial Community Analysis

Subsamples of soil were taken from each pot after the conditioning phase. A portion of each subsample was air-dried and used to examine soil nitrogen availability. Inorganic nitrogen was extracted by shaking 10g soil in 40mL 2M KCl for 1 hour. Extracts were passed through a Whatman #42 filter and frozen until

colorimetric analysis for ammonium and nitrate concentrations (Weatherburn 1967, Doane and Horwáth 2003).

The remainder of each subsample of soil was lyophilized and used to examine microbial community assembly under the influence of each plant species. For each sample, we extracted DNA from 500mg of soil, using the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA) following manufacturer instructions. After extraction, we purified DNA by incubating with 1% cetyltrimethyl ammonium bromide (CTAB) for 15 minutes at 65°C, re-extracting DNA in 24:1 chloroform:isoamyl alcohol, and washing with cold ethanol. Purified DNA was eluted in 1x TE buffer, and stored at a concentration of 20ng/uL at -80°C until use.

We characterized bacterial, fungal, and nitrogen-fixing communities by sequencing genes that are commonly used for the identification of these organisms. Samples were diluted to a concentration of 2ng/uL and specific gene targets were amplified simultaneously using Fluidigm 2 Step Access Array Amplification on a Fluidigm 48.48 Access Array IFC (Fluidigm Corporation, San Fransisco, CA), following manufacturer instructions. Unique nucleotide barcodes were added to PCR reactions to identify individual samples so that all PCR products could be pooled and sequenced together. We used primers 515F and 926F to amplify the V4-V5 region of the bacterial 16S rRNA gene (Walters et al. 2016), primers ITS3 and ITS4 to amplify the fungal second internal transcribed spacer region (ITS2) (White et al. 1990), and primers PolF and PolR to amplify the nitrogen-fixing gene, *nifH* (Poly et al. 2001). PCR products were confirmed on

a Fragment Analyzer (Advanced Analytics, Ames, IA) and pooled in equimolar concentrations. Pooled products were size-selected on a 2% agarose E-gel (Life Technologies, Carlsbad, CA) and extracted with Qiagen gel extraction kit (Qiagen, Hilden, Germany). Size selected products were run on an Agilent Bioanalyzer (Agilent, Santa Clara, CA) and pooled. Pooled amplicons were sequenced on an Illumina MiSeq V3 platform using a 2x250 base pair read configuration in Bulk Kit version 3 (Illumina, San Diego, CA, USA). PCR reactions and sequencing were carried out at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

Raw microbial sequence data was analyzed using a variety of platforms. We merged the forward and reverse reads of each paired-end sequence using Fast Length Adjustment of Short reads (FLASH) software (Magoč and Salzberg 2011), removing sequences that contained greater than 10% of bases with quality scores below 30 and ambiguous bases. For the remaining sequences, we used USEARCH (http://www.drive5.com/usearch/) to 1) de-replicate sequences and remove singletons, 2) remove chimeric sequences detected by the GOLD database for bacteria (Kyrpides 1999), the UNITE ITS database for fungi (Abarenkov et al. 2010), and a custom *nifH* database created by downloading sequences from the RDP FunGene website (Fish et al. 2013) and assigning taxonomic information using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990); and 3) cluster sequences by 97% similarity to form operational taxonomic units (OTUs). We aligned representative sequences for each bacterial OTU, and left fungal and *nifH* sequences unaligned. For

bacteria, we assigned taxonomic information using the RDP classifier in QIIME (Caporaso et al. 2010). For fungi and nitrogen fixers, we assigned taxonomic information using the BLAST algorithm (Altschul et al. 1990) and the same databases mentioned in step 2 above. Representative *nifH* OTUs that could not be matched to any taxonomic information in the database using the BLAST algorithm were removed.

Data Analysis

We calculated plant-soil feedback for each pair of plant species in our study using Bever's interaction coefficient, $I_s = \alpha_A - \alpha_B - \beta_A + \beta_B$ (Bever et al. 1997), where α_A is the growth of plant A with conspecific-conditioned microbes minus the growth of plant A in sterile soil ($\alpha_A = G_{A\alpha} - G_{A_\oslash}$), α_B is the growth of plant B with heterospecific-conditioned microbes minus the growth of plant B in sterile soil ($\alpha_B = G_{B\alpha} - G_{B_\oslash}$), β_A is the growth of plant A with heterospecific-conditioned microbes minus the growth of plant A in sterile soil ($\beta_A = G_{A\beta} - G_{A_\oslash}$), and β_B is the growth of plant B with conspecific-conditioned microbes minus the growth of plant B in sterile soil ($\beta_B = G_{B\beta} - G_{B_\oslash}$). Positive I_s means that the plant-soil feedback always favors one plant species over the other, while negative I_s means that plant-soil feedback can facilitate coexistence between the two plant species (Bever et al. 1997). We also interpreted these α and β terms as estimates of the beneficial or harmful effect of each microbial community on plant growth (i.e. negative α_A represents a net harmful effect of plant A's microbes on its own growth). We also compared the average ammonium and nitrate concentrations of each conditioning treatment. Analysis of variance (ANOVA)

was used to determine the significant influence of conditioning treatments on feedback plant biomass, soil ammonium, and soil nitrate. Post-hoc t-tests were performed to determine significant differences between individual treatment effects.

Processed sequence data was analyzed using the vegan() package (Oksanen et al. 2009) for the R statistical environment (R Core Development Team 2014). Bacterial, fungal, and nitrogen-fixing communities were analyzed individually. To determine if treatments explained a significant portion of variance in microbial community composition after conditioning, we performed permutational multivariate analysis of variance (perMANOVA) (Anderson 2001) using the adonis() function. We visualized the effects of conditioning treatments on microbial community composition using non-metric multidimensional scaling (NMDS) and the plotting function ordiplot(). Significant correlations between soil nitrogen and microbial community composition were determined using the function envfit() and were plotted onto ordinations. In order to identify microbial OTUs that were enriched by each conditioning treatment, we performed canonical correspondence analysis using the function cca() to explore the variation in microbial community composition that was due to plant identity. Axis scores that corresponded to the centroid of microbial OTUs clustered by plant identity were extracted, and the top 5% of OTUs were compared at the phyla (bacteria, fungi) or genus (*nifH*) level.

RESULTS

We found negative plant-soil feedback (that is, $I_S < 0$) between invasive *L. cuneata* and its native congener *L. virginica*, neutral feedback between *L. cuneata* and the native grass *P. virgatum*, and positive feedback ($I_S > 0$) between native plants *L. virginica* and *P. virgatum* **(Figure 2.1)**. Conditioning treatments influenced subsequent plant growth (ANOVA, F=22.09, $p<0.0001$, **Table 2.1**, **Figure 2.2**) and soil ammonium concentration (ANOVA, F=4.767, $p<0.014$, **Figure 2.3**). Soil conditioned by legumes contained significantly more ammonium than soil conditioned by *P. virgatum* (post-hoc T-tests, $p<0.05$). Overall, conditioning treatments did not significantly influence soil nitrate concentration (ANOVA, F=2.883, $p=0.068$), however, *P. virgatum* conditioned soil contained significantly less nitrate than *L. cuneata* conditioned soil (post-hoc T-test, $p<0.05$). *L. cuneata* plants grew larger in the presence of microbes from either native conditioning treatment, but they did not benefit from conspecific-conditioned soil (post-hoc T-tests, $p<0.05$). Similarly, *L. virginica* grew larger in the presence of native-conditioned microbes, but it experienced no benefit from soil microbes conditioned by *L. cuneata* (post-hoc T-tests, $p<0.05$). Conversely, microbes from every conditioning treatment hindered *P. virgatum* growth. Legume-conditioned soil was significantly more harmful for *P. virgatum* than conspecific-conditioned soil (post-hoc T-tests, $p<0.05$) **(Figure 2.2)**.

Plant conditioning treatments led to the development of distinct microbial community assemblages (perMANOVA, $p<0.05$)**(Figure 2.4)**. Soil ammonium after conditioning was correlated to bacterial and fungal community composition,

but not to nitrogen-fixing community composition. All conditioning treatments led to the enrichment of diverse bacterial communities, with key differences in enriched OTUs observed at the phyla level **(Figure 2.5)**. *P. virgatum* enriched for OTUs within the phyla Fibrobacteres, Chlamydiae, and Tenericutes, which were not enriched under either legume. Enriched OTUs under *P. virgatum* were also made up of proportionally more Proteobacteria, Armatimonadetes, and Verrucomicrobia, and proportionally less Chloroflexi and Actinobacteria than those of either legume. *L. virginica* enriched for proportionally more Gemmatimonadetes and Firmicutes, and less Acidobacteria than *P. virgatum* and *L. cuneata*. *L. cuneata* enriched for more Planctomycetes and fewer Bacteroidetes than *L. virginica* and *P. virgatum*.

Conditioning treatments also lead to the enrichment of distinct nitrogen-fixing bacterial OTUs. Diverse nitrogen-fixing communities were enriched for in all conditioning treatments **(Figure 2.6)**. Both legumes enriched for distinct nitrogen-fixing OTUs that were dominated by *Bradyrhizobium spp*. P. virgatum enriched for distinct nitrogen-fixing OTUs that were not dominated by *Bradyrhizobium spp*. No OTUs were found to be associated with multiple conditioning treatments.

L. cuneata enriched for fewer fungal OTUs within the phylum Basidiomycota than both *P. virgatum* and *L. virginica* **(Figure 2.7)**. Within these enriched Basidiomycota, *L. cuneata* selected for ectomycorrhizal fungi within the family Clavariaceae, *L. virginica* selected for ectomycorrhizal fungi within the families Clavariaceae, Cortinariaceae, and Ceratobasidiaceae, and *P. virgatum*

selected for ectomycorrhizal fungi within the families Clavariaceae, Cortinariaceae, Sebacinaceae, and Geminibasidiaceae **(Table 2.2)**. Additionally, *L. cuneata* enriched for the most diverse plant pathogenic basidiomycotal OTUs (*Entoloma crassicystidiatum*, *Hyphodermella rosea*, and *Sporobolomyces symmetricus)*. Native plants enriched for less diverse potentially pathogenic OTUs. *L. virginica* enriched for OTUs within the class Exobasidiomycetes, which contains plant pathogens. *P. virgatum* enriched for the plant pathogenic basidiomycotal OTU *Gliophorus europlexus*, and for OTUs that belonged to the genus *Cryptococcus*, which contains plant pathogens. *L. virginica* enriched for OTUs within the class Exobasidiomycetes, which contains plant pathogens. Interestingly, *P. virgatum* also enriched for basidiomycotal OTUs within the genus *Clitopilus*, which contains pathogen suppressive microbes.

DISCUSSION

As a first step toward understanding the role that plant-soil feedback may play in facilitating *L. cuneata* invasion, this study characterized feedback between *L. cuneata* and its native potential competitors, *L. virginica* and *P. virgatum*. We found neutral to negative feedback between *L. cuneata* and these native plant species**.** Our results suggest that plant-soil feedback should increase the likelihood of coexistence between invasive *L. cuneata* and its native congener *L. virginica*. However, by contributing to the heterogeneity of the soil environment and mutual invasibility of invasive and native populations (Burns and Brandt 2014), negative feedback may help to maintain low population densities of the invader that can increase in size if niche opportunities arise (Shea and Chesson

2002). Negative feedback has been shown to increase the population growth rates of rare species (Bever et al. 1997), and may therefore benefit invaders during the initial stage of invasion, when they are minor components of the plant community. Thus, the observed negative feedback between *L. cuneata* and *L. virginica* may facilitate invasion by helping *L. cuneata* to establish low population densities in a newly introduced range. This interpretation is supported by a previous observation that *L. cuneata* maintained low densities upon introduction into a restored prairie for years before experiencing a population boom that coincided with a decrease in soil nutrient resources (**Chapter 4**).

Conversely, positive plant-soil feedback was observed between the native plants *L. virginica* and *P. virgatum*, and the nature of this feedback favored the dominance of *L. virginica*. This finding is generally inconsistent with prior work, which has shown that most feedbacks that exist between native species are negative (Bever 1994, Klironomos 2002, Kulmatiski et al. 2008). Positive feedback has a destabilizing effect that can reinforce the competitive exclusion of one species (Bever 2003). Grasses are generally considered to have a competitive advantage over legumes for light and water (Haynes 1980). Grasses outcompete legumes in nitrogen-rich environments, but benefit from increased legume yields and nitrogen fixation in nitrogen-limited environments (Trannin et al. 2000). Positive plant-soil feedback supports *L. virginica* dominance may balance competitive disadvantages against *P. virgatum* and maintain sufficient *L. virginica* populations so that they can increase when nitrogen resources become depleted.

L. cuneata benefitted from soil that was conditioned by native plants, but did not experience any benefit from conspecific-conditioned soil. This is consistent with other studies that have documented increased exotic growth in soil conditioned by native plants (Nijjer et al. 2007, Scharfy et al. 2010, Suding et al. 2013), and those that suggest that native plants may play a role in facilitating exotic invasion (Smith et al. 2004). However, prior work on the *L. cuneata* system suggests that *in situ* soils conditioned by plant communities containing *L. cuneata* may increase *L. cuneata* yields (Coykendall and Houseman 2014, Crawford and Knight 2017). Both of these studies examined the growth of plants in soils conditioned by communities of plants either including *L. cuneata* or not, as opposed to soils conditioned by *L. cuneata* alone. Thus, the conditioning treatments in these experiments represent the sum of effects of all plant species on the soil microbial community. It is possible that the net effects of these mixed communities of plants was enough to overcome the negative impacts of *L. cuneata*'s own microbes that are documented in this study.

Similarly, *L. virginica* did not benefit from microbes in *L. cuneata*-conditioned soil, but did benefit from native-conditioning treatments. This lack of benefit in *L. cuneata* soil suggests that *L. cuneata* may disrupt the native symbiont community that is required to achieve high native plant yields. This interpretation is supported by prior evidence that the degradation of mutualist populations can limit native plant growth (Stinson et al. 2006, Vogelsang and Bever 2009). Because we sampled bulk soil, as opposed to soil that was directly associated with roots, our study may have been biased against finding important

arbuscular mycorrhizal symbionts, which require a plant host to survive (Kirk et al. 2004). Because of prior evidence that arbuscular mycorrhizal symbionts can increase *L. cuneata* growth under phosphorus limitation (Wilson 1988), future studies should be designed to identify arbuscular mycorrhizal fungi that may be important for plant-soil feedbacks in the *L. cuneata* system.

Our results showed that microbial communities associated with *L. cuneata* conditioned soil contained less diverse ectomycorrhizal fungal OTUs. Although grasses and forbs, like *L. cuneata* and the native plants examined in this study, do not typically associate with ectomycorrhizal fungi (Smith and Read 2010), a decrease in the diversity of this symbiont pool may have negative implications for native woody plants within this system. Prior work has shown that diverse ectomycorrhizal symbiont communities may benefit plant growth (Baxter and Dighton 2005). In addition to their roles as beneficial symbionts, ectomycorrhizal fungi actively decompose soil organic matter and release mineral nutrients from the parent material of soil (Smith and Read 2010, Landeweert et al. 2001). Ectomycorrhizal fungal strains differ in their ability to break down phenolic compounds (Court et al. 2006), and their tolerance of high nitrogen (Arnebrant 1994) and low pH (Hung and Trappe 1983) environments. Therefore, the association of less diverse ectomycorrhizal fungi with *L. cuneata* conditioning in this experiment may reflect distinct plant chemical or nutrient inputs. Shifts in nutrient acquisition by mycorrhizal communities can have large scale implications for carbon and nitrogen cycling within ecosystems (Phillips et al. 2013).

Additionally, we found that nitrogen-fixing bacteria within the genus *Bradyrhizobium* dominated communities that were associated with *L. cuneata*- and *L. virginica*-conditioned soil, but not soil conditioned by *P. virgatum*. *Bradyrhizobium* is the preferred nitrogen-fixing symbiont of *L. cuneata* (Busby et al. 2016) and has been shown to be more beneficial for *L. cuneata* yields than for *L. virginica* (Hu et al. 2014). Our observation of increased soil ammonium in legume-conditioned compared to *P. virgatum*-conditioned soil suggests that nitrogen fixation is occurring within these plant hosts and is sufficient to alter soil resources. An alternative explanation is that *P. virgatum* plants took up more soil nitrogen over the course of the conditioning phase. Because *L. cuneata* has been shown to increase soil nitrogen levels in the field (Lynd and Ansman 1993), it is likely that leguminous nitrogen fixation played at least a partial role in influencing soil nitrogen levels. *L. cuneata* and *L. virginica* enriched for unique *Bradyrhizobium* OTUs, which suggests that each of these plants attracts unique symbiont communities. Because *L. cuneata* has previously been shown to benefit more from *Bradyrhizobium* spp. inoculation than *L. virginica*, this specific attraction may be important for *L. cuneata* invasion. However, because both legumes performed equally well in soil conditioned by *P. virgatum*, which was not associated with *Bradyrhizobium*-dominated communities, and *L. virginica*, it seems unlikely that nitrogen-fixing symbiont availability influenced the plant-soil feedbacks observed in this study. Ten *Bradyrhizobium* OTUs were abundant in soil from all three conditioning treatments. These OTUs may function as important nitrogen-fixing symbionts, but would not have been classified as *P.*

virgatum-enriched with the methods used in this study. Additionally, some of the non-*Bradyrhizobium* nitrogen-fixing bacteria that were associated with *P. virgatum* conditioning may serve as sufficient symbionts for *L. cuneata*. There is some evidence that *L. cuneata* may be a promiscuous host (Gu et al. 2007). Future studies should examine the effect of individual symbiont strains on L. cuneata and native plant growth.

While both legumes observed in this study generally benefitted from the presence of soil microbes, *P. virgatum* was harmed by the presence of microbes from all conditioning treatments. *L. cuneata*-conditioned soil was most deleterious. As prior work has shown that *L. cuneata* also hinders the growth of the native grass *Sorghastrum nutans* (Coykendall and Houseman 2014), it may be that *L. cuneata* has a strongly negative impact on grasses in general. Future studies should be designed to examine the generality of feedbacks observed in this study. This negative influence of soil microbes on *P. virgatum* growth may have been driven by soil pathogens. *L. cuneata*-conditioned soil was associated with the most diverse fungal plant pathogens in this study. Switchgrass-conditioned soil was also associated with a fungus from the genus *Clitopilus*, which is known to produce pathogen-suppressing antibiotics (Kilaru et al.). This may explain why switchgrass yields were higher in switchgrass-conditioned soil compared to legume-conditioned soil.

We also observed differences in the types of cellulose- and lignocellulose-degrading bacteria that were associated with each conditioning treatment. Legume-conditioned soil was associated with more Actinobacteria and fewer

Bacteroidetes than *P. virgatum*-conditioned soil. *P. virgatum*-conditioned soil was also associated with Fibrobacteres OTUs. These types of organisms degrade cellulose and lignocellulose molecules in dead plant material (Pankratov et al. 2006, Ransom-Jones et al. 2012, Naas et al. 2014). Because cellulose-degrading bacteria influence the rate of litter decay in an ecosystem (Purahong et al. 2016) and because carbon made available during litter decomposition can stimulate the cycling of other nutrients in soil (Phillips et al. 2009), differences in the composition of these communities may have large-scale effects on soil environments. These shifts in cellulose-degrading bacteria may have long-term effects in the wild, where litter inputs occur seasonally or after plant senescence, although we would not expect to capture such effects on plant growth in this short-term greenhouse experiment.

Results from the microbial DNA sequencing data in this study are a necessary first step toward identifying the causal agents of feedback between *L. cuneata* and native potential competitors. We have shown that *L. cuneata* yields increase in the presence of native-conditioned microbial communities, and we have identified the types of organisms that may play a role in facilitating observed plant-soil feedbacks. In order to examine the ability of these identified organisms to function as casual agents of feedback, future studies should examine the influence of isolate inoculation on *L. cuneata* and native plant yields. A recent study suggests that plant-soil feedback between *L. cuneata* and native plants may be overwhelmed by interspecific competition (Crawford and Knight 2017). However, other studies have shown that the growth of some grassland plant

species is influenced by plant-soil feedback even in the presence of interspecific competition (Casper and Castelli 2007). Overall, these studies highlight the importance of examining plant-soil feedback and competition together. The influence that organisms identified as potential agents of feedback in this study have on plant growth should also be examined in the presence of interspecific competition.

In summary, *L. cuneata* benefits from native-conditioned soil, which should help it to achieve a foothold after introduction into a new site. By disrupting beneficial communities or increasing pathogen load, *L. cuneata* seems to promote microbial communities that are harmful for both conspecific and native plants.

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FIGURES

Figure 2.1: Pairwise plant-soil feedbacks between invasive *L. cuneata* and its native competitors *L. virginica* and *P. virgatum*. Bars represent feedback effect sizes as calculated with Bever's I-score $I_s = \alpha_A - \alpha_B - \beta_A + \beta_B$ (Bever et al. 1997).

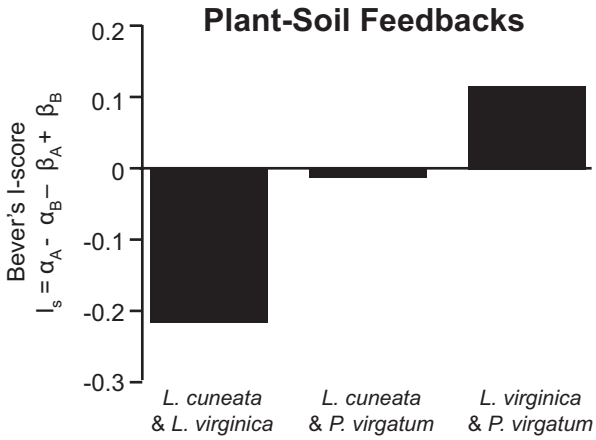


Figure 2.2: Influence of conditioned microbial communities on subsequent plant growth. Bars represent average plant biomass in live soil minus plant biomass in sterile soil. Error bars represent standard error. Significant differences between plant responses to each treatment are represented by letter codes (post-hoc Student's t-tests, $p<0.05$).

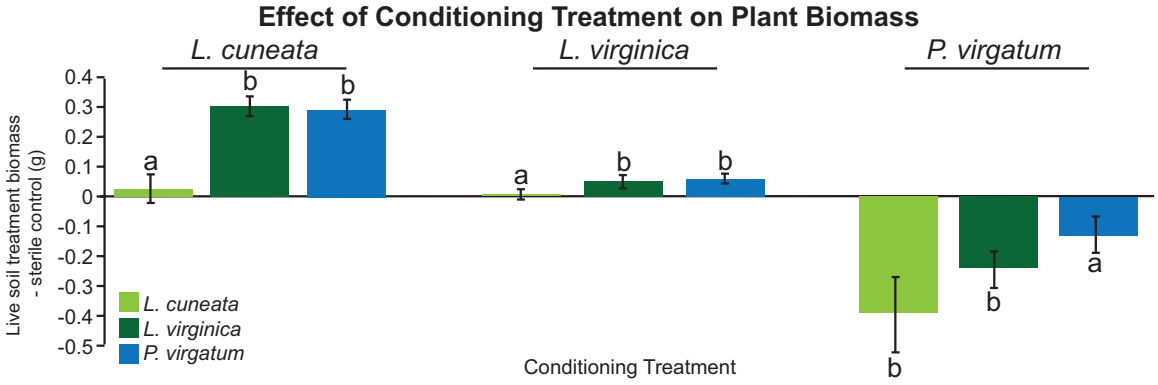


Figure 2.3: Influence of soil conditioning on soil nitrogen concentration. Bars represent average concentration (ppm) of ammonium or nitrate in conditioned soil. Error bars represent standard error. Significant differences between each treatment are represented by letter codes (post-hoc Student's t-tests, $p<0.05$).

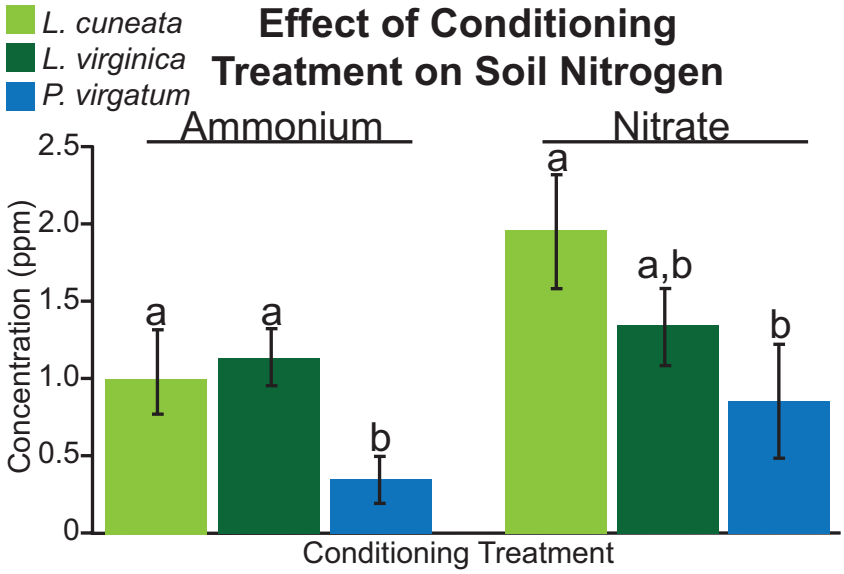


Figure 2.4: Influence of soil conditioning on soil bacterial, fungal, and nitrogen-fixing community composition. Ellipses represent the standard deviation of microbial community composition. Light green ellipses represent communities in *L. cuneata* conditioned soil (LC), dark green ellipses represent communities in *L. virginica* conditioned soil (LV), and blue ellipses represent communities in *P. virgatum* conditioned soil (PV). Red arrows indicate significantly correlated inorganic nitrogen vectors ($p<0.05$).

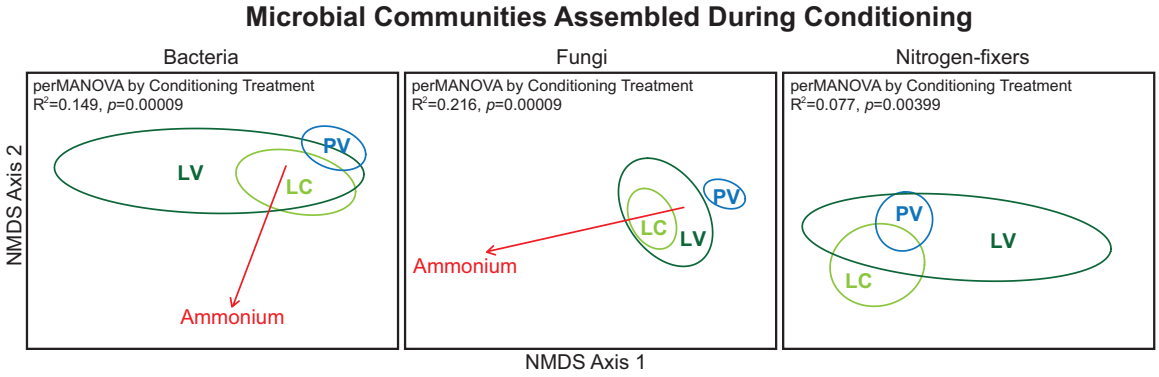


Figure 2.5: Summary of bacterial OTUs enriched by conditioning treatments. Proportion of OTUs enriched under each conditioning treatment that belong to listed bacterial phyla. Colored bars indicate OTU classifications denoted by the key.

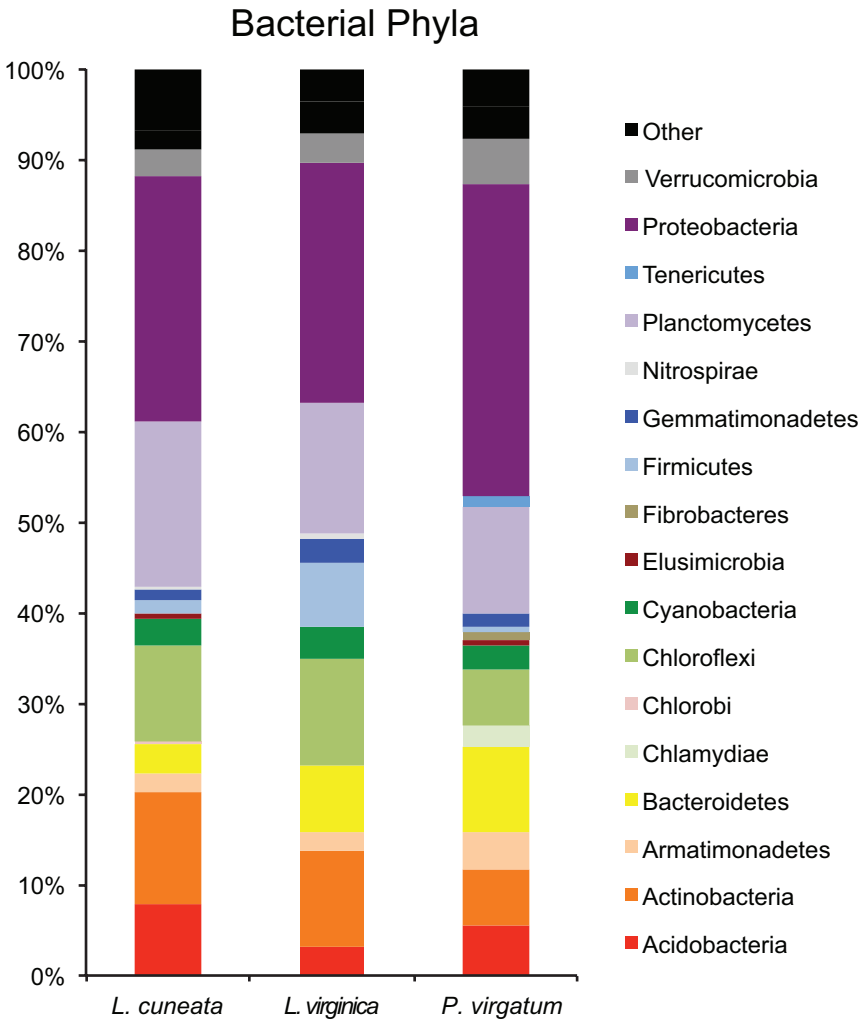


Figure 2.6: Summary of nitrogen-fixing bacterial OTUs enriched by conditioning treatments. Proportion of OTUs enriched under each conditioning treatment that belong to listed nitrogen-fixing bacterial genera. Colored bars indicate OTU classifications denoted by the key.

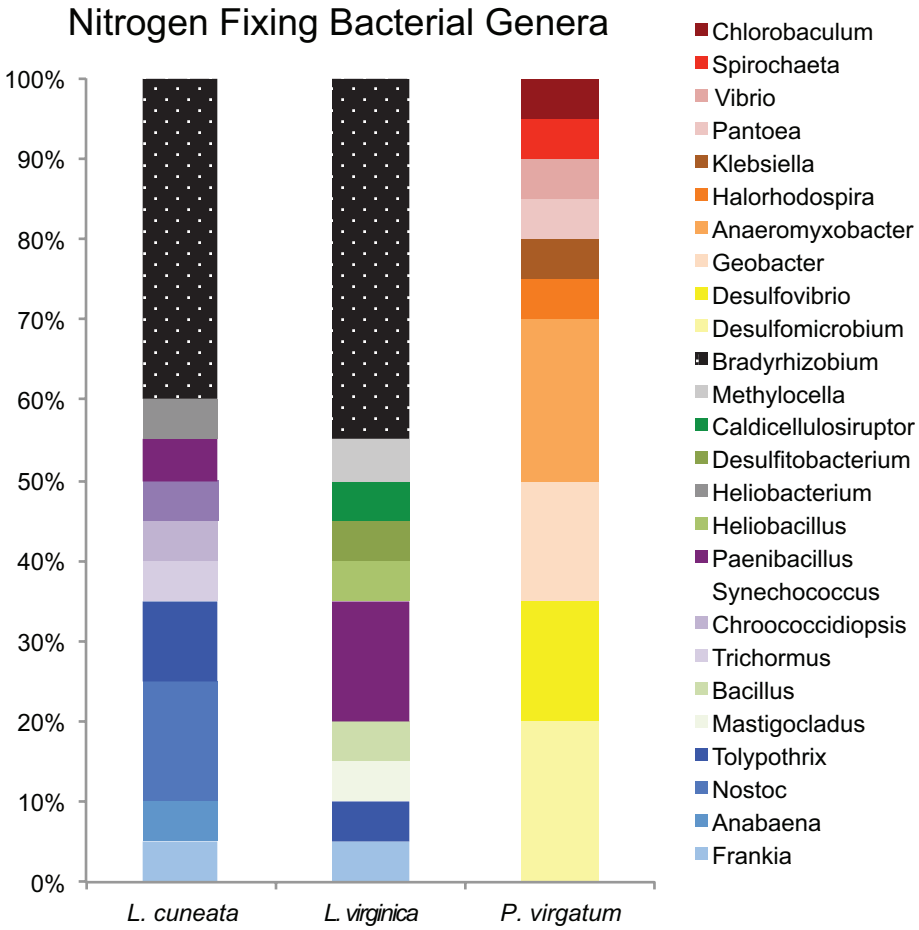
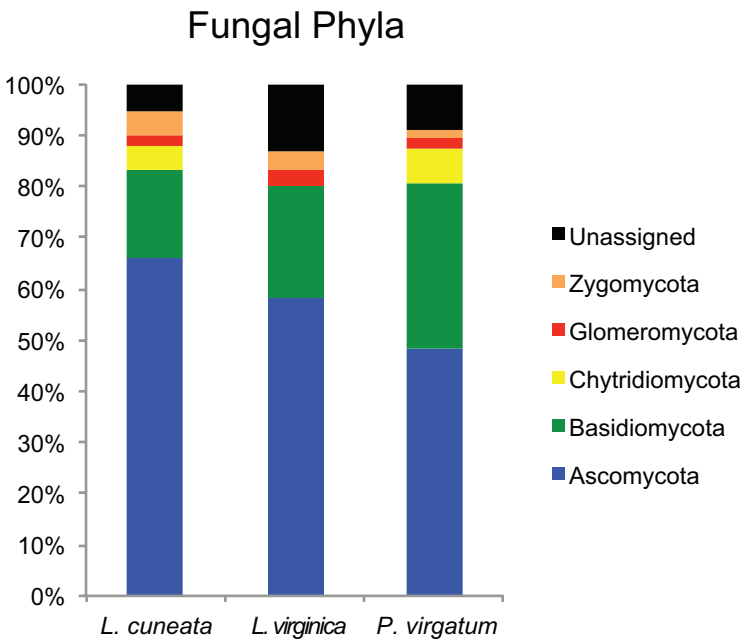


Figure 2.7: Summary of fungal OTUs enriched by conditioning treatments. Proportion of OTUs enriched under each conditioning treatment that belong to listed fungal phyla. Colored bars indicate OTU classifications denoted by the key.



TABLES

Table 2.1: Summary of results from two-way analysis of variance (ANOVA) test for significance of plant species, conditioning treatment, and interaction effects on plant biomass.

	Degrees of Freedom	Sum of Squares	Mean Square	F-value	*p*-value
Plant Species	2	2.7559	1.3780	126.81	$2x10^{-16}$*
Conditioning Treatment	2	0.4801	0.2400	22.09	$9.83x10^{-9}$*
Interaction	4	0.1721	0.0430	3.96	0.00493*
Residuals	105	1.1410	0.0109		

Table 2.2: Summary of enriched Basidiomycotal fungial OTUs under each conditioning treatment. Functional roles highlighted in green represent beneficial fungi, and those highlighted in red represent deleterious fungi.

Treatment	Class	Order	Family	Genus	Species	Function	Reference
L. cuneata	Agaricomycetes	Agaricales	Clavariaceae	*unidentified*	*unidentified*	ECM	(Birkebak et al. 2013)
			Entolomataceae	*Entoloma*	*crassicystidiatum*	pathogen	(Agerer and Waller 1993)
			Psathyrellaceae	*Coprinopsis*	*cinerea*	saprophyte	(Hoegger et al. 2004)
		Polyporales	Phanerochaetaceae	*Hyphodermella*	*rosae*	pathogen	(Telleria et al. 2010)
		Russulales	Stereaceae	*Acanthophysium*	*lividocaeruleum*	wood rot	(Lim et al. 2005)
	Microbotryomycetes	Sporidiobolales	*Incertae sedis*	*Sporobolomyces*	*symmetricus*	pathogen	(Xu et al. 2014)
L. virginica	Agaricomycetes	Agaricales	Clavariaceae	*unidentified*	*unidentified*	ECM	(Birkebak et al. 2013)
				Clavaria	*californica*	ECM	(Birkebak et al. 2013)
			Cyphellaceae	*Campanophyllum*	*proboscideum*	wood rot	(Gilbertson 1980)
			Cortinariaceae	*Cortinarius*	*unidentified*	ECM	(Bödeker et al. 2014)
		Cantharellales	Ceratobasidiaceae	*unidentified*	*unidentified*	ECM	(Yagame et al. 2012)
		Hymenochaetales	Schizopoaceae	*Hyphodontia*	*alutaria*	wood rot	(Greslebin and Rajchenberg 2000)
	Exobasidiomycetes	*Unidentified*	*unidentified*	*unidentified*	*unidentified*	pathogen	(Denchev and Moore 2009)
	Microbotryomycetes	Sporidiobolales	*Incertae sedis*	*Rhodotorula*	*phylloplana*	yeast	(Biswas et al. 2001)
P. virgatum	Agaricomycetes	Agaricales	Clavariaceae	*unidentified*	*unidentified*	ECM	(Birkebak et al. 2013)
			Cortinariaceae	*Cortinarius*	*sinapicolor*	ECM	(Bödeker et al. 2014)
			Entolomataceae	*Clitopilus*	*unidentified*	suppressor	(Kilaru et al. 2009)
			Hygrophoraceae	*Gliophorus*	*europerplexus*	pathogen	(Ainsworth et al. 2013)
		Auriculariales	*Incertae sedis*	*Auricularia*	*unidentified*	wood rot	(Worrall et al. 1997)
		Polyporales	Fomitopsidaceae	*Fomitopsis*	*meliae*	wood rot	(Han et al. 2016)
		Sebacinales	Sebacinaceae	*unidentified*	*unidentified*	ECM	(Weiss et al. 2004)
		Trechisporales	*unidentified*	*unidentified*	*unidentified*	wood rot	(Vohník et al. 2012)
	Tremellomycetes	Tremellales	*Incertae sedis*	*Cryptococcus*	*unidentified*	pathogen	(Xue et al. 2007)
	Wallemiomycetes	Geminibasidiales	Geminibasidiaceae	*Geminibasidium*	*unidentified*	ECM	(Nguyen et al. 2013)

CHAPTER 3*: LESPEDEZA CUNEATA* ROOT AND LITTER CHEMICALS DIFFERENTIALLY INFLUENCE PLANT-SOIL FEEDBACKS WITH NATIVE COMPETITORS

ABSTRACT

Chemical input from plant litter and roots may have distinct influences on soil microbial communities and downstream plant-soil feedback patterns. The objective for this study is to characterize plant-soil feedback that results from conditioning soil with distinct chemical fractions produced by invasive *Lespedeza cuneata*. I collected and characterized root exudates, root leachates, and litter leachates from *L. cuneata* plants, and I used these solutions to condition soil for a greenhouse experiment. I examined the effect that conditioning soil with each solution had on the development of soil microbial communities and on the growth of *L. cuneata* and native plant species, *L. virginica* and *Panicum virgatum*. Root and litter derived chemical inputs led to the development of distinct soil microbial communities and produced distinct plant-soil feedback patterns. Despite containing chemical components that attract and enriching for known nitrogen-fixing symbionts, soil conditioned with root exudates generally had the most negative effect on plant growth. Results suggest that *L. cuneata* root exudates may enrich for deleterious microbes that limit the growth of conspecific and native plants. *L. cuneata* chemical inputs may also have direct negative affects on native plant growth.

INTRODUCTION

Plant species produce tissues with unique chemical compositions, which are introduced into the soil via both passive leaching from living or dead plant

material and active root exudation. Once in the soil, these chemical introductions have the potential to influence soil microbial communities and may have different effects on plant-soil feedback patterns. Plant-soil feedbacks influence the outcome of plant competition, and can therefore have substantial impacts on the composition of plant communities (Bever 2003). Additionally, plant-soil feedbacks are thought to play an important role in facilitating or hindering plant invasion (Callaway et al. 2004, Suding et al. 2013).

Seasonal plant litter additions can have long-term effects on soil microbial communities. Litter-mediated plant-soil feedbacks are largely due to shifts in the quantity and quality of litter inputs, which drive nutrient cycling and resource availability for subsequent plant generations (Aerts 1997, Aerts et al. 2003). Litter decomposition dynamics may have important influences on plant competition for nutrient resources (Berendse 1998). High quality litter is nitrogen-rich, and tends to decompose quickly and stimulate nutrient mineralization. Conversely, low quality litter is nitrogen-poor and decomposes more slowly (Lavorel and Garnier 2002). Additionally, litter leachates often contain phenolic compounds that can slow decomposition and nutrient release rates (Kalburtji et al. 1999, Sariyildiz and Anderson 2003). The availability of nutrients in soil can influence the competitive dynamics between plant species (Wilson and Tilman 1991), and can therefore impact plant community composition. Additionally, litter leachates have been shown to influence soil microbial community composition (Fujii et al. 2004, Castells et al. 2005), which can influence nutrient cycling and mediate feedbacks for future generations of plants.

Continuous root exudation from plants can have rapid effects on soil microbial communities (Bais et al. 2006). Root exudate quantity and composition varies by plant species and with environmental conditions (Badri and Vivanco 2009), but they are typically composed of large quantities of ions, free oxygen, water, enzymes, mucilage, and carbon-based primary and secondary metabolites (Uren 2007). Labile carbon compounds in root exudates are readily utilized by soil microbes and lead to abundant microbial populations in plant rhizospheres (Bais et al. 2006). These labile carbon compounds can stimulate microbial nutrient cycling (Phillips et al. 2011, Meier et al. 2017). Therefore, root exudation may also contribute to plant-soil feedbacks related to nutrient cycling and resource availability.

Secondary metabolites in root exudates function as chemoattractants of specific microbes towards roots (Zheng and Sinclair 1996, Bacilio-Jiménez et al. 2003). The establishment of microbial mutualisms depends on this attraction and on subsequent complex chemical communication and microbial response to plant root exudates (Bais et al. 2006). Root exudate-mediated shifts in the abundance of beneficial and deleterious microbes may drive plant-soil feedbacks influencing future generations of plant communities (Bever et al. 1997). Negative feedbacks between invasive *Lespedeza cuneata* and its native congener, *L. virginica*, have been documented and may be driven by shifts in the relative abundance of soil mutualists **(Chapter 2)**. There is evidence that *L. cuneata* benefits more from participating in mutualisms with nitrogen-fixing bacteria (Hu et al. 2014) and may have a stricter preference for Rhizobiales bacteria than native congeners like *L.*

virginica (Busby et al. 2016). Additionally, there is evidence that *L. cuneata* may benefit from tri-partite symbioses with both nitrogen fixing bacteria and mycorrhizal fungi (Lynd and Ansman 1993). The ability of *L. cuneata* to enrich for beneficial symbionts at invasion sites likely depends on chemical communication with microbes through root exudation. *L. cuneata* has high phenol content and allelopathic compounds in its leaf tissue and root exudates (Kalburtji et al. 1999, 2001), and preliminary evidence shows that *L. cuneata* root exudates are chemically distinct from those of native congeners (Ringelberg et al. 2017). Both *L. cuneata* root exudates and litter leachates have been shown to decrease the germination or growth of native grass species (Kalburtji and Mosjidis 1992, 1993a, 1993b) and *L. cuneata* invasion has been shown to alter plant (Eddy and Moore 1998), bacterial and fungal community composition (Yannarell et al. 2011).

L. cuneata chemical inputs may play a role in facilitating plant-soil feedback in the *L. cuneata* system. Because litter mediated plant-soil feedbacks operate through influences in nutrient cycling and resource availability, they may be especially influential on communities of microbial decomposers. Because root exudate mediated plant-soil feedbacks operate through the differential attraction of beneficial and deleterious microbes, they may be especially influential on symbiont and pathogen communities. In this study, I collected multiple fractions of *L. cuneata* chemical inputs and submitted them for chemical analysis. I performed a greenhouse plant-soil feedback experiment to determine the influence that these chemical fractions had on soil microbial communities and

plant growth. I address the following research goals: 1) characterize the effects of *L. cuneata* root exudates, root leachates, litter leachates, and living plants on subsequent *L. cuneata*, *L. virginica*, and *Panicum virgatum* growth; 2) identify unique effects of *L. cuneata* root exudates, root leachates, and litter leachates on soil microbial community development; and 3) identify unique chemical components of *L. cuneata* root exudates, root leachates, and litter leachates that may facilitate differences in observed effects on plant yields.

METHODS

L. cuneata Leachate and Root Exudate Collection and Characterization

I grew four hundred *L. cuneata* plants from seed in equal parts fine vermiculite and coarse quartz sand, which allowed for both moisture retention and mechanical support. I watered plants with ¼ strength Hoagland's complete medium weekly for three months. After three months, I collected root exudates following the procedure of Phillips and colleagues (2009). Plants were carefully removed from the vermiculite/sand mixture with intact roots and transferred into hydroponic culture in ¼ strength Hoagland's medium for one week. This allowed plants to recover from the removal process and become acclimated to growth in a liquid solution. After acclimation, plants were transferred into pots containing 20mL1mM $CaSO_4$ for 3 hours in order to harvest root exudates. Exudates were filter sterilized, pooled, and stored at -20°C until use. After exudate collection from living plants, I divided them into aerial tissues (hereafter, "litter") and root portions for leachate collection. Litter and roots were sorted by tissue type, and shaken in a total of 8L (20mL per plant) room temperature water for 24 hours

(Castells et al. 2005). Plant tissue was removed and leachates were filter sterilized, pooled, and stored at -20°C until use.

I characterized pooled exudate and leachates by analyzing for dissolved organic carbon (DOC), dissolved inorganic nitrogen (DIN), dissolved organic nitrogen (DON). Inorganic carbon was removed from each solution by converting it to dissolved CO_2 (acidifying each solution to a pH of 2) and purging with inert gas. DOC and total nitrogen (TN) were measured simultaneously for each sample in triplicate on a Shimadzu TOC-L, TNM-L analyzer with ASI-L autosampler (Shimadzu Corporation, Kyoto, Japan). DOC was measured by combustion catalytic oxidation at 680 °C, and TN was measured by catalytic thermal decomposition at 720°C. DIN was measured on a SmartChem 200 Discrete Sampler (Unity Scientific, Milford, MA, USA). 1mL samples of each solution were analyzed for ammonium using SmartChem Method AMM-003-C and nitrate using SmartChem Method NO3-001-B. I quantified the DIN of each solution by adding measured ammonium and nitrate concentrations. I quantified the DON of each solution by subtracting DIN from the measured TN.

I also characterized the metabolites in exudate and leachate solutions by gas chromatography-mass spectrometry (GC/MS). 5mL of internal standard (hentriacontanoic acid (10 mg/uL); Sigma, USA) was added and solutions were derivatized with 80 mL methoxyamine hydrochloride (Aldrich, USA) (40 mg/mL in pyridine) for 60 minutes at 50°C and with 100 mL MSTFA+1% TMCS (Thermo Scientific, USA) at 70°C for 120 minutes. After heating, samples were incubated at room temperature for 2 hours. Solutions were analyzed using an Agilent 7890

Gas Chromatograph (Agilent Incorporated, Palo Alto, CA, USA) on a ZB-5MS (60m x 0.32 mm I.D., 0.25 mm film thickness) capillary column (Phenomenex, CA, USA), an Agilent 5975 Mass Selective Detector in positive electron impact mode (EI) at 69.9eV ionization energy in m/z 30-800 scan range, and an Agilent/HP 7683B Autosampler. Inlet and MS interface temperatures were 250°C, and the ion source temperature was adjusted to 230°C. 1mL of each solution was injected with the split ratio of 10:1, and the helium carrier gas maintained at a constant flow rate of 2 mL/min. Solutions underwent isothermal heating for 5 minutes at 70°C, a temperature increase to 310°C at a rate of 5°C per minute, and were held at 310°C for 10 minutes. GC/MS was performed at the Metabolomics Center of the Roy J. Carver Biotechnology Center, University of Illinois, Urbana, USA.

Greenhouse Experiment

Plant-soil feedback experiments consist of a conditioning phase, in which plants influence microbial community assembly, and a feedback phase, in which plant growth in the presence of conditioned microbial communities is measured (Bever 1994). In this chapter, I conducted a greenhouse experiment in order to examine the plant-soil feedbacks that are mediated by *L. cuneata* chemical inputs. I conditioned soil with *L. cuneata* root exudates, root leachates, litter leachates, living plants or water in order to document the microorganisms that respond to these different stimuli and quantify the impact of these microorganisms on plant growth. The water treatment served as a control that represented soil that did not receive any plant inputs. The living plant treatment

served as a control to represent plant-mediated effects that were not captured by individual chemical fractions.

I collected live soil from John English Memorial Prairie, Comlara Park, McLean County, IL, USA in the spring of 2016. This live soil was stored for 24 hours at 4°C before use as a 3% inoculum into autoclave-sterilized soil. Approximately 450 cubic centimeters of inoculated soil was placed into a total of 50 (n=10) Leonard Jar assemblies (Trung and Yoshida 1983), which could provide continuous wicking of the conditioning solution into the soil. 20mL of the appropriate solution (root exudate, litter leachate, root leachate, or water) was added to each assembly weekly, removing any remaining liquid from the previous week. For the living plant conditioning treatment, five *L. cuneata seeds* were added to each conditioning pot (n=10) at the start of the conditioning phase. Pots were thinned to one plant after two weeks, and given 20mL of water each week. The conditioning phase of this experiment lasted two months.

After conditioning, we removed *L. cuneata* plants from the living plant treatment. For all conditioning pots, soil was homogenized and a subsample was taken for microbial community analysis; these soil samples were lyophilized and stored at -80°C until use. The remaining soil from each conditioning pot was divided into three sterile pots in order to quantify soil feedback effects on three different plant species (*L. cuneata*, *L. virginica*, *Panicum virgatum*). Feedback pots were filled with sterile soil to a total volume of approximately 450 cubic centimeters. Five *L. cuneata*, *L. virginica*, or *P. virgatum* seeds were added to each feedback pot. After two weeks of growth, pots were thinned to one plant

each. Plants were watered weekly and no nutrient solution was added. After three months of growth, the aboveground portion of each plant was collected and air-dried to a constant weight.

Soil Microbial Community Analysis

Subsamples of soil taken from each pot after conditioning were used to examine microbial community assembly under the influence of each conditioning treatment. I extracted DNA from 500mg of each lyophilized soil sample, using the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA) following manufacturer instructions. I purified extracted DNA by incubating with 1% cetyltrimethyl ammonium bromide (CTAB) for 15 minutes at 65°C, re-extracting DNA in 24:1 chloroform:isoamyl alcohol, and washing with cold ethanol. Purified DNA was eluted in 1x TE buffer and stored at a concentration of 20ng/mL at -80°C until use.

I amplified specific gene targets from extracted DNA using Fluidigm 2 Step Access Array Amplification on a Fluidigm 48.48 Access Array IFC (Fluidigm Corporation, San Fransisco, CA), following manufacturer instructions. Unique barcodes were added to each sample so that PCR products could be pooled for sequencing. I used primers 515F and 926F to amplify the V3-V4 region of the bacterial 16S rRNA gene (Caporaso et al. 2011, Lane 1991), primers ITS3 and ITS4 to amplify the fungal second internal transcribed spacer region (ITS2) (White et al. 1990), and primers PolF and PolR to amplify the nitrogen-fixing gene, *nifH* (Poly et al. 2001). PCR products were confirmed on a Fragment Analyzer (Advanced Analytics, Ames, IA) and pooled in equimolar

concentrations. Pooled products were size selected on a 2% agarose E-gel (Life Technologies, Carlsbad, CA) and extracted with Qiagen gel extraction kit (Qiagen, Hilden, Germany). Size selected products were run on an Agilent Bioanalyzer (Agilent, Santa Clara, CA) and pooled. Pooled amplicons were sequenced on an Illumina MiSeq V3 platform using a 2x250 base pair read configuration in Bulk Kit version 3 (Illumina, San Diego, CA, USA). PCR reactions and DNA sequencing were carried out at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

I analyzed raw microbial sequence data on a variety of platforms. Forward and reverse reads of each paired-end sequence were merged using Fast Length Adjustment of Short reads (FLASH) software (Magoč and Salzberg 2011). Sequences that contained greater than 10% of bases with quality scores below 30 were removed. I processed the remaining sequences in USEARCH (http://www.drive5.com/usearch/) to 1) de-replicate sequences and remove singletons; 2) remove chimeric sequences detected by the GOLD database for bacteria (Kyrpides 1999), the UNITE ITS database for fungi (Abarenkov et al. 2010), and a custom *nifH* database created by downloading sequences from the RDP FunGene website (Fish et al. 2013) and assigning taxonomic information using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990); and 3) cluster sequences by 97% similarity to form operational taxonomic units (OTUs) (Rodriguez et al. 2016, Gradoville et al. 2017). I aligned representative sequences for each bacterial OTU, and left fungal and *nifH* sequences unaligned. For bacterial sequences, I used the RDP classifier in

QIIME (Caporaso et al. 2010) to assign taxonomic information. For fungi and nitrogen fixers, I assigned taxonomic information using the BLAST algorithm (Altschul et al. 1990) and the same databases mentioned in step 2 above. I removed representative fungal and *nifH* OTUs that could not be matched to any taxonomic information using the BLAST algorithm.

Data Analysis

I quantified the beneficial or harmful effect of each conditioning treatment on *L. cuneata*, *L. virginica*, or *P. virgatum* growth by subtracting the average growth of each plant in water-conditioned soil. I considered conditioning treatments to be beneficial if they led to plants being larger than those grown in water-conditioned soil. Conversely, I considered conditioning treatments to be harmful if they led to plants being smaller than those grown in water-conditioned soil. Analysis of variance (ANOVA) was used to determine significance influence of conditioning treatments on feedback plant biomass. *Post-hoc* t-tests were performed to determine significant differences between individual treatment effects on each plant species.

I visualized differences in the nutrient content (DON, DIN, DOC) of each solution as bar graphs. I categorized metabolites identified by GC-MS as being present in root exudates-only, root leachates-only, litter leachates-only, or present in all three solutions. In order to identify metabolites that were significantly related to plant biomass, I fit linear models with plant biomass as the response variable and individual metabolite concentration as the predictor variable.

I analyzed sequences from bacterial, fungal, and nitrogen-fixing communities separately. To determine if conditioning treatments explained a significant portion of variance in microbial community composition after conditioning, I performed permutational multivariate analysis of variance (Anderson 2001) using the function adonis() in the R statistical environment (R Core Development Team 2014). I visualized the effect of each conditioning treatment on microbial community composition with non-metric multidimensional scaling (NMDS). To identify microbial OTUs that were enriched under each conditioning treatment, I performed canonical correspondence analysis using the function cca() to explore the variation in microbial community composition that was due to conditioning treatment only. The most extreme 5% of axis scores that corresponded to the centroid of microbial OTUs clustered by conditioning treatment were considered to be enriched by that treatment.

RESULTS

Conditioning soil with root exudates, root leachates, litter leachates, and living *L. cuneata* plants had distinct effects on the three plant species examined in this study (ANOVA, F=6.152, p=0.0006)**(Figure 3.1)**. For *L. cuneata*, conditioning with a living *L. cuneata* plant did not influence biomass during the feedback phase, but conditioning with root exudates, root leachates, and litter leachates all led to decreased biomass measurements. All three chemical fraction treatments lead to significantly less *L. cuneata* biomass than the living plant treatment (Student's t-test, p<0.05). Nine metabolites (arabinose, butanoic acid-3-hydroxy, decanoic acid, glycerol, maltose, phosphoric acid, sucrose,

tetradecanoic acid, and xylulose) were negatively related to *L. cuneata* biomass, and one metabolite (benzoic acid-2,4-dihydroxy) was positively related to *L. cuneata* biomass **(Table 3.1)**. Similarly, *L. virginica* biomass was not influenced by soil conditioned with living *L. cuneata* plants. Soil conditioned by root exudates and litter leachates lead to *L. virginica* plants that were significantly smaller than those grown in soil conditioned by root leachates (Student's t-test, $p<0.05$), but were not significantly different from those grown in soil conditioned by living plants (Student's t-test, $p>0.05$). Twenty-one metabolites (alanine, benzoic acid-2-methyl, butanoic acid-3-hydroxy, decanoic acid, fructose glucaric acid, gluconic acid, glucose, glycerol, hexadecanol, inositol myo, lactic acid, maltose, mannose, phosphoric acid, pyroglutamic acid, ribose, succinic acid, sucrose, urea) were negatively related to *L. virginica* biomass, and four metabolites (benzoic acid-2,4-dihydroxy, butanoic acid-2-hydroxy, butanoic acid-3-methyl-2-oxo, pipecolic acid) were positively related to *L. virginica* biomass. Soil conditioned with living *L. cuneata* plants was significantly more harmful for *P. virgatum* growth than soil conditioned with *L. cuneata* root exudates (Student's t-test, $p<0.05$). Both root leachates and litter leachates benefitted *P. virgatum* growth. Twenty-six metabolites (2-methyl succinic acid, 4-aminobutanoic acid, adipic acid, arabinose, arabitol, azelaic acid, benzene-1,2,4,-triol, benzoic acid-2,5-dihydroxy, butanoic acid-4-hydroxy, decanoic acid, erythritol, ethanolamine, glutaric acid, glycerol, maltose, mannitol, octadecanol, pentadecanoic acid, phosphoric acid, quinic acid, shikimic acid, tetradecanoic acid, trehalose, xylose,

xylulose) were negatively related to *P. virgatum* biomass. No metabolites were positively related to *P. virgatum* biomass.

Soil conditioning treatments led to the development of distinct microbial assemblages (perMANOVA, $p<0.05$)**(Figure 3.2)**. For all three microbial communities (bacteria, fungi, nitrogen-fixers), living plants influenced microbial communities most distinctly. Chemical conditioning treatments led to similar taxonomic distributions of enriched bacterial OTUs **(Figure 3.3a)**, with a few key differences. Litter leachate-enriched OTUs had proportionally more Cyanobacteria and fewer Firmicutes than the other treatments. OTUs enriched by both leachates contained proportionally more Armatimonadetes than those conditioned by root exudates or living plants. Conditioning treatments also led to similar taxonomic distributions of enriched fungal OTUs **(Figure 3.3b)**. Root exudates enriched OTUs contained proportionally fewer Ascomycota and more Basidiomycota and Zygomycota than the other treatments. All conditioning treatments enriched for OTUs within the order Agaricales (Birkebak et al. 2013) **(Table 3.2)**. Root exudates and root leachates also enriched for OTUs within the order Sebacinales (Weiß et al. 2016). Living plants, root exudates, and litter leachates enriched for potentially pathogenic OTUs within the fungal genus *Entoloma* (Agerer and Waller 1993 , Kobayashi and Hatano 2001). Root exudates also enriched for potentially pathogenic OTUs within the fungal genus *Hygrocybe* (Halbwachs et al. 2013). All three *L. cuneata* solutions lead to the enrichment of more nitrogen-fixing Cyanobacteria, Firmicutes, and Gamma-Proteobacteria OTUs than the living plant treatment **(Figure 3.3c)**. Root

exudates enriched for the greatest number of *Bradyrhizobium* OTUs, but did not enrich for any *Burkholderiales* OTUs.

L. cuneata solutions differed in nutrient content and chemical make-up. Root exudates and root leachates had less dissolved organic carbon (DOC) than litter leachates. Root exudates contained more dissolved inorganic nitrogen (DIN) and less dissolved organic nitrogen (DON) than either leachate **(Figure 3.4)**. Root exudates contained twelve unique metabolites that were not present in either leachate (2-Methylsuccinic acid, 4-Aminobutanoic acid, Adipic acid, Azelaic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybutanoic acid, Erythritol, Mannitol, Quinic acid, Shikimic acid, Trehalose, Xylose). Root leachates contained 2 unique metabolites that were not present in either root exudates or litter leachates (3-methyl-2-oxobutanoic acid, Pipecolic acid). Litter leachates contained 16 unique metabolites that were not present in either root exudate or root leachates (1-Benzylglucopyranoside, 1-Ethylglucopyranoside, 1-methyl-a-D-glucopyranoside, 3,4-Dihydroxybutanoic acid, 3-methyl-4-hydroxybutanoic acid, 1,2,3-trihydroxybutane, 3-methyl-3-hydroxybutanoic acid, Erythronic acid, Erythrose, Galactose, Methyl-Inositol 2, Methyl-Inositol 3, Sedoheptulose, Serine, Tartaric acid, Threonine). Five metabolites were found in high concentrations in all three solutions (Ethanolamine, Glycerol, Glyoxylic Acid, Lactic Acid, Phosphoric Acid)**(Table 3.3)**.

DISCUSSION

The main goal of this study was to characterize plant-soil feedbacks mediated by different *L. cuneata* chemical inputs. I found that conditioning soil

with root exudates, root leachates, litter leachates, and living plants had different effects on plant biomass that depended on plant species **(Figure 3.1)**. Soil conditioned with *L. cuneata* root exudates was generally more harmful for plant growth than root leachates or litter leachates, when compared to plant growth in water-conditioned soil. This suggests that root exudates either enrich for deleterious microbes or do a poor job at enriching for beneficial microbes. Differential enrichment of microbes under root exudate conditioning may have been caused by metabolites that were present in only root exudates **(Table 3.3)**.

Some of the metabolites found in root exudates only are exuded by plants in response to stress from nutrient limitation (2-Methyl succinic acid (Lipton et al. 1987)), oxygen availability (4-aminobutanoic acid, 4-hydroxybutanoic acid (Bouche and Fromm 2004)) and pathogens (Azelaic acid and 2,5-dihydroxybenzoic acid (Bellés et al. 1999, Zoeller et al. 2012)). The lack of nutrient addition throughout the course of conditioning and feedback phases may have lead to nutrient limitation stress in this study. However, because the water control was also grown without nutrient addition and was used to compare plant growth in experimental treatments to, this should not have influenced the results of this study. The presence of exudates associated with oxygen stress likely reflects the hydroponic growth phase of root exudate collection in this experiment. Exudates associated with response to pathogen stress may have been caused by the enrichment of OTUs within the pathogenic genera *Entoloma* and *Hygrocybe* **(Table 3.1)**. Future studies should examine the pathogenesis of these types of organisms on *L. cuneata* and native competitors.

The remaining metabolites that were found in root exudates only may play an important role in the interactions between *L. cuneata* and mutualistic bacteria. Mannitol and xylose have been shown to function as chemoattractants for Rhizobium (Bowra and Dilworth 1981), and Quinic acid, shikimic acid, and adipic acid have been shown to function as chemoattractants for Bradyrhizobium (Parke et al. 1985). Interestingly, these compounds have been shown to be stronger attractants for Bradyrhizobium than Rhizobium (Parke et al. 1985). Therefore, the presence of these metabolites in root exudates only may explain the heightened Bradyrhizobium enrichment observed in soil conditioned by root exudates **(Figure 3.3c)**. Additionally, these three compounds have been shown to stimulate exopolysaccharide production by Bradyrhizobium (Tully 1988), which plays an important role in the early stages of legume nodulation (Djordjevic et al. 1987). Trehalose has also been shown to protect Bradyrhizobium from desiccation (Streeter 2003), and may therefore help to maintain symbiont populations near the rhizosphere. Erythritol may also play an important role in the nodulation process. The ability of a rhizobial strain to utilize erythritol as a carbon source influences that strain's ability to compete for nodule space on plant hosts (Yost et al. 2006), and thus may determine which strains are able to successfully associate with *L. cuneata*. Future studies should examine the influence that these chemical components have on the development of nitrogen-fixing communities in soil. The apparent ability of *L. cuneata* root exudates to attract nitrogen-fixing symbionts does not explain the negative effect that soil conditioned with root exudates had on conspecific and native plant growth.

Similar potential mycorrhizal symbionts were also enriched by each conditioning treatment **(Table 3.1)**. This suggests that the observed negative impact of root exudate conditioned soil on plant growth may have resulted from the enrichment of deleterious microbes (fungal genera Entoloma, Hygrocybe), as opposed to the inability to enrich for beneficial microbes.

Metabolites in these chemical fractions may also directly influence plant growth. Relationships between individual metabolites and plant biomass were overwhelmingly negative for all plant species; however, the biomass of both native plants was negatively related to more metabolites than the biomass of *L. cuneata* plants **(Table 3.1)**. This suggests that *L. cuneata* chemical solutions may be having direct negative influences that are stronger for native plants than for congeners. *L. cuneata* residues have been previously shown to have allelopathic affects that reduce the growth of native grasses (Kalburtji and Mosjidis 1992, 1993a, b). The influence that individual metabolites identified from *L. cuneata* chemical fractions in this study have on native plant growth should be examined in greater detail in future work.

Conditioning with living *L. cuneata* plants was less harmful for *L. cuneata* and more harmful for *P. virgatum*. This suggests that there is some additional characteristic of the living plant that may buffer or exacerbate the influence of microbial recruitment based on exudate and leachate chemistry. Living plant conditioned soil microbial communities were most distinct from other conditioning treatments **(Figure 3.2).** The living *L. cuneata* plant conditioning treatment produced results that were consistent with the experiment conducted in **Chapter**

2, even though I compared feedback biomass to that of unconditioned live soil instead of sterile soil in this current experiment. This suggests that the influence of *L. cuneata* plants on soil microbial community assembly may be consistent over time in similar environmental conditions.

There were many similarities in the taxonomic distributions of microbial OTUs enriched by all chemical fractions and by living plants **(Figure 3.3)**. Some metabolites identified in this study occurred in high concentrations in all three of the examined solutions (ethanolamine, glycerol, glyoxylic acid, lactic acid, phosphoric acid). Some of these compounds have been shown to influence microbial attraction and function, and therefore may play an important role in shaping community development. Ethanolamine and glycerol have been shown to elicit a chemotactic response from bacteria (Repaske and Adler 1981, Zhulin et al. 1997). Additionally, glyoxylic acid has been shown to be important for plant defense (Dubey et al. 2013), and may also influence patterns of microbial virulence (Dunn et al. 2009). This suggests that these compounds may have facilitated similarities in the enrichment of microbial communities under conditioning by *L. cuneata* root exudates, root leachates, and litter leachates. The common occurrence of lactic acid in all three chemical fractions may indicate that *L. cuneata* experienced oxygen stress in growing or root exudate collection conditions (Jones 1998). Lactic acid and phosphoric acid may have influenced microbial communities indirectly by the pH of soil environment, which has been shown to be one of the biggest drivers of microbial community composition (Fierer and Jackson 2006).

I observed large variation in the nitrogen fixing OTUs that were enriched under each conditioning treatment **(Figure 3.3c)**. Root exudates, root leachates, and litter leachates all enriched for more nitrogen-fixing OTUs within the phyla Cyanobacteria, Firmicutes, and within the class gamma-Proteobacteria, than conditioning with living plants. I did not observe an enrichment of Cyanobacteria in my 16S rRNA analysis of the whole bacterial community, which suggests that the nitrogen fixing members of this taxonomic phyla may respond to different stimuli than their non-fixing relatives. Previous work suggests that symbiotic cyanobacteria are attracted toward sugars in root exudates from both host and non-host plants (Nilsson et al. 2006).

Litter leachates contained more dissolved organic carbon (DOC) than root exudates and leachates **(Figure 3.4)**. This is consistent with previous work that shows that litter leachates contain large amounts of DOC that enter the soil system during litter decomposition (Don and Kalbitz 2005). This input of DOC can serve as an easily metabolized energy source for soil microbes (Qualls and Haines 1992), which can lead to rapid change in soil microbial community composition (Cleveland et al. 2007). Additionally, root exudates contained more dissolved inorganic nitrogen (DIN) and less dissolved organic nitrogen (DON) than root and litter leachates **(Figure 3.2)**. This is consistent with the results from another study that found higher levels of inorganic nitrogen than organic nitrogen in legume root exudates (Paynel et al. 2001). This prior study also showed that nitrogen in legume root exudates can be directly taken up by competing plant species. However, I found that root exudate conditioned soil led to decreases in

the biomass of the three plant species examined in this study **(Figure 3.1)**, regardless of this potential positive effect on nitrogen availability for plants. This suggests that microbial communities conditioned by root exudates may have had sufficiently negative effects on plant growth to limit the benefit from nitrogen addition.

One limitation of this study is that it did not compare root exudates and litter leachates produced by *L. cuneata* to those produced by native competitors. Preliminary work suggests that *L. cuneata* root exudates are chemically distinct from exudates of native potential competitors (Ringelberg et al. 2017), but this needs to be examined in greater detail. Future studies should identify metabolites that are present in *L. cuneata* only, and determine the influence that these metabolites may be having on invaded ecosystems. Future studies should focus on mapping specific metabolites to individual microbial taxa.

In summary, results from this experiment show that *L. cuneata* litter and root derived chemical inputs influence soil microbial communities differently and produce distinct plant-soil feedback patterns. *L. cuneata* chemical inputs may also negatively influence native plant growth. Root exudates produced the most negative effects on conspecific and native plant growth, potentially due to pathogen enrichment.

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FIGURES

Figure 3.1: Influence of conditioned microbial communities on subsequent plant growth. Bars represent average plant biomass in treatment conditioned soil minus plant biomass in water conditioned soil. Blue bars represent plant growth in soil conditioned by root exudates, grey bars represent plant growth in soil conditioned by root leachates, light green bars represent plant growth in soil conditioned by litter leachates, and dark green bars represent plant growth in soil conditioned by living *L. cuneata* plants. Error bars represent standard error. Significant differences between plant responses to each treatment are represented by letter codes (post-hoc Student's t-tests, $p<0.05$).

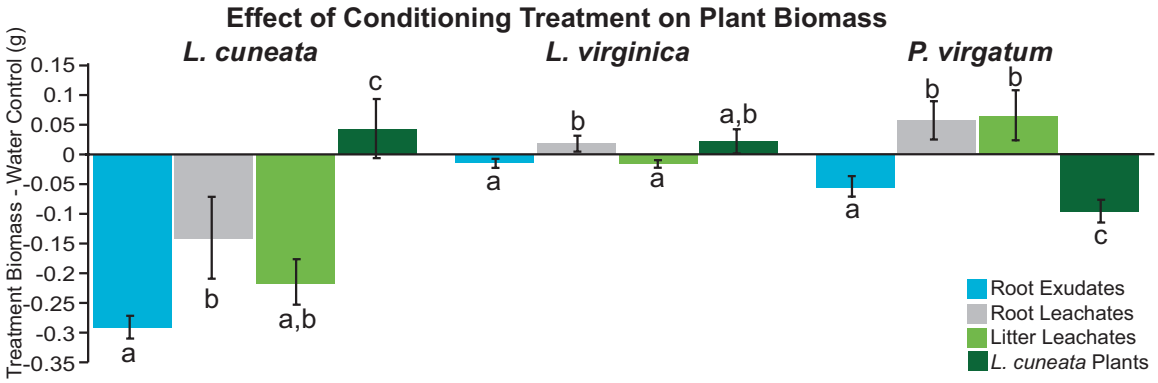


Figure 3.2: Influence of soil conditioning on soil bacterial, fungal, and nitrogen-fixing community composition. Ellipses represent the standard deviation of microbial community composition. Blue ellipses represent communities in root exudate conditioned soil, grey ellipses represent communities in root leachate conditioned soil, light green ellipses represent communities in litter leachate conditioned soil, and dark green ellipses represent communities in living *L. cuneata* plant conditioned soil.

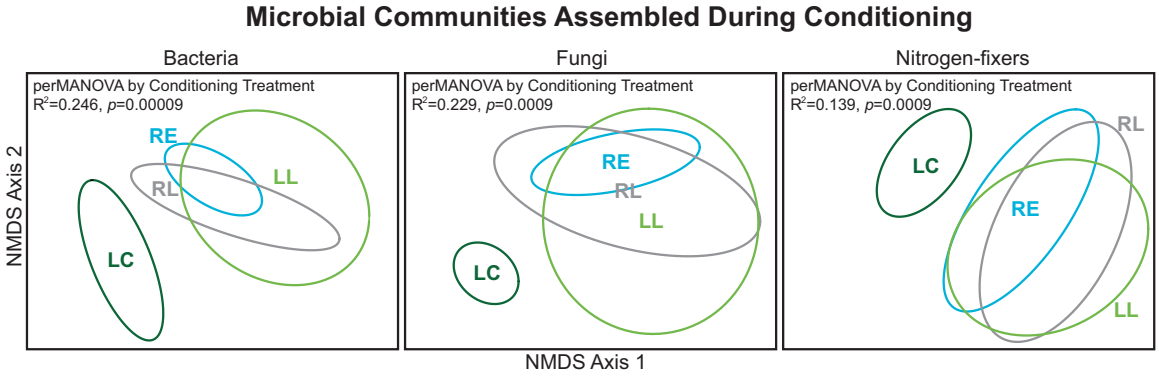


Figure 3.3: Summary of microbial OTUs enriched by conditioning treatments. Proportion of OTUs enriched under living *L. cuneata* plant, root exudate, root leachate, and litter leachate conditioning treatments that belong to the listed a) bacterial, b) fungal, and c) nitrogen-fixing bacterial taxonomic groups. Colored bars indicate OTU classifications denoted by the key.

Figure 3.3 (a)

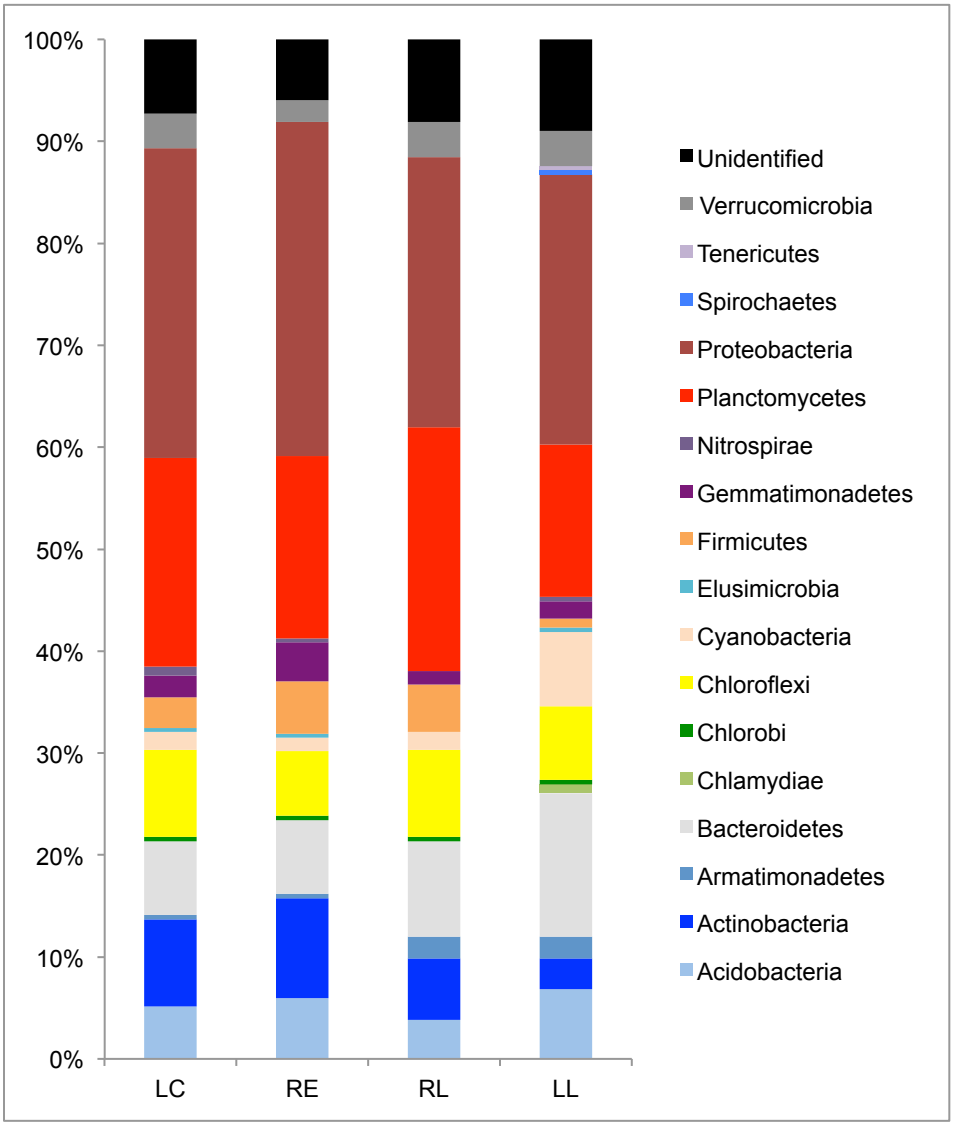
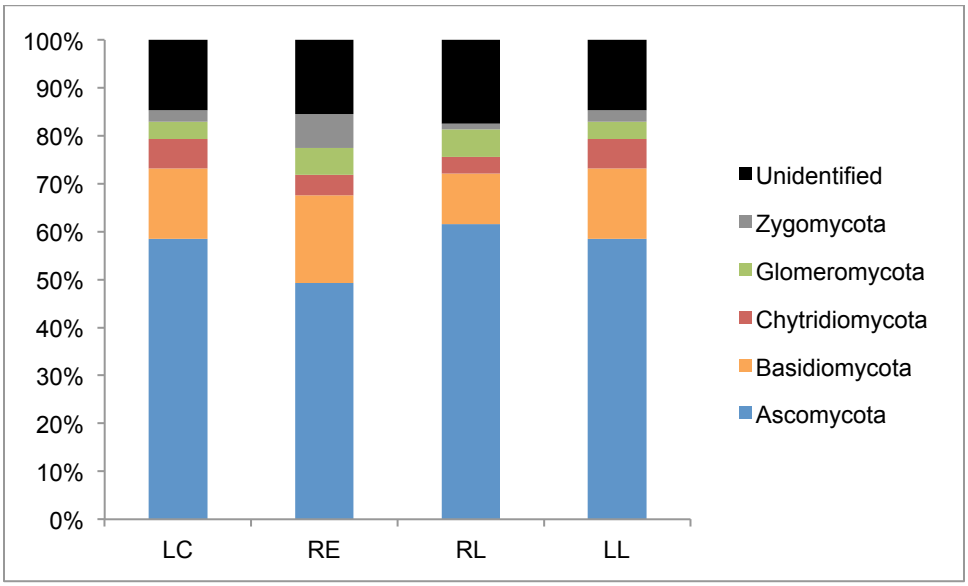


Figure 3.3 (b)


100%
90%
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70%
60%
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10%
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LC
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RL
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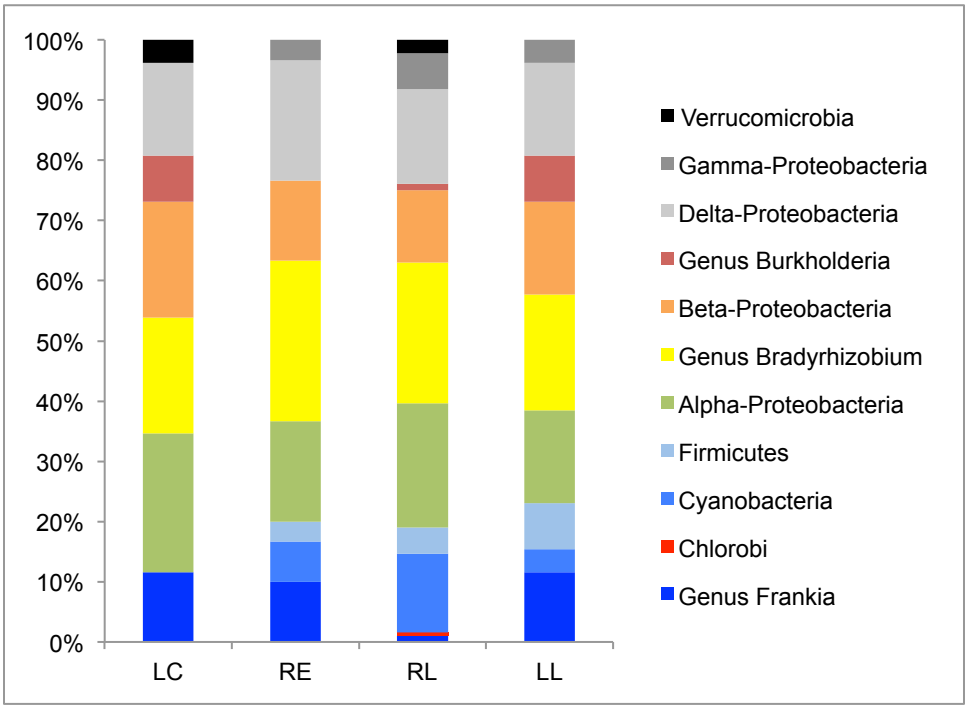
Unidentified
Zygomycota
Glomeromycota
Chytridiomycota
Basidiomycota
Ascomycota

Figure 3.3 (c)


100%
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10%
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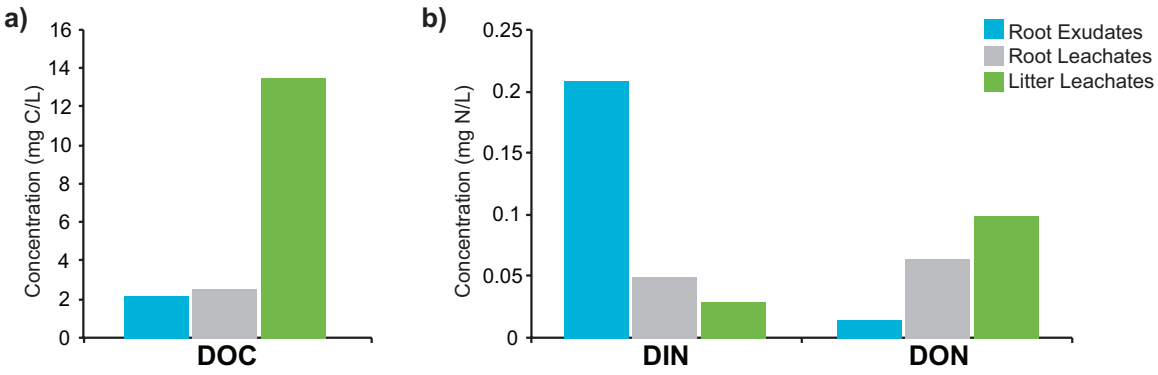
LC
RE
RL
LL
Verrucomicrobia
Gamma-Proteobacteria
Delta-Proteobacteria
Genus Burkholderia
Beta-Proteobacteria
Genus Bradyrhizobium
Alpha-Proteobacteria
Firmicutes
Cyanobacteria
Chlorobi
Genus Frankia

Figure 3.4: Nutrient content of *L. cuneata* root and litter solutions. Bars represent concentration of a) dissolved organic carbon or b) dissolved inorganic nitrogen and dissolved organic nitrogen in mg/L. Blue bars represent root exudates, grey bars represent root leachates, and green bars represent litter leachates.



TABLES

Table 3.1: Metabolites with significant relationships to plant biomass. R^2 and *p*-values corresponding with linear models with plant biomass as the response variable and metabolite concentration as the predictor variable. All metabolites listed in this table were significantly related to plant biomass.

Plant	Metabolite	R^2	*p*-value	Direction
L. cuneata	Benzoic acid-2,4-dihyrdoxy	0.1142	0.0381	Positive
	Arabinose	0.1073	0.04322	Negative
	Butanoic acid-3-hydroxy	0.1039	0.04597	Negative
	Decanoic acid	0.1297	0.02868	Negative
	Glycerol	0.1173	0.03599	Negative
	Maltose	0.1282	0.02947	Negative
	Phosphoric acid	0.1279	0.02981	Negative
	Sucrose	0.1276	0.02981	Negative
	Tetradecanoic acid	0.09997	0.04936	Negative
	Xylulose	0.1119	0.03972	Negative
L. virginica	Alanine	0.2519	0.00277	Negative
	Benzoic acid-2,4-dihydroxy	0.2706	0.001896	Positive
	Butanoic acid-2-hydroxy	0.2558	0.002563	Positive
	Butanoic acid-3-hydroxy	0.2805	0.001543	Negative
	Butanoic acid-3-methyl-2-oxo	0.2857	0.001385	Positive
	Decanoic acid	0.2258	0.004658	Negative
	Fructose	0.2611	0.002299	Negative
	Glucaric acid	0.2361	0.003804	Negative
	Gluconic acid	0.146	0.02124	Negative
	Glucose	0.2852	0.0014	Negative
	Glycerol	0.1077	0.04286	Negative
	Glyoxylic acid	0.1816	0.01091	Negative
	Hexadecanol	0.122	0.033	Negative
	Inositol myo	0.2806	0.001541	Negative
	Lactic acid	0.2057	0.006889	Negative
	Maltose	0.2351	0.003878	Negative
	Mannose	0.2017	0.007438	Negative
	Phosphoric acid	0.1562	0.01758	Negative
	Pipecolic acid	0.2857	0.001385	Positive
	Pyroglutamic acid	0.1293	0.005292	Negative

Table 3.1 (cont'd): Metabolites with significant relationships to plant biomass. R^2 and *p*-values corresponding with linear models with plant biomass as the response variable and metabolite concentration as the predictor variable. All metabolites listed in this table were significantly related to plant biomass.

L. virginica	Ribose	0.1046	0.04534	Negative
	Succinic acid	0.1829	0.01065	Negative
	Sucrose	0.1538	0.01836	Negative
	Urea	0.2424	0.003354	Negative
P. virgatum	2-Methyl succinic acid	0.2058	0.006878	Negative
	4-amino butanoic acid	0.2058	0.006878	Negative
	Adipic acid	0.2058	0.006878	Negative
	Arabinose	0.1999	0.007699	Negative
	Arabitol	0.2054	0.006927	Negative
	Azelaic acid	0.2058	0.006878	Negative
	Benzene-1,2,4-triol	0.1907	0.009178	Negative
	Benzoic acid-4-hydroxy	0.2058	0.006878	Negative
	Decanoic acid	0.1178	0.03566	Negative
	Erythritol	0.2058	0.006878	Negative
	Ethanolamine	0.1063	0.04397	Negative
	Glutaric acid	0.2049	0.00699	Negative
	Glycerol	0.1906	0.0092	Negative
	Maltose	0.1088	0.04204	Negative
	Mannitol	0.2058	0.006878	Negative
	Octadecanol	0.1873	0.009801	Negative
	Pentadecanoic acid	0.1966	0.008199	Negative
	Phosphoric acid	0.1681	0.01407	Negative
	Quinic acid	0.2058	0.006878	Negative
	Shikimic acid	0.2058	0.006878	Negative
	Sucrose	0.1694	0.01373	Negative
	Tetradecanoic acid	0.2036	0.00717	Negative
	Trehalose	0.2058	0.006878	Negative
	Xylose	0.2058	0.006878	Negative
	Xylulose	0.1964	0.00824	Negative

Table 3.2: Summary of enriched Basidiomycotal fungal OTUs under each conditioning treatment. Functional roles highlighted in green represent beneficial fungi, and those highlighted in red represent deleterious fungi.

Conditioning Treatment	Class	Order	Family	Genus	Species	Ecology	Reference
Living plants	Agaricomycetes	Agaricales	Clavariaceae	unidentified	unidentified	ECM	(Birkebak et al.)
	Agaricomycetes	Agaricales	Entolomataceae	Entoloma	undatum	pathogen	(Agerer and Waller, Kobayashi and Hata
	Agaricomycetes	Agaricales	Inocybaceae	Crepidotus	applanatus	wood rot	(Hesler and Smith)
	Agaricomycetes	Agaricales	Psathyrellaceae	Lacrymaria	lacrymabunda	common in soil	(Cortez and Coelho)
	Agaricomycetes	Polyporales	Meruliaceae	Steccherinum	unidentified	wood rot	(Yuan and Wu)
	Agaricostilbomycetes	Agaricostilbales	Chionosphaeraceae	Kurtzmanomyces	unidentified	yeast	(Zhang et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Rhodotorula	lamellibrachiae	AMF Stimulator	(Fracchia et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Rhodotorula	unidentified	AMF Stimulator	(Fracchia et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Sporobolomyces	poonsookiae	pathogen suppression	(Bashi and Fokkema
	Tremellomycetes	Tremellales	Incertae sedis	Dioszegia	takashimae	AMF Associated	(Renker et al.)
Root exudates	Agaricomycetes	Agaricales	Agaricaceae	Agaricus	unidentified	common saprophyte	(Bas 1991)
	Agaricomycetes	Agaricales	Clavariaceae	Clavaria	californica	ECM	(Birkebak et al.)
	Agaricomycetes	Agaricales	Entolomataceae	Entoloma	unidentified	pathogen	(Agerer and Waller, Kobayashi and Hata
	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe	mollis	pathogen	(Halbwachs et al.)
	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	unidentified	wood rot	(Hoegger et al.)
	Agaricomycetes	Agaricales	Strophariaceae	Gymnopilus	eucalyptorum	wood rot	(Ratkowsky and Gat
	Agaricomycetes	Auriculariales	Incertae sedis	Auricularia	unidentified	wood rot	(Looney et al.)
	Agaricomycetes	Sebacinales	Sebacinaceae	Efibulobasidium	albascens	ECM	(Weiß et al.)
	Agaricomycetes	Sebacinales	Sebacinaceae	unidentified	unidentified	ECM	(Weiß et al.)
	Agaricostilbomycetes	Agaricostilbales	Agaricostilbaceae	Bensingtonia	ciliata	yeast	(Wang et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Rhodotorula	pallida	AMF Stimulator	(Fracchia et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Sporobolomyces	lactophilus	pathogen suppression	(Bashi and Fokkema
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Sporobolomyces	linderae	pathogen suppression	(Bashi and Fokkema
	Tremellomycetes	Tremellales	Incertae sedis	Hannaella	luteola	yeast	(Landell et al.)
	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	Pseudozyma	flocculosa	yeast	(Piątek et al.)
Root Leachates	Agaricomycetes	Agaricales	Agaricaceae	unidentified	unidentified	saprotroph	(Richter et al.)
	Agaricomycetes	Agaricales	Clavariaceae	unidentified	unidentified	ECM	(Birkebak et al.)
	Agaricomycetes	Agaricales	Strophariaceae	unidentified	unidentified	wood rot	(Barrasa et al.)
	Agaricomycetes	Polyporales	Polyporacheae	Dichomitus	squalens	wood rot	(Rytioja et al.)
	Agaricomycetes	Sebacinales	Sebacinaceae	unidentified	unidentified	ECM	(Weiß et al.)
Litter Leachates	Agaricomycetes	Agaricales	Clavariaceae	unidentified	unidentified	ECM	(Birkebak et al.)
	Agaricomycetes	Agaricales	Entolomataceae	Entoloma	undatum	pathogen	(Agerer and Waller, Kobayashi and Hata
	Agaricomycetes	Agaricales	Inocybaceae	Crepidotus	applanatus	wood rot	(Hesler and Smith)
	Agaricomycetes	Agaricales	Psathyrellaceae	Lacrymaria	lacrymabunda	common in soil	(Cortez and Coelho)
	Agaricomycetes	Polyporales	Meruliaceae	Steccherinum	unidentified	wood rot	(Yuan and Wu)
	Agaricostilbomycetes	Agaricostilbales	Chionosphaeraceae	Kurtzmanomyces	unidentified	yeast	(Zhang et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Rhodotorula	lamellibrachiae	AMF Stimulator	(Fracchia et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Rhodotorula	unidentified	AMF Stimulator	(Fracchia et al.)
	Microbotryomycetes	Sporidiobolales	Incertae sedis	Sporobolomyces	poonsookiae	pathogen suppression	(Bashi and Fokkema
	Tremellomycetes	Tremellales	Incertae sedis	Dioszegia	takashimae	AMF Associated	(Renker et al.)

Table 3.3: Chemical metabolites identified by GC/MS in *L. cuneata* root and litter solutions. Values represent relative concentration of each metabolite per 2mL of solution. Metabolites highlighted in blue indicate those that were found in root exudates only. Metabolites highlighted in grey indicate those that were found in root leachates only. Metabolites highlighted in green indicate those that were found in litter leachates only. Metabolites highlighted in orange indicate those that were found in all solutions.

	Root Exudates	Root Leachates	Litter Leachates
1,3-DIAMINOPROPANE	0.14	0.13	0.00
1-Benzylglucopyranoside	0.00	0.00	0.62
1-Ethylglucopyranoside	0.00	0.00	13.58
1-Methyl-a-D-glucopyranoside	0.00	0.00	54.50
2-Methylsuccinic acid	0.08	0.00	0.00
3,4-Dihydroxybutanoic acid	0.00	0.00	0.63
3-Methyl-4-hydroxybutanoic acid	0.00	0.00	0.76
4-Aminobutanoic acid	0.17	0.00	0.00
Adipic acid	0.54	0.00	0.00
Alanine	4.05	2.26	4.97
arabinose	2.95	0.24	0.60
arabitol	1.06	0.00	0.02
Azelaic acid	0.23	0.00	0.00
benzene-1,2,4-triol	0.37	0.11	0.00
Benzoic acid	0.50	0.44	1.41
Benzoic acid, 2,4-dihydroxy	0.40	2.46	0.85
Benzoic acid, 2,5-dihydroxy	0.17	0.00	0.00
Benzoic acid, 2-methyl	0.13	0.00	0.18
Butane, 1,2,3-trihydroxy	0.00	0.00	16.90
Butanoic acid, 2-hydroxy	0.09	0.28	0.00
butanoic acid, 3-hydroxy	0.39	0.00	0.34
Butanoic acid, 3-methyl-2-oxo-	0.00	0.18	0.00
Butanoic acid, 3-methyl-3-hydroxy	0.00	0.00	0.09
butanoic acid, 4-hydroxy	0.09	0.00	0.00
decanoic acid	1.06	0.00	0.61
Erythritol	1.99	0.00	0.00
Erythronic acid	0.00	0.00	0.72
ERYTHROSE	0.00	0.00	0.31
ethanolamine	75.57	71.54	62.70
Fructose	4.52	0.37	6.25
Galactonic acid	0.27	0.00	7.83
Galactose	0.00	0.00	0.24
Glucaric acid	0.50	0.00	0.84
gluconic acid	0.29	0.00	0.91
glucose	15.27	0.91	14.75
Glutaric acid	0.65	0.10	0.12
glycerol	41.88	19.99	24.95
Glycine	1.29	1.73	6.16
Glycolic acid	0.96	1.83	5.00
glyoxylic acid	11.85	9.90	14.54

Table 3.3 (cont'd): Chemical metabolites identified by GC/MS in *L. cuneata* root and litter solutions. Values represent relative concentration of each metabolite per 2mL of solution. Metabolites highlighted in blue indicate those that were found in root exudates only. Metabolites highlighted in grey indicate those that were found in root leachates only. Metabolites highlighted in green indicate those that were found in litter leachates only. Metabolites highlighted in orange indicate those that were found in all solutions.

	Root Exudates	Root Leachates	Litter Leachates
hexadecanol	0.20	0.00	0.80
Inositol, myo	0.52	0.00	0.61
INOSITOL, scyllo	9.78	0.00	179.11
lactic acid	80.64	39.05	123.53
Maltose	3.76	0.00	2.29
MANNITOL	1.24	0.00	0.00
MANNOSE	0.38	0.02	0.77
Methyl-inositol #1	34.84	0.70	2618.71
Methyl-inositol #2	0.00	0.00	3.10
Methyl-inositol #3	0.00	0.00	6.41
octadecanol	0.43	0.22	0.12
Pentadecanoic acid	2.36	0.58	0.00
Phosphoric acid	19.86	4.83	10.32
Pipecolic acid	0.00	0.33	0.00
Propanoic acid, 3-hydroxy	0.07	0.02	0.33
Pyroglutamic acid	0.67	0.00	1.25
quinic acid	0.17	0.00	0.00
ribonic acid	0.19	0.00	4.44
ribose	0.89	0.67	1.78
Sedoheptulose	0.00	0.00	1.64
Serine	0.00	0.00	1.76
SHIKIMIC ACID	0.47	0.00	0.00
Succinic acid	1.64	1.25	2.17
SUCROSE	2.08	0.24	0.90
Tartaric acid	0.00	0.00	0.34
Tetradecanoic acid	4.01	1.34	1.53
threonic acid	0.47	0.00	7.02
THREONINE	0.00	0.00	0.45
Trehalose	0.37	0.00	0.00
urea	3.18	0.00	5.13
xylose	0.92	0.00	0.00
Xylulose	11.82	0.00	2.06

CHAPTER 4: *LESPEDEZA CUNEATA* INVASION HAS LASTING INFLUENCES ON SOIL MICROBIAL COMMUNITIES *IN SITU*

ABSTRACT

Lespedeza cuneata, an east Asian legume with an invaded range that spans the eastern and Midwestern United States, can alter soil microbial communities by means of its unusual litter chemistry and its microbial mutualisms. Alterations to soil microbial communities may facilitate *L. cuneata* invasion and mediate its influence on soil chemistry. The objectives for this study are 1) to examine the influence of *L. cuneata* on soil microbial communities in the field, 2) to identify microbial taxonomic groups as potential symbionts, pathogens, and decomposers that are important in *L. cuneata* dominated systems, and 3) to identify shifts in soil chemistry that coincide with the progression of *L. cuneata* invasion. I conducted a multi-year study that examined *L. cuneata* biomass, soil microbial community composition, and soil chemistry at an invaded prairie in McLean County, Illinois, USA. *L. cuneata* biomass was correlated to microbial community composition to identify microbial taxa that were enriched by *L. cuneata* invasion. Results suggest that the impact of invasion is long-lasting and more complicated than measures of current biomass might suggest. Severe invasion was associated with distinct decomposer, symbiont, and pathogen communities that may be important for mediating both invasive success and ecosystem impacts.

INTRODUCTION

Lespedeza cuneata, an invasive legume introduced from Asia in the late 1800's, dominates native plant communities and disrupts natural grassland

ecosystems across the United States (Eddy and Moore 1998). The invasive success of *L. cuneata* is due to a variety of plant traits, such as high seed production and population growth rates (Woods et al. 2009), shading-out of neighboring plants (Allred et al. 2010), and allelopathic inhibition of competitor seed germination (Kalburtji and Mosjidis 1992, 1993a, b).

Additionally, participation in mutualistic relationships with soil microbes may contribute to the invasive success of *L. cuneata*. As a legume, *L. cuneata* associates with nitrogen-fixing bacteria that enable its growth in nutrient poor conditions (Brandon et al. 2004, Houseman et al. 2014). *L. cuneata* has been shown to benefit more from symbiotic nitrogen fixation than its native counterpart, *L. virginica* (Hu et al. 2014), and *L. cuneata* may also have a stronger affinity for associating with Rhizobiales bacteria than several of its native North American congeners (Busby et al. 2016). Results from **Chapter 2** suggest that nitrogen fixation in *L. cuneata* may increase soil ammonium concentrations.

Plant-soil feedback may also influence the invasive success of *L. cuneata* in the field. Plant-soil feedback is a density dependent force that results from plant-mediated shifts in the relative abundance of beneficial and deleterious soil microorganisms (Mills and Bever 1998, Bever 2002). Plant-soil feedback influences the growth of future conspecific and heterospecific plants, and alters the outcome of plant-plant competition (Bever 2003). In **Chapter 2**, I showed that negative plant-soil feedback exists between *L. cuneata* and its native congener, *L. virginica*. This negative plant-soil feedback may be important for the early stages of *L. cuneata* invasion. Results from this study suggest that *L. cuneata*

enriches for deleterious microbes and may alter communities of microbial decomposers in soil. *L. cuneata* litter and root exudates contain large quantities of phenolic compounds that directly decrease the germination and growth of native grasses (Kalburtji and Mosjidis 1992, 1993b, a). These types of compounds have been shown to slow decomposition and rates of nutrient release in soil (Kalburtji et al. 1999, Sariyildiz and Anderson 2003). Large amounts of *L. cuneata* litter that are introduced when the invader dominates native ecosystems may enrich for microbial decomposers that are well-suited to degrade high lignin content plant litter. For example, white-rot fungi have previously been shown to play an important role in the breakdown of lignin in *L. cuenata* litter (Gamble et al. 1996).

A plant's ability to alter soil microbial communities is a pre-requisite for facilitating plant-soil feedback. Recent work has shown that *L. cuneata* has unique interactions with soil microbes. *L. cuneata* has been shown to alter bacterial and fungal community composition at invasion sites (Yannarell et al. 2011). Additionally, there is some evidence that *L. cuneata* growth may increase in soil with a history of *L. cuneata* invasion into a native grassland community (Coykendall and Houseman 2014, Crawford and Knight 2017). However, results from greenhouse studies reported in **Chapter 2 and 3** of this dissertation suggest that *L. cuneata* itself may not be enriching for beneficial microbes, but instead *L. cuneata* may benefit from microbes enriched by native plant communities.

This current chapter was designed as a follow-up observational study to see if similar results could be obtained in the field. I will address the following

specific research questions: 1) Does *L. cuneata* invasion have a lasting influence on soil microbial community composition *in situ*? 2) Is *L. cuneata* invasion correlated with the enrichment of potential symbionts, pathogens, and specialist decomposers? and 3) Is *L. cuneata* invasion correlated with specific changes in soil chemistry?

METHODS

Sampling and Soil Nutrient Analyses

Vegetation and soil sampling were conducted at John English Memorial Prairie, Comlara Park, Mclean County, IL, USA. This prairie was restored from abandoned farmland in the 1970's by seeding with Illinois grasses for one year and then with locally collected forbs thereafter. In 2006, 108 permanent 1 m^2 sampling plots were established to characterize plant community composition (Borowicz and Armstrong 2012). *Lespedeza cuneata* invasion was first noted in John English Prairie in 2006, and started to become increasingly dominant in 2011. In the present study, we examined a subset of the original plots that has observations in each of the study years (2006, 2009, 2010, 2011, and 2016; n = 59). *L. cuneata* biomass was measured in late August of all sampling years, and soil chemistry and soil microbial community composition was examined in 2011 and 2016 only, at the same time as vegetation sampling. *L. cuneata* biomass was measured by clipping all aboveground vegetation from the center 0.25m^2 of each plot. *L. cuneata* tissue was dried to constant weight and total *L. cuneata* biomass was recorded. Multiple variables were created to quantify the long term effects of *L. cuneata* invasion: Cumulative *L. cuneata* biomass was calculated by

summing yearly biomass recordings and the duration of *L. cuneata* invasion represented the number of years since *L. cuneata* had first been recorded in that plot. Soil samples were collected from a depth of 0-5cm to examine soil chemistry and soil microbial community composition.

Soil inorganic nitrogen was extracted from each soil sample by shaking 10g soil in 40mL 2M KCl for 1 hour. Shaken samples were passed through a Whatman #4 filter and frozen until undergoing colorimetric analysis for ammonium and nitrate concentrations (Weatherburn 1967, Doane and Horwáth 2003). The pH of each sample was recorded after 1-hour incubations of air-dried soil in 10mM $CaCl_2$.

Soil microbial community characterization

For each soil sample, I extracted DNA from 500mg of field fresh soil using the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA) following manufacturer instructions. After extraction, I purified extracted DNA by incubating with 1% cetyltrimethyl ammonium bromide (CTAB) for 15 minutes at 65°C, extracting DNA from bound CTAB with 24:1 chloroform:isoamyl alcohol, precipitating with 100% ice cold ethanol, washing twice with 70% ethanol, and finally dissolving purified DNA in 65°C 1x TE buffer. I quantified DNA yields by absorbance at 260nm and diluted samples to an ending concentration of 20ng/μL and stored samples at -80°C until use.

I characterized microbial communities by sequencing genes that are commonly used for the identification of bacteria, fungi, and nitrogen-fixing organisms. Samples were diluted to a concentration of 2ng/uL and specific gene

targets were amplified simultaneously using Fluidigm 2 Step Access Array Amplification on a Fluidigm 48.48 Access Array IFC (Fluidigm Corporation, San Fransisco, CA), following manufacturer instructions. Unique nucleotide barcodes were added to PCR reactions to identify individual samples so that all PCR products could be pooled and sequenced together. I used primers 515F and 926F to amplify the V4-V5 region of the bacterial 16S rRNA gene (Walters et al. 2016), primers ITS3 and ITS4 to amplify the fungal second internal transcribed spacer region (ITS2) (White et al. 1990), and primers PolF and PolR to amplify the nitrogen-fixing gene, *nifH* (Poly et al. 2001). PCR products were confirmed on a Fragment Analyzer (Advanced Analytics, Ames, IA) and pooled in equimolar concentrations. Pooled products were size selected on a 2% agarose E-gel (Life Technologies, Carlsbad, CA) and extracted with Qiagen gel extraction kit (Qiagen, Hilden, Germany). Size selected products were run on an Agilent Bioanalyzer (Agilent, Santa Clara, CA) and pooled. Pooled amplicons were sequenced on an Illumina MiSeq V3 platform using a 2x250 base pair read configuration in Bulk Kit version 3 (Illumina, San Diego, CA, USA). PCR reactions and sequencing were carried out at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

I processed raw microbial sequence data using multiple platforms. Forward and reverse reads of each paired-end sequence were merged using Fast Length Adjustment of Short reads (FLASH) software (Magoč and Salzberg 2011). During this step, I also removed sequences that contained greater than 10% of bases with quality scores below 30 and ambiguous bases. For the

remaining sequences, I used USEARCH (http://www.drive5.com/usearch/) to 1) de-replicate sequences and remove singletons, 2) remove chimeric sequences detected by the GOLD database for bacteria (Kyrpides 1999), the UNITE ITS database for fungi (Abarenkov et al. 2010), and a custom *nifH* database created by downloading sequences from the RDP FunGene website (Fish et al. 2013) and assigning taxonomic information using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990); and 3) cluster sequences by 97% similarity to form operational taxonomic units (OTUs). I aligned representative sequences for each bacterial OTU, and I left fungal and *nifH* sequences unaligned. For bacteria, I assigned taxonomic information using the RDP classifier in QIIME (Caporaso et al. 2010). For fungi and nitrogen fixers, I assigned taxonomic information using the (BLAST) algorithm (Altschul et al. 1990) and the same databases mentioned in step 2 above.

Data Analysis

I visualized the progression of *L. cuneata* invasion at John English Memorial Prairie by plotting *L. cuneata* biomass on top of spatial coordinates of sampling plots for each year. In order to examine the site-wide changes that occurred during the greatest increase in *L. cuneata* invasion severity, I compared the average *L. cuneata* biomass, soil ammonium, soil nitrate, and soil pH in 2011 and 2016. Statistically significant differences between years were determined with Student's T-tests. I used linear regression to determine which of these variables was related to *L. cuneata* biomass in 2016.

I examined microbial community composition using processed sequence data in the vegan() package (Oksanen et al. 2009) for the R statistical environment (R Core Development Team 2005). Bacterial, fungal, and nitrogen-fixing communities were examined individually. I visualized variation in microbial community composition that could be explained by sampling year using non-metric multidimensional scaling (NMDS) and the plotting function ordiplot(). Significant correlations (p<0.05) between environmental data (yearly *L. cuneata* biomass, duration of *L. cuneata* invasion, cumulative *L. cuneata* biomass, soil ammonium, soil nitrate, pH, space) and microbial community composition were plotted onto ordinations. Significance of correlations was determined by comparison to permutations of environmental variables using the function envfit().

In order to identify microbial OTUs that were associated with severe L. cuneata invasion, I modeled 2016 L. cuneata biomass as a function of microbial community composition using partial least squares regression (PLSR) (Carrascal et al. 2009). I considered the top 2% of the OTU loadings along the first PLSR latent variable as being associated with high 2016 *L. cuneata* biomass, and the bottom 2% of these OTU loadings as being associated with low 2016 *L. cuneata* biomass.

RESULTS

L. cuneata invasion expanded drastically between the years of 2011 and 2016 **(Figure 4.1)**. During this time, there was also a significant increase in average soil pH (from 5.2 to 5.57) and decrease in average soil ammonium and

nitrate concentrations **(Figure 4.2)**. Despite the site-wide decrease in average soil ammonium concentration, there was a positive relationship between 2016 *L. cuneata* biomass and soil ammonium concentration **(Figure 4.3)**.

Sampling year (2011 or 2016) explained a significant portion of the variance in bacterial, fungal and nitrogen-fixing community turnover **(Figure 4.4)**. In 2011, the north-west and south-east portions of the prairie had distinct bacterial and fungal community composition. By 2016, bacterial and fungal community composition became more similar across the prairie **(Figure 4.4a, b)**. For bacteria, a gradient from early *L. cuneata* biomass to 2016 and cumulative *L. cuneata* biomass was significantly correlated to bacterial community turnover. Soil pH and nitrate gradients were also significantly correlated to bacterial community turnover. For fungi, gradients of early, 2016, and cumulative *L. cuneata* biomass were correlated to community turnover. No measures of soil chemistry were significantly correlated to fungal community turnover. Nitrogen-fixing bacterial community composition was distinct by year **(Figure 4.4c)**. This distinct turnover was correlated to changing soil inorganic nitrogen concentrations and soil pH. In addition to this separation based on year, a gradient from early to 2016 *L. cuneata* biomass was significantly correlated to nitrogen-fixing bacterial community turnover.

There were coarse differences in the taxonomic distributions of bacterial, fungal, and nitrogen-fixing bacterial OTUs that were associated with high 2016 *L. cuneata* biomass compared to OTUs associated with low 2016 *L. cuneata* biomass **(Figure 4.5)**. Bacterial OTUs associated with high 2016 *L. cuneata*

biomass represented proportionally more Chloroflexi, Proteobacteria, and Acidobacteria and proportionally less Verrucomicrobia and Actinobacteria than OTUs associated with low 2016 *L. cuneata* biomass **(Figure 4.5a)**. Within the phylum Proteobacteria, OTUs associated with high 2016 *L. cuneata* biomass, but not low 2016 *L. cuneata* biomass, represented the orders Caulobacterales (genus *Phenylobacterium*), Sphingomonadales (genus *Kaistobacter*), and Enterobacteriales (genus *Erwinia*) **(Table 4.1)**. OTUs associated with high 2016 *L. cuneata* biomass represented proportionally more Burkholderiales and Xanthomonadales (families Sinobacteraceae and Xanthomonadaceae) and proportionally less Rhizobiales and Desulfomonadales (genus *Geobacter*) compared to OTUs associated with low 2016 *L. cuneata* biomass.

Fungal OTUs associated with high 2016 *L. cuneata* biomass represented proportionally more Basidiomycota than those associated with low 2016 *L. cuneata* biomass. OTUs associated with low 2016 *L. cuneata* biomass included the phylum Zygomycota (genera *Mortierella* and *Mucor*), but OTUs associated with high 2016 *L. cuneata* biomass did not **(Figure 4.5b)**. Within the phylum Basidiomycota, OTUs associated with both high and low 2016 *L. cuneata* biomass included the order Agaricales **(Table 4.2)**. Fungal OTUs associated with high 2016 *L. cuneata* biomass also included the orders Hymenochaetales and Trechisporales. OTUs associated with low 2016 *L. cuneata* biomass also included the orders Sebacinales and Thelephorales. High and low 2016 *L. cuneata* biomass was associated with taxonomically distinct nitrogen-fixing bacterial communities, however both sets of associated OTUs included similarly

large amounts of *Bradyrhizobium* OTUs **(Figure 4.5c).** Different *Bradyrhizobium* OTUs were associated with high and low *L. cuneata* biomass environments.

DISCUSSION

The first goal of this observational study was to determine whether *L. cuneata* invasion has a lasting influence on soil microbial community composition in the field. A long-term influence on soil microbial community composition is a necessary pre-requisite for plant-soil feedbacks that have been observed in the greenhouse setting to be applicable in the real world. I found that long-term measures of *L. cuneata* biomass were correlated with turnover in bacterial, fungal and nitrogen-fixing bacterial communities. This suggests that the impact of *L. cuneata* invasion is long-lasting and more complicated than measures of current biomass might suggest. This interpretation is supported by prior evidence that the long-term effects of invasion may be more influential on soil microbial communities than current shifts in vegetation (Elgersma et al. 2011). The ability of *L. cuneata* to cause long-term shifts in microbial community composition in the field suggests that plant-soil feedback observed between *L. cuneata* and native plants in the greenhouse **(Chapters 2 and 3)** may be playing a role in *in situ* dynamics.

Bacterial and fungal community composition was less variable in 2016 compared to 2011. Because *L. cuneata* invasion increased drastically between 2011 and 2016, this decrease in community variability may be at least partially due to increased *L. cuneata* biomass across the prairie. Plants have species specific influences on microbial communities, and *L. cuneata* has been shown to

produce root exudates that are chemically distinct from native prairie plant species (Ringelberg et al. 2017, in press). The dominance of *L. cuneata* throughout the prairie may have lead to less variable plant chemical inputs across the site, and a less variable influence on soil microbes. Distinct composition of nitrogen-fixing bacterial communities by year was correlated to gradients of soil pH and nitrate. This suggests that nitrogen-fixing bacteria may be more sensitive to changes in edaphic factors than the whole bacterial community. Previous work has shown that high levels of soil nitrogen decrease nitrogen fixation by free-living (Castillo and Cardenas 1982) and symbiotic bacterial nitrogen fixers (Bisseling et al. 1978). Therefore, shifts in nitrogen availability may influence nitrogen-fixing communities.

The second goal of this study was to determine if *L. cuneata* invasion was associated with the enrichment of potential pathogens, symbionts, and decomposers. Severe *L. cuneata* invasion is associated with OTUs within the genus Erwinia, which contains known pathogens of *Lespedeza biocolor*, another Asian *Lespedeza* congener (Zhang and Nan 2014). As pathogenesis has not yet been documented on *L. cuneata*, the influence of *Erwinia spp.* on *L. cuneata* warrants future examination.

Severe *L. cuneata* invasion was also associated with particular microbial symbiont populations. Potential symbionts within the bacterial orders Sphingomonadales (genus *Kaistobacter*), Burkholderiales, and Xanthomonadales (families Sinobacteraceae and Xanthomonadaceae) were associated with severe *L. cuneata* invasion. Little is known about the function of

genus *Kaistobacter*, but there is evidence that this genus may be a plant endophyte (Zebin et al. 2016). Sinobacteraceae, Xanthomonadales, and Burkholderiales, bacteria have all previously been shown to inhabit *L. cuneata* nodules (Gu et al. 2007, Palaniappan et al. 2010, Hu et al. 2014, Busby et al. 2016), and thus may participate in or influence active nitrogen-fixing symbioses. OTUs from the nitrogen-fixing genus *Bradyrhizobium* were associated with both high and low *L. cuneata* biomass. Previous work has shown that *Bradyrhizobium* is a preferred symbiont of *L. cuneata* (Busby et al. 2016), and that *L. cuneata* benefits more from Bradyrhizobium nodulation than its native congeners (Hu et al. 2014). Therefore, *Bradyrhizobium* abundance in soil may be important for the invasive success of *L. cuneata*. Different Bradrhizobium OTUs were associated with high and low *L. cuneata* environments. This suggests that *L. cuneata* may enrich or specific *Bradyrhizobium* strains, and is consistent with results obtained from the greenhouse experiment in **Chapter 2**.

Previous work has shown that *L. cuneata* also associates with arbuscular mycorrhizal fungal symbionts (Wilson 1988). This study did not find any evidence of arbuscular mycorrhizal symbionts; however, because arbuscular mycorrhizal fungi are obligate symbionts that are tightly clustered around plant roots (Kirk et al. 2004), the bulk soil sampling conducted here may have biased the results against finding these types of fungus. Future studies should be designed to identify arbuscular mycorrhizal fungi that may influence *L. cuneata* growth and invasion.

This study found evidence that high *L. cuneata* biomass was associated with less diverse ectomycorrhizal fungi within the phylum Basidiomycota. These results are consistent with greenhouse results from **Chapter 2**. Grasses and forbs do not associated with ectomycorrhizal fungi (Smith and Read 2010), but a decrease in the diversity of this pool of symbionts may have negative consequences for native woody plants in invaded ecosystems. Diverse ectomycorrhizal symbionts have been shown to benefit plant growth (Baxter and Dighton 2005). This decreased diversity may also have implications for decomposition and nutrient cycling at invasion sites. Ectomycorrhizal fungi actively decompose soil organic matter (Smith and Read 2010), but differ in their ability to break down phenolic compounds (Court et al. 2006) and tolerate a range of nitrogen (Arnebrant 1994) and pH conditions (Hung and Trappe 1983). The decrease in diversity of ectomycorrhizal fungi may therefore reflect a change to high phenolic content *L. cuneata* litter inputs and changing site conditions. Because mycorrhizal communities can have large implications for carbon and nitrogen cycling (Phillips et al. 2013), this shift may have detrimental effects for invaded ecosystems.

L. cuneata invasion may also enrich for populations of decomposers that are efficient at breaking down lignin and other phenolic compounds found in *L. cuneata* litter. OTUs from the bacterial order Caulobacterales (genus Phenylobacterium)**(Table 4.1)** and the fungal orders Hymenochaetales and Trechisporales **(Table 4.2)** were associated with severe *L. cuneata* invasion and play important roles in decomposition. *Phenylobacterium* is common in upper soil

horizons and degrades phenolic compounds (Baldrian et al. 2012). Increased abundances of *Phenylobacterium* in high *L. cuneata* environments may be driven by high phenol contents in *L. cuneata* litter (Langdale and Giddens 1967, Kalburtji et al. 1999), and may therefore be responding to changes in soil chemistry that accompany *L. cuneata* invasion. Hymenochaetales and Trechisporales are white rot fungi (Greslebin and Rajchenberg 2000, Nagy et al. 2016), which are known to play an important role in the later stages of decomposition and specialize in the breakdown of lignin (Lundell et al. 2014). The association of these fungi with severe *L. cuneata* invasion may be another symptom of the condensed tannins in *L. cuneata* litter. The breakdown of lignin in *L. cuneata* litter by white rot fungi has been previously documented and may play an important role in the release of litter bound nitrogen into the ecosystem (Gamble et al. 1996).

These specialist decomposers may not be enriched in environments with less *L. cuneata* litter. The Zygomycotal genera *Mortierella* and *Mucor* were associated with low *L. cuneata* biomass. These fungal saprobes are active in multiple stages of decomposition (Thormann et al. 2003), but despite their ability to degrade lignin, they are relatively inefficient decomposers (Allison et al. 2009). Thus, their lack of association with high *L. cuneata* biomass suggests that they may not be well suited to decompose *L. cuneata* litter.

The final goal of this study was to determine if *L. cuneata* invasion was associated with specific changes in soil chemistry. Concurrent with the increase in *L. cuneata* invasion, I observed an increase in average soil pH and a decrease

in soil inorganic nitrogen content. These changing edaphic factors may have encouraged *L. cuneata* invasion. Due to its ability to obtain nitrogen through bacterial symbioses, *L. cuneata* often invades nutrient depleted sites (Brandon et al. 2004, Houseman et al. 2014). However, the positive relationship between *L. cuneata* biomass and soil ammonium observed here suggests that *L. cuneata* may be adding nitrogen to soil once it has become a dominant member of the plant community. Previous work has shown that *L. cuneata* has the capacity to increase soil nitrogen concentrations in nutrient depleted soil over time (Lynd and Ansman 1993).

Overall, the results from this study suggest that *L. cuneata* may be enriching for similar microbial communities in both greenhouse and field settings. *L. cuneata* invasion enriched for symbiont, pathogen, and unique decomposer communities that may influence plant-soil feedback with native plants and the impact of invasion on ecosystem properties.

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FIGURES

Figure 4.1: Progression of *L. cuneata* invasion over ten years at John English Memorial Prairie. Circle size indicates *L. cuneata* biomass at each sampling plot according to the key.

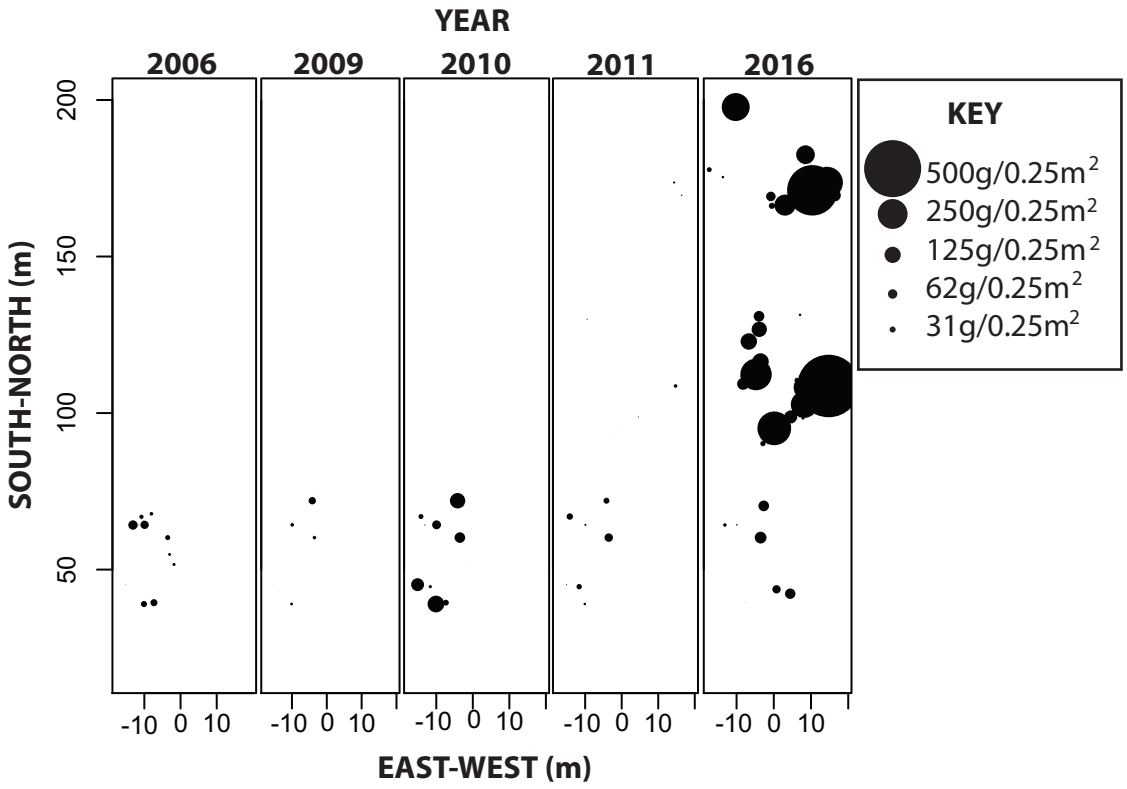


Figure 4.2: Change in *L. cuneata* biomass and soil chemistry over time. Bars represent average *L. cuneata* biomass, soil pH, soil ammonium concentration or soil nitrate concentration in 2011 (blue bars) or 2016 (red bars). Error bars represent standard error. Asterisks indicate significant differences between yearly averages (Student's t-test, $p<0.05$).

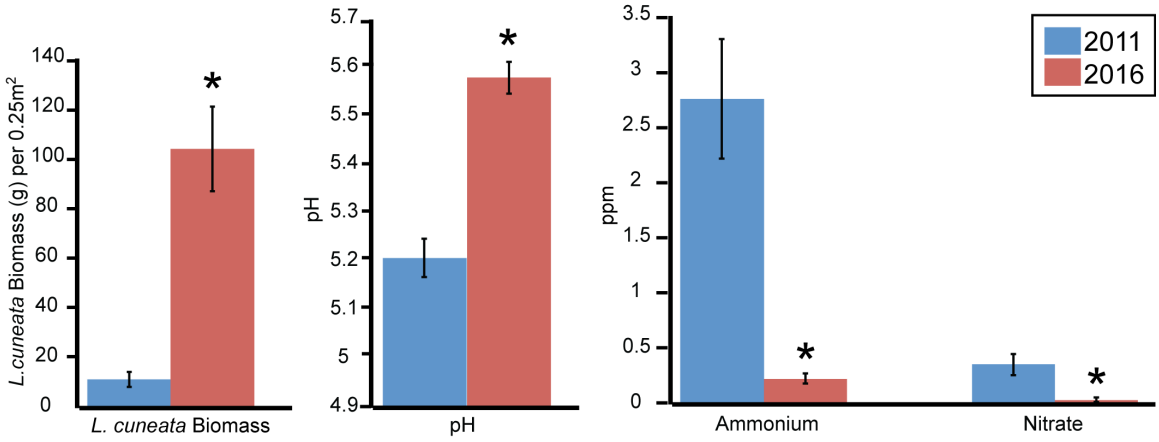


Figure 4.3: Relationship between *L. cuneata* biomass and soil chemistry. Scatterplots of individual measures of soil ammonium, soil nitrate or soil pH against individual measures of *L. cuneata* biomass in 2011 and 2016. Asterisks represent statistically significant linear trendlines ($p<0.05$).

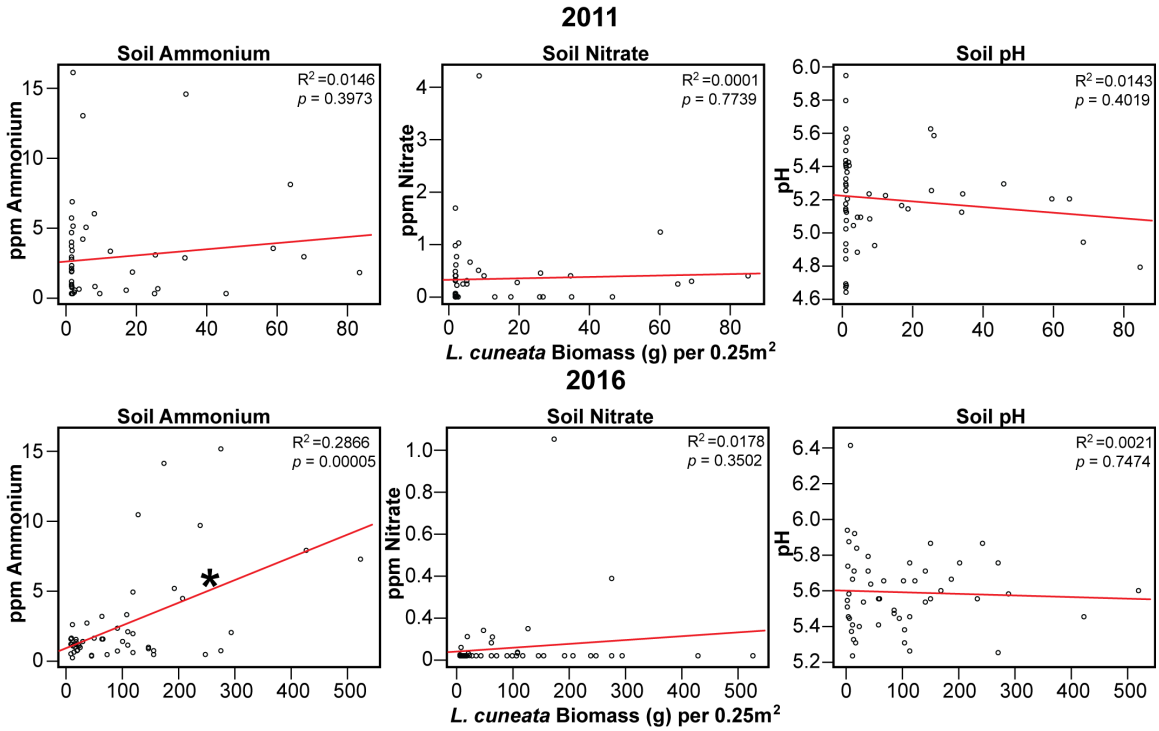


Figure 4.4: Environmental variables correlated with turnover in soil microbial community composition. Non-metric multidimensional scaling plots representing turnover in a) bacterial, b) fungal, or c) nitrogen-fixing bacterial communities by year. Each circle indicates the microbial community associated with one soil sample. Red arrows indicate significantly correlated environmental vectors (p<0.05).

Figure 4.4 (a)

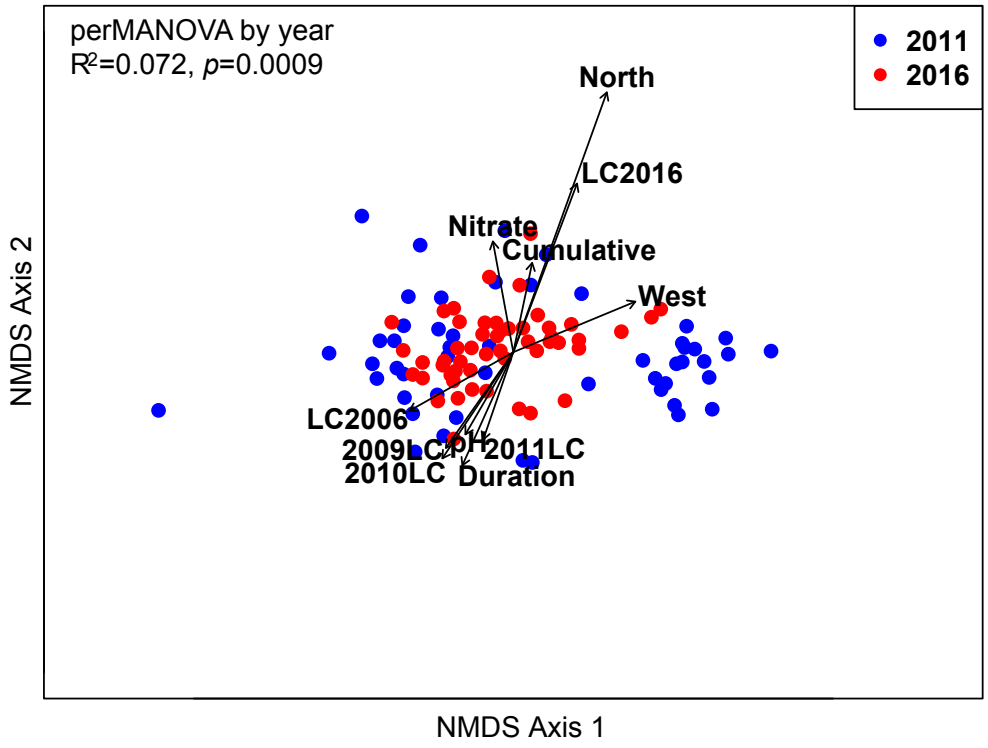


Figure 4.4 (b)

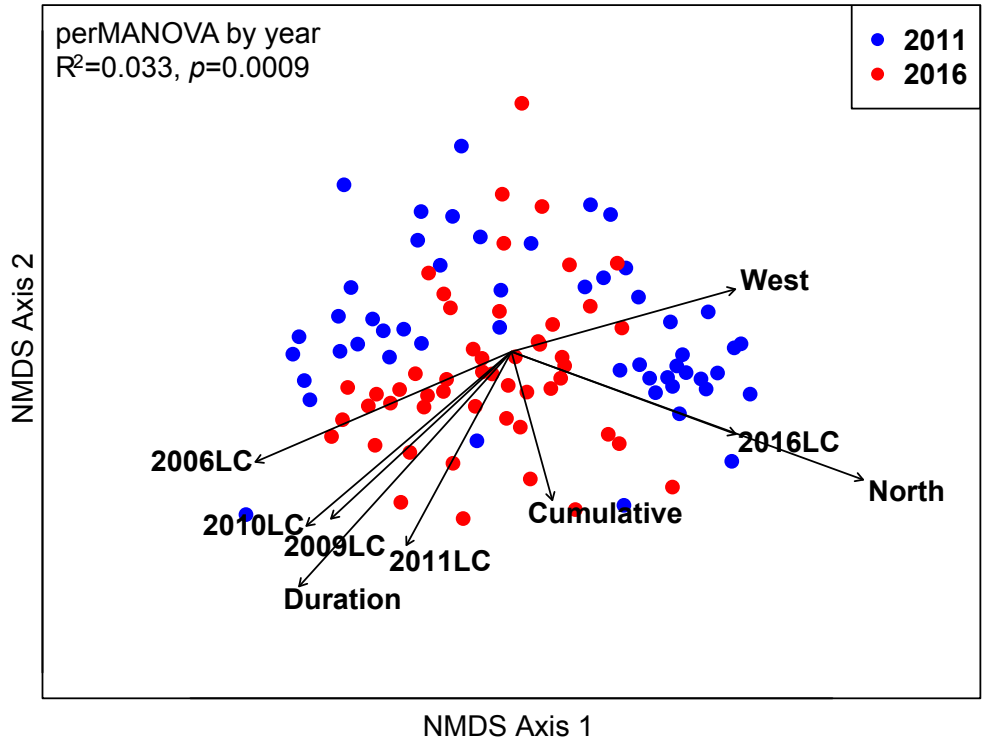

perMANOVA by year
R²=0.033, p=0.0009
2011
2016
NMDS Axis 2
NMDS Axis 1
West
2016LC
North
Cumulative
2006LC
2010LC
2009LC
2011LC
Duration

Figure 4.4 (c)

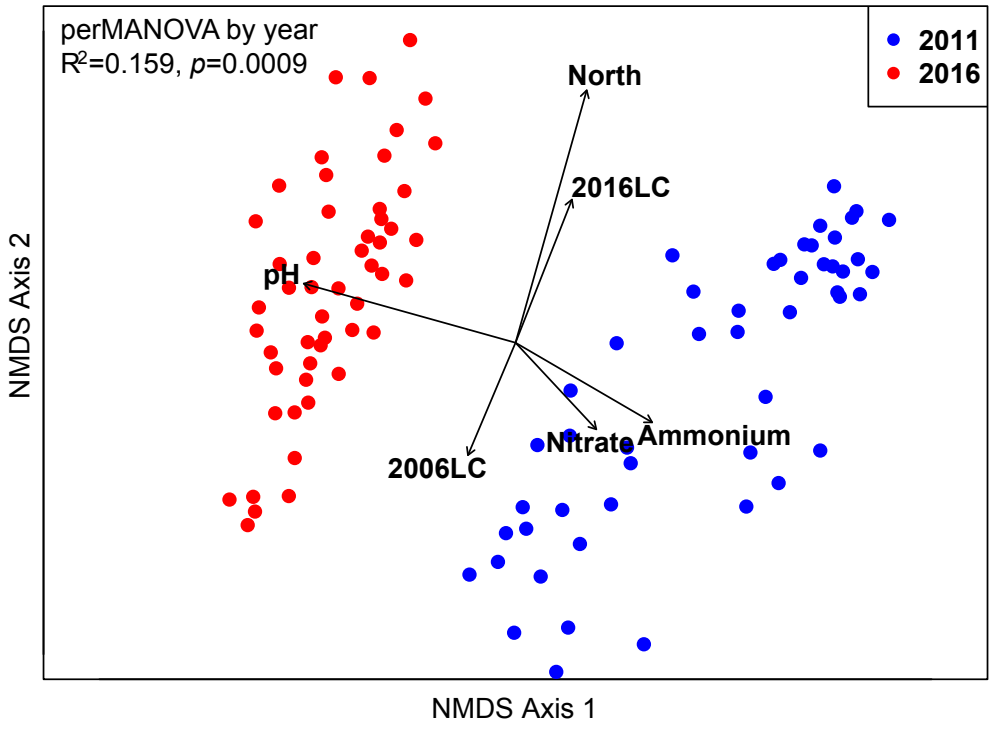

perMANOVA by year
R²=0.159, p=0.0009
2011
2016
North
2016LC
pH
Nitrate
Ammonium
2006LC
NMDS Axis 2
NMDS Axis 1

Figure 4.5: Summary of OTUs associated with high or low *L. cuneata* biomass in 2011 and 2016. Proportion of OTUs associated with high or low *L. cuneata* biomass that belong to listed a) bacterial phyla, b) fungal phyla or c) nitrogen-fixing bacterial genera. Colored bars indicate OTU classifications as designated by the appropriate key.

Figure 4.5 (a)

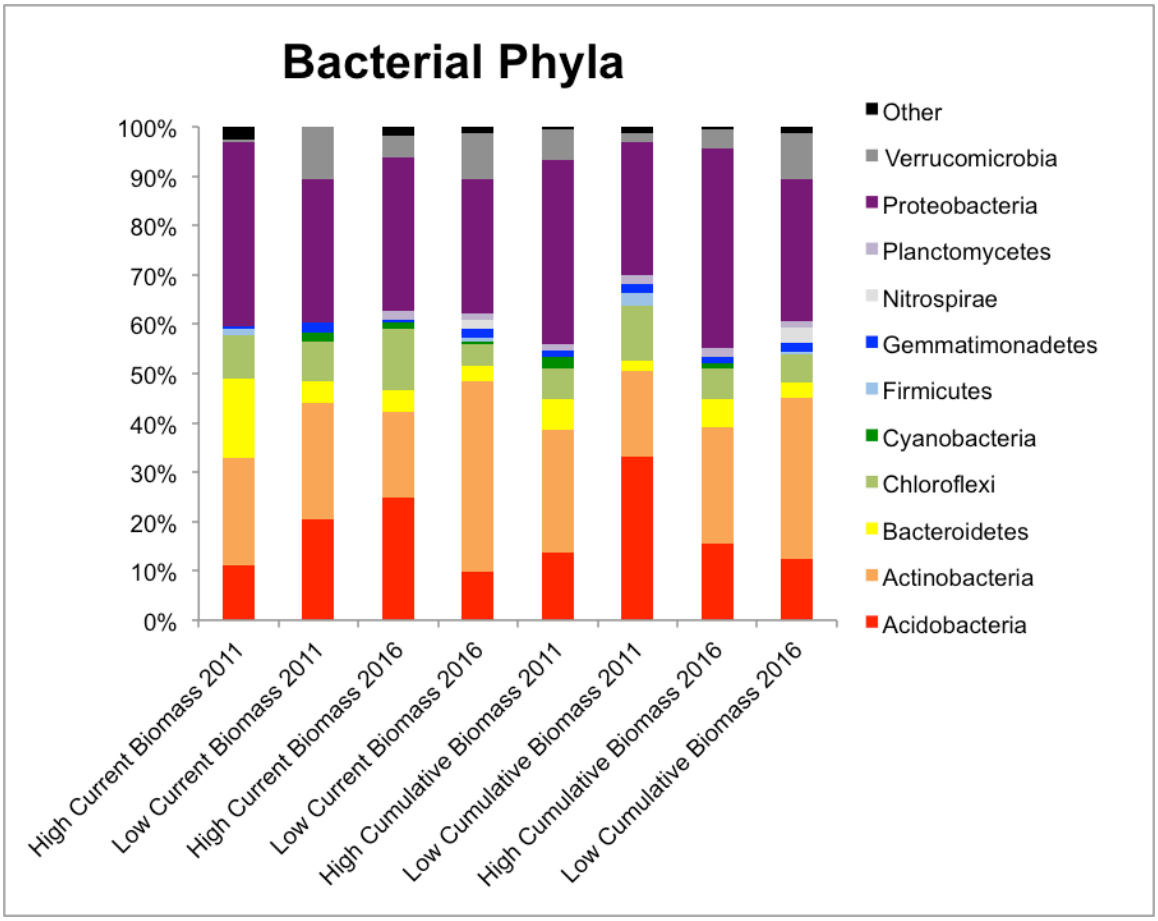


Figure 4.5 (b)

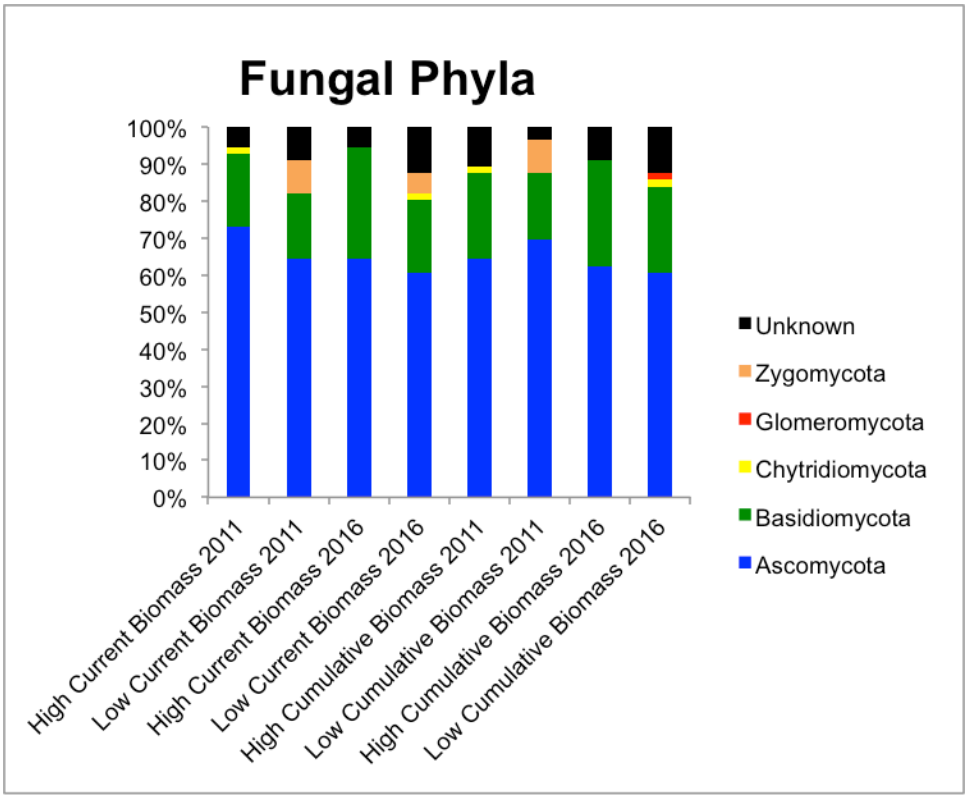
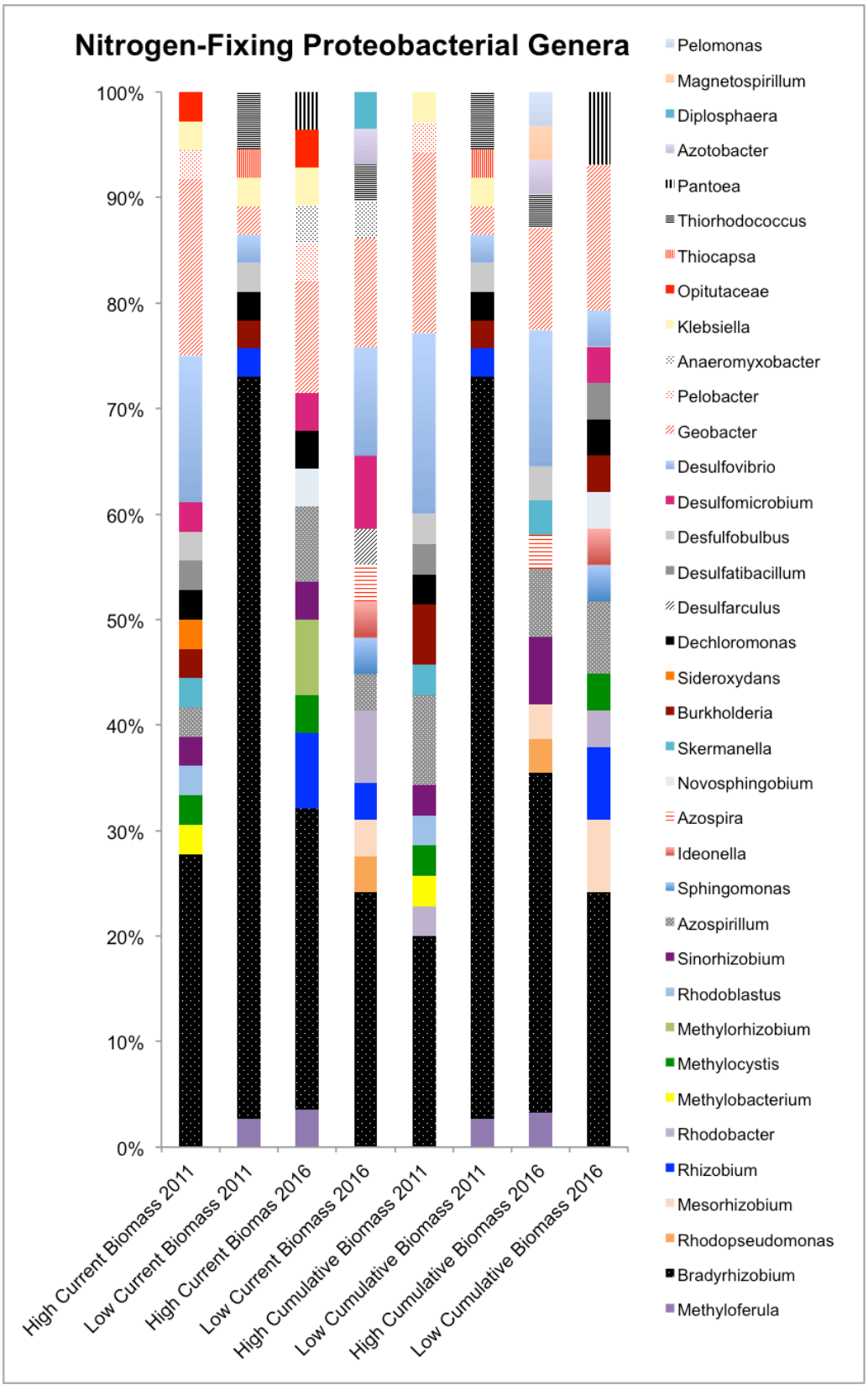

Fungal Phyla
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
High Current Biomass 2011
Low Current Biomass 2011
High Current Biomass 2016
Low Current Biomass 2011
High Cumulative Biomass 2011
Low Cumulative Biomass 2011
High Cumulative Biomass 2016
Low Cumulative Biomass 2016
Unknown
Zygomycota
Glomeromycota
Chytridiomycota
Basidiomycota
Ascomycota

Figure 4.5 (c)


Nitrogen-Fixing Proteobacterial Genera
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
High Current Biomass 2011
Low Current Biomass 2011
High Current Biomas 2016
Low Current Biomass 2016
High Cumulative Biomass 2011
Low Cumulative Biomass 2011
High Cumulative Biomass 2016
Low Cumulative Biomass 2016
Pelomonas
Magnetospirillum
Diplosphaera
Azotobacter
Pantoea
Thiorhodococcus
Thiocapsa
Opitutaceae
Klebsiella
Anaeromyxobacter
Pelobacter
Geobacter
Desulfovibrio
Desulfomicrobium
Desfulfobulbus
Desulfatibacillum
Desulfarculus
Dechloromonas
Sideroxydans
Burkholderia
Skermanella
Novosphingobium
Azospira
Ideonella
Sphingomonas
Azospirillum
Sinorhizobium
Rhodoblastus
Methylorhizobium
Methylocystis
Methylobacterium
Rhodobacter
Rhizobium
Mesorhizobium
Rhodopseudomonas
Bradyrhizobium
Methyloferula

TABLES

Table 4.1: Bacterial OTUs associated with severe *L. cuneata* invasion. Proteobacterial orders represented by OTUs associated with high 2016 *L. cuneata* biomass. Percentages represent the percentage of the associated community that is made up OTUs belonging to each individual order.

	2016 *L. cuneata* Biomass	
Proteobacterial Order	**High**	**Low**
Caulobacterales	4%	0%
Rhizobiales	8%	31.82%
Rhodospirillales	6%	4.55%
Sphingomonadales	6%	0%
Burkholderiales	18%	2.27%
Desulfuromonadales	6%	18.18%
Myxococcales	8%	2.27%
Syntrophobacterales	2%	2.27%
Enterobacteriales	4%	0%
Pseudomonadales	0%	0%
Xanthomonadales	18%	2.27%
Unknown Alphaproteobacteria	8%	0%
Unknown Betaproteobacteria	12%	36.36%

Table 4.2: Fungal OTUs associated with severe *L. cuneata* invasion. Basidiomycotal orders represented by OTUs associated with high 2016 *L. cuneata* biomass. Percentages represent the percentage of the associated community that is made up OTUs belonging to each individual order.

	2016 *L. cuneata* Biomass	
Basidiomycotal Order	**High**	**Low**
Agaricales	75%	63.64%
Hymenochaetales	6.25%	9.09%
Polyporales	0%	0%
Sebacinales	0%	9.09%
Thelephorales	0%	9.09%
Filobasidiales	0%	9.09%
Trechisporales	18.75%	0%

CHAPTER 5: CONCLUSIONS

SUMMARY OF FINDINGS

In this dissertation, I examined the influence of *L. cuneata* on soil microbial communities and plant-soil feedback patterns. I characterized plant-soil feedback between *L. cuneata* and native plants in multiple greenhouse experiments, and ground truthed my results with an observational study.

In **Chapter 2**, I conducted a greenhouse experiment that allowed *L. cuneata* and native plants to individually influence soil microbial community assembly and subsequent plant growth. I found negative plant-soil feedback between *L. cuneata* and *L. virginica* that may facilitate population growth in the early stages of invasion (Bever et al. 1997, Bever 2002). Microbial communities that assembled under *L. cuneata* had no effect on the growth of itself or its native congener *L. virginica*, but they decreased the growth of the native grass *Panicum virgatum*. Conversely, *L. cuneata* and *L. virginica* benefitted from the presence of microbial communities that assembled under either native plant species. This suggests that *L. cuneata* disrupts native-selected microbial communities that may be beneficial for its own growth and that of some of its native competitors. These results differ from prior work that suggests that *L. cuneata* invasion may benefit future *L. cuneata* growth (Coykendall and Houseman 2014, Crawford and Knight 2017). Because these studies measured the growth of plants in soil conditioned by whole communities, as opposed to individual plants, it is possible that the net effects of these mixed plant communities was sufficient to overcome the individual effects of *L. cuneata*'s own microbes documented here.

My results suggest that *L. cuneata* enriches for unique strains of *Bradyrhizobium* symbionts, but because *L. cuneata* grew the least in *L. cuneata* conditioned soil, these enriched symbionts may not be the most beneficial. *L. cuneata* also enriched for the plant pathogenic OTUs *Entoloma crassicystidiatum*, *Hyphodermella rosae* and *Sporobolomyces symmetricus*. Either through the disruption of beneficial microbial communities or the enhancement of plant pathogen populations, *L. cuneata* seems to promote microbial communities that are harmful for both conspecific and native plants. *L. cuneata* growth benefitted from native-conditioned microbial communities.

In **Chapter 3**, I conducted a greenhouse experiment to examine differences in *L. cuneata* root and litter mediated effects on plant-soil feedbacks. I found that root exudates, root leachates, litter leachates, and living plants influenced subsequent plant growth distinctly. This suggests that *in situ* plant-soil feedback may be mediated by complex sums of multiple chemical inputs that differentially effect microbial community composition and plant growth. Individual metabolites identified in *L. cuneata* solutions were mostly negatively related to the growth of *L. cuneata* and native plants.

Root exudates were generally more harmful for plant growth than either leachate, and therefore may enrich for deleterious microbes or be unable to enrich for sufficient beneficial microbes. *L. cuneata* root exudates contained a number of metabolites that are known chemoattractants of Rhizobium and Bradyrhizobium (mannitol, xylose, quinic acid, shikimic acid, adipic acid) (Bowra and Dilworth 1981, Parke et al. 1985), and were not found in either leachate.

Root exudate conditioned microbial communities were dominated by nitrogen-fixers within the genus Bradyrhizobium. Living plants, root exudates, and litter leachates enriched for the pathogenic fungal genus *Entoloma*, and root exudates also enriched for the pathogenic fungal genus *Hygrocybe*. Results from this chapter suggest that root exudates may play important roles in structuring communities of nitrogen-fixing symbionts and pathogens.

The living plant conditioning treatment in **Chapter 3** also functioned as an independent replication of the experiment conducted in **Chapter 2**. The results from these independent experiments mirrored one another, and thus suggest that plant-soil feedback experiments are repeatable under controlled environmental conditions.

In **Chapter 4**, I conducted an observational experiment to determine the applicability of results from greenhouse studies in the real world. I examined the *in situ* invasion of *L. cuneata* into a restored prairie containing native plants over a period of ten years, and I examined microbial community composition before and after a large increase in invasion severity. I found that *L. cuneata* invasion has lasting influences on soil microbial community composition, which may be sufficient to facilitate plant-soil feedback in the field. Consistent with results from my greenhouse experiment in **Chapter 2**, I found evidence that *L. cuneata* enriches for potential plant pathogens, different strains of *Bradyrhizobium* symbionts, and different communities of decomposers when compared to soils predominantly conditioned by other plants. *L. cuneata*'s high phenol content litter

may require specific white-rot fungi for the breakdown and release of nutrients into soil.

I found that the increase in *L. cuneata* invasion coincided with a decrease in average soil nitrogen across the prairie. As *L. cuneata* often invades nutrient depleted sites (Houseman et al. 2014), this decrease in soil nitrogen availability may have facilitated expansion.

FUTURE DIRECTIONS

Through this dissertation, I have identified taxonomic groups of potential symbionts, pathogens, and decomposers that may be important in *L. cuneata* invaded ecosystems. Future studies should directly examine the influence of these types of microbes on *L. cuneata* and native plant growth. No arbuscular mycorrhizal fungi were identified as being enriched within this work. The examination of only bulk soil may have biased my results against identifying arbuscular mycorrhizal symbionts, which require a plant host to survive. Future studies should examine shifts in microbial communities associated with plant roots during plant-foil feedback experiments and throughout the progression of *L. cuneata* invasion *in situ*.

This dissertation work has also lead to the identification of secondary metabolites present in *L. cuneata* root exudates that may be important for structuring communities of soil mutualists. Future work should examine the influence of mannitol, xylose, quinic acid, shikimic acid, and adipic acid on the development of nitrogen-fixing bacterial communities. Examining differential exudation of these metabolites at different stages of *L. cuneata* invasion and

under changing environmental conditions may increase our understanding of how root exudates may mediate *L. cuneata* growth and invasion. Chemotaxis of nitrogen-fixing bacteria toward *L. cuneata* root exudates should be examined in detail. I have also identified metabolites that may directly inhibit native plant growth. The influence of these individual metabolites on plant growth should be examined.

This work has shown that native plants may mediate different effects on soil microbial communities than invasive *L. cuneata*, however, this work did not compare the chemical composition of root exudates between plant species. Preliminary work outside of this dissertation suggests that *L. cuneata* root exudates are chemically distinct from the root exudates of native competitors (Ringelberg et al. 2017). These differences should be documented and examined in future studies, and can lead to a better understanding of how native plants structure soil communities.

CONCLUSIONS

The results from this dissertation provide a novel examination of plant-soil feedback in the *L. cuneata* system. Overall, this work suggests that *L. cuneata* may enrich for largely deleterious microbial communities that limit the growth of conspecifics and native plants. The benefit from native-selected microbes may support *L. cuneata* dominance and expansion. *In situ* plant-soil feedback patterns are likely mediated through complex sums of plant inputs.

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