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Plant Species and Soil Type Effects on Root Characteristics, Microbiota and Plant-soil Feedback Responses in Four Prairie Species

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Literature Review (Chapter 1)

Mycorrhizae refers to the groups of fungi that form symbiotic relationships with plants through external or internal connections with the root zone. Two distinct forms of mycorrhizae have been described, endomycorrhizae and ectomycorrhizae.

Ectomycorrhizae (EMF) are most commonly associated with tree and shrub species of the families Pinaceae, Fagaceae, Salicaceae, Cupressaceae, Betulaceae, Dipterocarpaceae, and Myrtaceae. Due to the nature of the hyphal structure EMF are not known to be associated with aquatic plants. Species of plants containing ectomycorrhizae often occur in temperate forests, or tropical forests with limited soil nutrient availability, with low species richness. Worldwide species richness of ectomycorrhizae may be more than 5,000 species mostly from the Basidiomycetes and some from the Ascomycetes. Bruns et al. (2007); (in Bergemann et al. 2007) suggest that competition and dispersal play an important role in determining the structure of ectomycorrhizal communities. Smaller island habitats were correlated with lower EMF species richness.

Ectomycorrhizae do not penetrate living cells but instead surround them. They facilitate nutrient transfer, sometimes directly from leaf litter (Malloch et al. 1980). Up to 86% of host nitrogen may be provided by the EMF (Hobbie and Hobbie 2006). EMF contributed uptake of phosphorus may also facilitate increased plant growth rate (Durall et al. 1991) and increase total plant phosphorus uptake ability (Durall et al. 1998, Antonovics et al. 1996).

Ectomycorrhizae serve as the main nutrient uptake component in many host plants and many species are dependent on them for survival (Boddy et al. 2004). EMF mycelia from one host plant can infect neighboring plants and ultimately form common mycorrhizal networks (CMNs) connecting mother plants to seedlings allowing seedlings connected to the network to uptake more nitrogen than seedlings not connected to a mother plant. The extent of these networks in the field is largely unknown. CMNs may connect both conspecific and interspecific plants, and may include multiple ectomycorrhiza species (Kazuhide, 2006).

It is understood that carbon, nitrogen, and phosphorus exchange occurs within CMNs, but the bi-directional component is less understood. While a source-sink relationship may occur in a laboratory setting, bidirectional field components are more difficult to assess. Durall et al. (1997) demonstrated that net carbon gain does occur in the field. Through carbon isotope labeling it is suggested that carbon exchange is occurring directly through the hyphal network.

Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) are root zone symbionts that are monophyletic belonging to Glomeromycota. AMF have had little to no morphological change in the past 400 million years, and are often called ancient asexuals. AMF research has been slowed by the inability to grow the fungus without the plant hosts and the recognition that the symbiotic relationship is exceedingly complex. AMF form a relationship with a multitude of host plants that

is estimated to include 70-90% of terrestrial plant species over a wide range of environments (Parinske 2008). Species distribution and richness is contingent on a multitude of environmental factors. AMF may help plants to handle a variety of environmental stresses. They display unique mechanisms for nutrient uptake and exchange as well as contribute to a variety of plant processes.

Unlike EMF, AMF penetrate host cells forming subcellular structures known as arbuscules. The life histories for the majority of AMF species have not yet been accurately described, due to the inability to culture most AMF species in the lab. It has also been observed that spores cultured *in vitro* may differ from naturally occurring spores. Charvat et al. (1999) demonstrated that spores of the genus *Glomulus* produced *in vitro* were smaller with thicker spore walls than their naturally occurring counterparts.

A germinating spore extends its hyphae outwards in search of a host. Chemical communication signals released by the hyphae cause encountered plant root cells to temporarily suspend immune responses. The plant cells then begin preparing their intracellular environment (Denison and Kiers 2011). Although association of AMF does not seem to be host dependent, it has been shown that sporulation rate in the laboratory may be host specific (Grace et al. 1987, Hung and Sylvia 1988) Antonovics et al. (1996) have confirmed host-dependent sporulation rates in the field. It has been suggested that a host dependent mechanism may also positively influence spore and hyphal propagation rates.

Reproduction

Spore formation mechanisms are still little understood. Nuclei are transferred through the cytoplasm forming spores that contain 100-1,000 nuclei. Spore nuclei may come from both migration and meiosis (Pawlowska, 2005). The origins, genetic makeup, and reasons for high number of spore nuclei is poorly understood. The choice of which nuclei are expressed is also an area that may need further research. Individual cells, including spores, may contain hundreds of nuclei. Due to the genetic differences contained within spores and individuals the mechanisms of natural selection and evolution do not require sexual reproduction or normal population dynamics. For example, a single individual AMF contains the genetic diversity a population of other organisms may possess.

Environmental conditions may influence which nuclei are expressed by an AMF individual. Multiple nuclei produce wide phenotypic variety in a single AMF species. Reproduction is asexual and no sexual reproduction has been observed. However genetic material has been observed to be exchanged, fused, segregated, transferred and recombined between very closely related species thus simulating sexual reproduction in an asexual species (Parinske 2008, Denison and Kiers 2011). Genetic recombination within populations may be a common occurrence (Avio et al. 2004). Spores can germinate without host plants, but AMF are obligate biotrophs and usually need living photosynthetic organisms to complete the life cycle and produce new spores (Parinske, 2008).

Spore dispersal methods are not well understood, but it has been observed that spores can survive ingestion and be dispersed by grazing animals

including small mammals and arthropods. The impact of this dispersal is not yet known. Other organisms such as amoeba are known to consume AMF spores. The role of parasitism on spore population dynamics is also little understood (Fitter and Garbaye, 1994).

The fungal structure within the plant cell is surrounded by a plant structure known as the periarbuscular membrane (PAM), which is a structure continuous with the plant plasma membrane. This structure separates the AMF from the plant cell cytoplasm. The fungal plasma membrane is separated from the PAM by the periarbuscular space (PAS). The PAS comprises both plant and fungal materials. Structures that form the arbuscules are thought to be responsible for the nutrient exchange between host and symbiont (Parinske 2008). Arbuscules that are formed typically only last 4-5 days, and quickly collapse after nutrient exchange is complete. It is hypothesized that arbuscular collapse is a means for a plant to ensure sufficient phosphorus is being exchanged. Carbon exchange to AMF may also be stopped when phosphorus exchange is not ideal. AMF lack the ability to assimilate carbon without a plant host (Denison and Kiers 2011).

Hyphae extend outwards from the root zone forming the fungal hyphal network. The hyphae can be classified into three main groups: penetration, runner, and absorbing. The penetrating hyphae create the arbuscules, only penetrating the plant cell wall and not the membrane. Runner hyphae are thick-walled and the largest of the hyphae types. They extend away from roots in search of other roots to contact. Runner hyphae are the source of both penetration and absorbing hyphae. Absorbing hyphae form a fan-like network

and are most likely solely responsible for nutrient uptake from the surrounding soil (Allen 1996). These networks can be as dense as 100 m of hyphae per cubic centimeter of soil (Jastrow et al. 1995). Germinating spores do not always need direct access to roots and arbuscular formation in order to begin receiving carbon from a host plant. Germinating hyphae can connect to AMF-host networks to access carbon (Fortuna et al. 2011).

Host Search

AMF are constantly in search of new hosts and are able to infect a new host plant while simultaneously engaging in nutrient exchange with another host. Fungal hyphae may grow 100 times larger than a root hair allowing them to concurrently search for nutrients and new hosts (Denison and Kiers, 2011). The ability of AMF to form hyphal networks between two distinct species of host has also been demonstrated. The ability of AMF to link within different networks of genetically similar individuals may increase fungal fitness. The evolutionary history and importance of multi-nucleated cells to natural selection are areas that require greater exploration. Having multiple hosts at once may also increase fungal fitness and allow AMF to selectively choose which plants to exchange nutrients with based on amount of carbon exchanged. This may allow AMF to be selective in terms of host once multiple networks are established (Croll et al. 2009).

Phosphorus and water are the main nutrients supplied to the host plant by the hyphal network. The plant provides carbohydrates in exchange for nutrients.

A meta-analysis concluded that plants colonized by AMF have better growth and reproductive response when under water deficient conditions. It was also concluded that perennials responded more favorably than annuals (Jayne and Quigly 2014). The mechanisms for AMF alleviation of drought stress are beginning to be understood. The osmotic stress placed upon both leaves and roots is reduced by increased mycorrhizal production of metabolites that serve as osmolytes. Increased osmolyte production results in the lowering of leaf water potential. A lower water potential, even in drought conditions, allows a plant to maintain turgor pressure and internal cellular structure, especially in relation to photosynthetic organelles. Root osmotic potential is also lowered by similar mechanisms.

Oxidative stress caused by drought conditions is countered by AMF production of antioxidant compounds. AMF root colonization can increase the roots' own ability to uptake water as well as increase area of water uptake due to the extent of the hyphal network. Hyphae can grow where roots may not be able to grow, thus increasing potential water uptake. Also hyphal mats positively alter soil structure for better water retention (Peñuelas and Rapparini 2014).

AMF may also play a role in stimulating drought-related plant genes such as the aquaporins. Aquaporins have even been observed within the fungal cell structure, possibly explaining AMF's ability to contribute to plant aquaporin gene regulation (Amodeo 2009). The ability of AMF to mediate drought conditions has many agricultural and restoration possibilities that still need to be explored. AMF has also been suggested as a possible use as biofertilizer, reducing the need for

manufactured fertilizers. As noted before, the AMF ability to stabilize soil structure also has possible agricultural and restoration applications, especially as drought becomes a more common occurrence worldwide.

Phytoremediation

Other soil-related stresses such as heavy metals, soil compaction, and salinity may also reduced by the presence of AMF (Miransari 2010). Heavy metal toxicity in polluted areas can be detrimental to plant populations. It has been demonstrated that certain AMF-host relationships can improve tolerance to heavy metal contaminated areas (Shen et al. 2014, Biondi et al. 2014). Phytoremediation describes the use of plants to treat terrestrial, air, or aquatic problems to remove contaminants. The depth of knowledge of AMF as a facilitator in phytoremediation is still in its infancy, but may hold promise (Freitas et al. 2012). Areas such as mine tailings where heavy metals concentrate at the surface are in need of a solution to deal with metal toxicity. It has been shown that the addition of composted olive waste (COW) and AMF to *Tetraclinus articulata* allowed the plants to thrive in heavy-metal contaminated soil. This is an example of possible AMF use in phytoremediation (Borie et al. 2014). However it has also been noted that under certain conditions AMF colonization is greatly decreased by the presence of soil pollution (Deram et al. 2011). Barley (*Hordeum vulgare*) plants were tested under environmental sewage pollutant conditions. Plants with AMF showed greater resilience, ability to detoxify, and create new leaf and plant biomass than plants without AMF (Bartha et al. 2010). While

phytoremediation may be possible with AMF, the applications may be limited by a host of factors such as plant-AMF species association, pollutant type, and pollutant concentration.

Soil Structure

The effects of AMF on soil structure is a topic with relatively little current research. Mummey and Rillig (2006) showed that AMF contribute to soil structure at three hierarchical levels: plant community, individual root and soil mycelium. AMF influence the soil aggregation process at a physical and biochemical level. AMF interaction with other soil organisms, hyphal production and absorption of nutrients alters the structure, size, and nutrient composition of soil.

Jastrow and Miller (2000) also demonstrated that AMF contribute to the formation of macroaggregates in prairie soil systems. Hyphae and glomulin production entangle soil particles contributing to soil structure stability. Jastrow and Miller also found that the structure of the microorganism community may influence the extent to which AMF contributes to macroaggregate formation.

Salinity

The accumulation of salt in soils can have detrimental effects on agriculture and can often render soil unproductive. As much as 20% of worldwide irrigated cropland is decreased by stress from salinity in soils. AMF has been shown to improve photosynthetic processes as well as water regulation efficiency by regulation of aquaporins. Also AMF influence plant use of antioxidant

enzymes that counter oxygen free radicals existing in high saline conditions (Aroca et al. 2012).

Ion toxicity can inhibit plant growth and yield. Studies have shown that adding AMF to salt rich soil may allow for better plant productivity and growth (Al-Karaki 2000, Dixon et al. 1989). Al-Karaki et al. (2001) demonstrated that tomato plants under higher salinity conditions inoculated with *Glomus mosseae* were more productive than non-inoculated plants. Inoculated plants had higher shoot and root yields and leaf area. Phosphorus and other micro-nutrient content were also significantly higher in inoculated plants.

AMF Influences on Plant Growth

AMF can influence the growth of various host plants and their tissues. Most likely these effects are due to the enhanced uptake of nutrients facilitated by the AMF. Vascular tissue function, pollen production, fruit production, and flower production were shown to be positively influenced by the presence of AMF (Daft and Okusanya, 1973). AMF-inoculated legume species were also shown to grow larger and have larger internal stores of phosphorus than non-inoculated plants (Crush 1974). Green pepper plant seedlings inoculated with five separate species of AMF had increases in shoot and root dry weight and higher tissue concentrations of phosphorus and zinc. AMF-infected plants also flowered earlier than non-inoculated plants (Akpinar 2011). It was also shown that from year to year there was a difference in seedling growth based on species of AMF.

Aquatic Plant Colonization

Although AMF is most commonly associated with terrestrial plants, it has the ability to colonize aquatic plants with developed root systems. However, only a small number of aquatic plants have been described that host AMF. It cannot be assumed that aquatic AMF relationships are the same as terrestrial, and the role of AMF facilitation of phosphorus and micronutrient uptake is not yet completely understood, nor is diversity of AMF species that may be associated with aquatic plant species (Anderson et al. 1984). Riparian zone species have also shown colonization by AMF and riparian sediment redox potential may play a role in mycorrhizal development (Beck-Nielsen and Madsen, 2001).

AMF colonization can also vary across a soil-moisture gradient. Anderson et al. (1984) demonstrated that *Glomus caledonium* had a higher association with plants located in dryer soil with poor nutrient availability. *Gigaspora giganta* was found on plants located in wetter, nutrient rich soils.

Flooding is known to decrease the number of AMF spores in soil, but the correlation between spore density and colonization rate of wet soils is unknown. Wetland flooding has been shown to only partially reduce the colonization ability of AMF. Flood tolerance of specific species of AMF has yet to be categorized. AMF colonization also changes along a hydrological gradient. A higher water table is associated with lower colonization rates (Miller 2000).

Carbon Sequestration

AMF decreases the quantity of organic carbon that is respired from soil carbon sources back into the atmosphere in the form of CO_2. It has recently been suggested that root-associated (both ecto- and endomycorrhizal) fungal species may be the main component of large-scale carbon sequestration processes. A ^{14}C bomb-carbon model used chronsequences from a boreal forest, involving fungal biomarkers, that allowed quantification of carbon sequestration over a time frame between hundreds and thousands of years. This method determines the age since C fixation and then uses a mathematical model to calculate vertical organic matter profiles, resulting in an estimate of root derived carbon accumulation. Models based on the carbon content of multiple organic soil and humus layers were used to determine that 50-70% of sequestered carbon was stored in the roots and root-associated microorganisms in a Swedish boreal forest. Globally boreal forests account for 11% of land surface cover (Bahr et al. 2013). Results from this and similar studies suggest that the humus layer mainly comprises root and below ground derived materials suggesting that mycorrhizal fungus is an important regulator of CO_2 sequestration.

AMF may be able to help mitigate rising $C0_2$ levels caused by global environmental and climate change by promoting AMF hyphal network growth. Larger hyphal networks have the ability to sequester increased amounts of carbon. AMF hyphae grew three times as large with the predicted 2050 levels of 550ppm than the 2009 ambient level of 360ppm (Alguacil et al 2009). AMF has the ability to help convert increased amounts of atmospheric CO_2 into biomass.

Active AMF management programs may be required to utilize the potential of mycorrhiza as a means for atmospheric carbon sequestration. It is unknown if mass inoculation or natural promotion of AMF-rich systems will produce a viable soil carbon sink for elevated atmospheric CO_2.

AMF and Bacterial interactions

The role and influence of bacterial populations in the life cycle, colonization process, and rhizosphere ecosystem is another interesting area of AMF research. Various studies from the 1960s, 1970s, and early 1980s reported findings that AMF and bacterial populations were interacting in ways that stimulated mutual development and/or plant growth. Most of the early studies were done in the lab with selected microbial populations that did not necessarily reflect natural conditions. A 1986 study revealed that AMF formation was influencing bacterial equilibrium in the rhizosphere but not the rhizoplane (Linderman and Meyer 1986).

The idea of Mycorrhizal Helper Bacteria (MHB) appeared in the 1990s. The exact way MHB are able to influence and affect the rhizosphere ecosystem is not understood. However what is known is that MHB populations consistently encourage the propagation and growth of mycorrhizal species (Garbaye 1994). Bothe et al. (2006) demonstrated that MHB are capable of completely stimulating the AMF species *Glomus intraradices* through its entire life cycle, including sporulation, without the need for a plant host. The old ideas that carbon can only be gained by AMF through a host plant and that arbuscles are the only means of

nutrient exchange are now being challenged *G. intaradices* was able to receive nutrition produced by the bacterium *Paenibacillus validus.* The researchers believe it will be possible to identify factors that can stimulate AMF growth and nutrition without the need for any plant partner.

It has also been observed that the spores and mycelium of the AMF *Gigaspora margarita* harbor a bacterial endosymbiont. The bacterial endosymbiont was observed in all life cycle stages of *G. margarita* and could also help facilitate nutrient exchange with host plants. Although the functional extent is not known, this research suggests a third layer of symbiosis may be another complex component to AMF survival strategies (Bandi et al. 1996).

Rhizosphere and rhizoplane interactions between AMF and other microbes can exist in a variety of forms. Relationships can be inhibitory, mutualistic, stimulating or competitive. AMF interactions with a host plant also change the host-microbial relationships in the soil. The microbiome structure and systematic influences between AMF and bacteria are complex and require more extensive research before interactions can be understood and predicted (Fitter and Garbaye 1994).

AMF are an important component of the plant-soil feedback system. Plant-soil feedback involves plant influenced soil changes that alter the physical and chemical properties of the soil. These changes can positively or negatively affect the plant itself and/or other plants around it. The patterns and mechanisms of plant-soil feedback are complex and more studies are needed (Bentivenga et al. 2008). Bezemer et al. (2006) showed that plant-soil feedback can have

influence on community structure and can also change over time. During the first growth periods early and mid-successional species did well independent of whether the soil type was early, mid, or late successional. However, during the second growth period conditions changed. Early-successional species showed negative feedback, mid-successional neutral feedback, and late-successional positive feedback, in terms of shoot biomass. Biotic factors influenced feedback more than abiotic factors, as fungal and bacterial biomass also increased over periods of succession.

AMF presence in plants has been shown to increase resistance of plants to pathogenic soil fungi. The exact levels and mechanisms of bioprotection provided by AMF are still unclear (Vierheilig 2004). The role of AMF in reducing possible shoot pathogens is another interesting area of research still in its infancy.

AMF in Prairies

Grasslands are estimated to cover up to one-fifth of the earth's land surface and AMF are believed to form partnerships with almost every perennial plant species in native grasslands (Miller et al. 2012). As in other ecosystems AMF influence soil structure in tallgrass prairies. Disturbances such as conversion to cropland, invasion by non-native plant species, and human development may change the AMF community structure of grasslands (Jastrow and Miller 2000).

AMF diversity in prairies is thought to be quite high. The co-existence of multiple species may possibly be explained by spatial niche partitioning. AMF species may only be competing for space regardless of the specific plant host (Fitzsimons et al. 2008). Diversity of AMF species in a prairie may not heavily influence tallgrass prairie community structure. The specific community structure of AMF found within a prairie ecosystem may be responsible for influencing plant community structure (Bever et al. 2006). Plant host may play an important role in sporulation and fungal growth rates. Twenty-three species of AMF were identified in a 75-m^2 plot and this number may be an underestimate of true species richness. Many factors such as season or optimal temperature may affect fungal growth in a given system (Antonovics et al. 1996).

The effects of AMF on soil within tallgrass prairie in Illinois are not yet known. AMF is an important and integral part of tallgrass prairie systems and should considered when formulating management plans for prairie restoration. Although AMF help increase species diversity of a prairie system, diversity may not be the best tool to assess ecosystem structure and function, especially in relation to invasive exotic species. It has been suggested that dominant species and not richness in tallgrass systems are the best indicator of a system's ability to resist invasion by non-native plant species (Kelly et al. 2004). A complex system of interactions is at work in tallgrass prairie systems with AMF playing a crucial role on many levels.

To better understand how to restore and produce high quality prairie patches, the role of AMF must be understood on a deeper level. Restoration of

prairie should include consideration of microbial communities, including AMF and soil pathogens to promote diversity and stability (Fitzsimons & Miller 2010). It has been shown that after tilling in an agricultural site has stopped and tallgrass prairie restoration begins, fungi biomass increases over time (Allison et al. 2005). These beneficial relationships in the rhizosphere can be an indicator of optimal soil and plant community structure. Soil management in any restoration project must pay attention to AMF (Barea et al. 2003). The effects of inoculation with AMF before restoration are not clearly understood. Studies have shown both beneficial and negligible results of inoculating before restoration begins. However these studies were not able to measure the long term effects of inoculation on tallgrass prairie restoration sites. AMF inoculation is expected to beneficial in restoration sites that are low in soil phosphorus or that have sparse remnant AMF communities (Charvat et al. 2008). AMF suppression may be more important in prairie restoration that is done in a site with many dominant C_4 grasses. AMF suppression was shown to reduce the dominance of C_4 grasses and allow forb species to establish, thus increasing plant species diversity (Blair et al. 2011). AMF influence on tallgrass prairie systems is complex and needs to be better understood. Responses of individual species to AMF colonization are an important component of understanding fungal ecology in tallgrass prairies.

Hickman et al. (2012) demonstrated that *A. gerardii* AMF colonization was diminished in soils where competition from invasive species occurred. Invasion suppressed and altered microbial communities reducing biomass of native

grasses. It may be necessary to restore pre-invasive soil conditions when attempting to restore native tall-grass prairie.

Prairie ecosystems may show plant response and adaptations to AMF populations within the community. The origin of the plant, soil, and AMF may influence community dynamics in prairie systems. Native plants grown in native soil, with native AMF had the highest above ground biomass and more inflorescences than plants under different treatment conditions where one part of the treatment (e.g. soil, AMF, plant) was non-native to the system (Munzbergova et al. 2011).

It must also be noted that there is currently debate on the effect prescribed burns might have on AMF densities and fungal inoculum potential. It was noted that AMF might help invasive exotics, which tend to be highly mycorrhizal, establish in disturbed areas (Haskins and Gehring 2004).

AMF Systematics and Species

The system of classification suggested by Benny and Morton (1990) split AMF into genera and families that were used to name AMF species until new molecular techniques showed these groupings to need major revision. Original classifications were based on morphology. Hyphal size, shape, color, structures, and reaction to staining were used for species identification.

The number of AMF species is at present unknown and no universal classification system yet exists. A complete overhaul of AMF taxonomical structure has been suggested as molecular data and comparisons become more

available. Taxonomic rankings and species classification are at present challenging and open for debate (Young 2012).

The traditional methods of identification have proven to be inaccurate and difficult during field studies that attempt to describe large scale ecological interactions between multiple AMF species and the communities in which they interact. Field identification is prone to errors and should be coupled with genetic markers to ensure correct identification. Morphological characters of most species of AMF have not yet been adequately characterized. Spores do not have enough reliable difference to accurately classify species using this method. Spores can also form multiple morphological types making identification impossible. Identification through sequencing of small subunit rRNA (SSU), individual internal transcribed tracer regions (ITS), mitochondrial region DNA, and nuclear rDNA have been attempted but as of yet do not allow for clear phylogenetic identification to species level. Kruger et al (2012) propose that the combined use of long sequence DNA from the collection of all ITS regions and the 5' portion of the nuclear large subunit rRNA gene (LSU) can provide a template to identify AMF species by means of genetic markers and they suggest new names for species based on more recent molecular comparisons. However it will take time before the phylogenetic tree of AMF can be constructed and universal naming of species can be accomplished.

Root Morphologies

In 1975 Baylis observed that plants with a genotype for coarse roots are more dependent on AMF than plants that have genotypes for fine roots. This relationship is most likely the product of coevolution and AMF's influence on a plant's ability to uptake nutrients (Baylis 1975).

Warm season grasses that dominate tallgrass prairies may have more root plasticity than cool season grasses. Higher colonization levels of AMF were negatively correlated with root branching of C_4 grasses. Warm season grasses were able to change root architecture depending on AMF colonization. However C_3 grasses appear to have non-plastic root architecture comprising a more fibrous root system, thus relying less on AMF for supplementing nutrient uptake (Hetrick and Wilson 1991).

Root morphology changes due to AMF have also been observed in other species. Atkinson et al. (1992) demonstrated that root colonization by AMF had significant effects on root morphology of poplar trees.

AMF colonization is also related to specific root length (SRL : ratio of root length to root dry weight). Higher SRLs are correlated with decreased reliance on AMF. For example, C_4 grasses have a smaller SRL than cool season C_3 grasses (Hetrick and Wilson 1991). Atkinson et al. (1995) showed that the SRL of *Prunus cerasifera* L. decreased with inoculation of AMF.

Andropogon gerardii

A. gerardii is a dominant C_4 perennial prairie grass that is widely planted in restoration. *A. gerardii* and other dominant prairie species drive prairie

ecosystem structure and function. They also contribute heavily to nutrient, especially nitrogen, cycling and are a major source of primary photosynthetic production. (Auen et al. 1993).

Baer et al. (2011) found that artificial cultivars of *A. gerardii* had higher net photosynthesis, stomatal conductance, and water use efficiency when compared to wild type. This suggests that prairie restoration using cultivars instead of wild type may result in unforeseen ecosystem consequences (Baer et al. 2011). Seed provenance of *A. gerardii* used during restoration may also influence the structure of the restored community and the dynamics of ecosystem functions. Local plants were more competitive against non-local and *vice versa* (Gibson et al. 2004). These studies illustrate that the dynamics of local ecosystem restoration are more complicated than species selection.

Bowker et al (2010) showed that geographic specific evolution may be at work among *A. gerardii* and the AMF community present in its native soil. Fitness of *A. gerardii* was greater among plants grown with AMF that likely co-evolved in local soils. Therefore restoration is more complex than introducing plants into a system. The soil and the microbiome must also be considered.

An earlier study by Bever et al. (2001) showed the *A. gerardii* ecotype from Illinois had developed a more highly branched root system in comparison to an ecotype from Kansas, which had a coarser root system. These plants grew better in their native soils, also indicating an adaption for local ecosystems. More root branching, in general, is an adaption that allows for less dependence on

AMF, which the Illinois ecotype showed by measure of decreased carbon allocation to the AMF.

A 1993 field study by Jastrow and Miller found that AMF colonization *of A. gerardii* was higher when plants had forb instead of conspecific neighbors. Root densities and inter-species interactions were possible reasons for the differences found. However, with new research it is possible that common mycorrhizal networks could also produce these results.

CMNs (common mycorrhizal networks) may play a role in monospecific assemblages of *A. gerardii* by influencing asymmetric competition belowground. Janos and Weremijewicz (2013) suggest that CMN contribute to size inequality of seedlings by means of positive feedback based on percent AMF colonization. AMF inoculated plants within CMN networks were significantly larger than inoculated plants not connected with a CMN. However, neighbors of larger plants within a CMF network were often a smaller size than individuals not found in the network. This indicates the CMN may allocate carbon to specific individuals based on need and play a role in plant competition within species.

Prairie forbs

Parthinium integrifolium (PI, wild quinine) is frequently found in prairie ranging from dry to mesic. PI prefers loamy or sandy soil and can also be associated with upland savannas and woodlands. PI can be found in most tallgrass prairies in the US, but is less frequent in the northeast (Ladd 1995).

Aster laevis (Smooth Blue Aster) is encountered occasionally in mesic to dry tallgrass prairies and also in upland savannas (Ladd 1995). *Aster novae-angliae* (New England Aster) is frequently found in all regions of wet to mesic tallgrass prairie (Ladd 1995).

Very few studies have been done focusing on these three forb species and none that could be found focusing on association with AMF. However, root architecture differences within Asteraceae make them interesting subjects to explore AMF associations within prairie systems.

The future of AMF research will need to rely heavily on new genetic techniques to identify species at both the AMF and microbial levels. The various interactions occurring at the rhizosphere and rhizoplane need to be better understood. The ability to construct a working model of AMF community dynamics is the next step in understanding how AMF is structured within various ecosystems. The AMF-soil-plant feedback loop is intricate. Understanding these loops better may allow for breakthroughs in areas such as food and crop production, ecosystem restoration, and bioremediation. As mentioned, it has been demonstrated that AMF can trigger plant gene expression in order to conserve water. This leads to questions on which other plant genes mycorrhizae might be able to regulate.

AMF is notoriously difficult to cultivate in a lab setting and a breakthrough in technique, possible through the use of MHBs, would further aid in the advancement of AMF research. As of yet no large scale experiments have attempted to determine if AMF is truly selective in host choice, due to both

identification and cultivation problems. The root plasticity demonstrated by *A. gerardii* in relation to the presence of AMF would be interesting to investigate for various other species.

It appears obvious that incorporating use of AMF in prairie, and other ecosystems, restoration is a component that should not be ignored. However, there is not yet a clear cut plan of attack to properly incorporate AMF into restoration processes.

Synthesis of Research (Chapter 2)

Abstract

Arbuscular mycorrhizal fungi (AMF) form a relationship with a multitude of host plants that is estimated to include 70-90% of terrestrial plant species over a wide range of environments. AMF and other microbiota, as well as abiotic factors are an important component of plant-soil feedback systems. The effects of plant species and soil type on root characteristics, plant-soil feedback and influence on microbiota and arbuscular mycorrhizal colonization were assessed through a manipulative laboratory study. *Aster laevis*, *Parthenium integrifolium*, *Aster novae-angliae* and *Andropogon gerardii* were grown in sterilized control soil and soil collected from beneath monocultures of *A. laevis*, *P. integrifolium*, and *A. gerardii*. Soil type (heterospecific and conspecific sources) was expected to have the largest influence on root characteristics and AMF colonization. Microbial community carbon metabolism became more homogenous after the study was completed. It was found that both plant and soil had similar influence. Negative feedback was expected for plants grown in *A. gerardii* soil, but the opposite was observed. The relationships between AMF colonization and fine root branching, and between AMF and specific root length were hypothesized to be negative. Specific root length was found to be negatively correlated to proportion mycorrhizal colonization, but fine root size and branching characteristics were not correlated with AMF colonization. Total biomass was positively correlated with proportion mycorrhizal colonization. It was found that mycorrhizal dependence of the four prairie species studied was high and plant death only occurred in

sterilized control soils. No net feedback was observed for *A. laevis*, *P. Integrifolium*, and *A. gerardii*.

Key Words: plant-soil feedback • arbuscular mycorrhizal fungi • *Andropogon gerardii* • *Aster laevis* • *Aster novae-angliae* • *Parthenium integrifolium* • microbiota

Introduction

Grasslands are estimated to cover up to one-fifth of the earth's land surface and arbuscular mycorrhizal fungi are believed to form partnerships with almost every perennial plant species in native grasslands (Johnson et al. 2012). Over 99% of tallgrass prairie in Illinois has been lost to development and agriculture (Anderson 1991). In order to restore and produce high quality prairie, the role of soil biota must be better understand. Restoration of prairie should include consideration of microbial communities, including arbuscular mycorrhizal fungi (AMF) and soil pathogens to promote diversity and stability (Barea et al. 2003, Fitzsimons and Miller 2010). After tilling in an agricultural site has stopped and tallgrass prairie restoration begins, fungal and bacterial biomass increases over time. Beneficial relationships in the rhizosphere resulting from increased microbial biomass may play a role in soil nutrient cycling and stable community structure (Allison et al. 2005).

Soil biota, including bacteria, fungi, and other microorganisms play key roles in plant community structure and function as they are instrumental to processes such as nitrogen fixation, litter decomposition, nutrient cycling, and carbon sequestration. Functioning of the soil biota in tallgrass prairie is not well

understood. Identifying plant and microbial associations beyond that of AMF amongst prairie species is also important (Jordan and Larson 2006).

Plant-soil feedback involves plant influenced soil changes that alter the physical and chemical properties of the soil (Bentivenga et al. 2008). The alteration of soil community and structure by a particular plant species can alter the performance, survival, and characteristics of plants of its own or different species (Callaway et al. 2008). Plant-soil feedbacks can be positive, enhancing performance, or negative, reducing performance (Kulmatiski et al. 2008). Community diversity is increased through negative feedback while positive feedback may produce more homogeneity within communities (Bever et al. 2012). Although mycorrhizal fungi serve as a carbon sink to a host plant, phosphorus and nitrogen feedback are also involved in allocation of resources by the host plant to the symbiont. The host plant may withhold carbon if phosphorus and nitrogen levels are too low. AMF may also slow phosphorus and nitrogen exchange if carbon given is not sufficient. Degrees of host or symbiont control over this feedback loop are not yet known (Jastrow et al. 2002).

Both negative and positive plant-soil feedback have been observed in studies of AMF. Bever 2002 observed negative feedback of AMF related to *Plantago* and *Panicum sphaerocarpon*. *Plantago* responded better to AMF contained within soil that had hosted *Panicum sphaerocarpon* than to AMF in its own soil. Positive feedback has been observed in AMF-colonized compared to non-colonized species of *Acacia auriculiformis* (Giri et al. 2003)

The relationships and possible feedback loops of soil biota associated with forb and grass species of Illinois tallgrass prairie systems have not yet been completely investigated. Negative plant-soil feedback is at work during seedling establishment of tropical tree seedlings, allowing co-existence of species. Temperate tree species had less prevalent negative plant-soil feedback than tropical species (Kobe and McCarthy-Neumann 2010a, Kobe and McCarthy-Neumann and Kobe 2010b). Bezemer et al. 2006 observed that conspecific and heterospecific plant growth was affected by soil type grown in and plant species alteration of the rhizosphere may play a role in plant-soil feedback. In general, plant species grown did better in soil conditioned by forbs than soil conditioned by grasses. Sandy soils used in the study had nutrient levels altered by study plants, but microbiota was altered in chalky soils. Due to accumulation of soil pathogens, conspecific plants may have lower fitness and reduced ability to compete when trying to establish in conspecific soils (Fergus et al. 2008).

AMF colonization is also related to specific root length (SRL: ratio of root length to root dry weight). Higher SRLs are correlated with decreased reliance on AMF. For example, C_4 grasses tend to have lower SRL than cool season C_3 grasses (Hetreck and Wilson 1991). Atkinson et al. (1995) showed that the SRL of *Prunus cerasifera* decreased with inoculation of AMF. Roots with high SRL are believed to be less energetically expensive to produce (Withington et al. 2006), allowing plants to increase the unit of soil explored per unit of biomass, and making reliance on AMF for additional nutrient uptake less necessary.

Warm season grasses that dominate tallgrass prairies may have more root plasticity than cool season grasses. Higher colonization levels of AMF were negatively correlated with root branching of C_4 grasses, and warm season grasses were able to change root architecture depending on AMF colonization. C_3 grasses, however, appear to have non-plastic root architecture comprising a more fibrous root system, thus relying less on AMF for supplementing nutrient uptake (Hetrick and Wilson 1991).

The purpose of this study was to compare physical characteristics, microbial community structure and mycorrhizal symbiosis of three prairie forbs, *Aster laevis* (AL), *Parthenium integrifolium* (PI), *Aster novae-angliae* (AN) and one dominant grass ,*Andropogon gerardii* (AG), in order to investigate possible differences in plant-soil feedback systems. Forbs were chosen because their reliance on AMF is relatively unknown. The three forb species were selected because they belong to the same family (Asteraceae) yet exhibit different root morphologies. Superficially AL, AN, and AG exhibit no taproot system. A taproot is present in PI. From previous studies AG is known to be highly mycorrhizal. The study species occupy similar geographic ranges and occur together in Illinois tallgrass prairie systems. *A. gerardii* was chosen as it is a dominant C_4 tallgrass. As a dominant prairie plant *A. gerardii* serves as a "matrix" species that is able to outcompete smaller forbs. Matrix species are widely distributed, stable, and the most abundant. Within the grass matrix species such as forbs can be found in local patches, are less abundant, and less predictable than matrix species (Fay and Hartnett 1998). Soil microbiota associated with *A. gerardii* may play a role in

this species' dominance in the tallgrass prairie ecosystem through effects on root structure, AMF colonization and biomass of AL, AN, and PI. In this study, it was hypothesized that soil type would have a larger influence on root structure, AMF colonization and biomass than plant species growing in the soil.

Seedlings of study species were grown in field soil collected from under conspecific plants or from under plants of other study species (heterospecific), or in sterilized soil that served as a control treatment. I investigated if soil treatments or plant species growing in the soil influenced certain physiological and microbial characteristics to a larger degree. It is possible that soil factors resulting from the species of inhabiting plant influence microbiomes and AMF colonization of differing species. The extent to which this may be true was investigated.

Response variables measured included total plant biomass, proportion AMF colonization of roots, and several variables describing root structure. Correlations among variables were quantified to test the hypothesis that an inverse relationship exists between SRL and proportion AMF colonization of roots. Soil microbial community structure changes were also investigated to explore possible plant species treatment effects. It was expected that microbial community structure would change between pre and post study. I hypothesized that SRL is negatively correlated with proportion mycorrhizal colonization of roots. I also expected proportion AMF colonization of roots to differ between species and between soil treatments and to see interactions between plant species and soil type. The expectation was to see the largest negative change

(negative feedback) in total biomass and root characteristics of forb species altered when grown in the soil that had been collected from AG (except for control soils).

Materials and Methods

Experimental Design

The experiment was a 4 x 4 factorial design with soil and plant species as the two treatment factors, and four levels of each factor. The four soil treatment levels were AG, PI, AL (i.e., soil collected from beneath each of the three plant species) and sterilized soil. AG, PI, AL and AN were the four plant species grown from seeds obtained from Prairie Moon Nursery, Witoka, MN.

Soil Collection

Soil was collected from a tallgrass prairie remnant in University Park, Illinois (41.450^0 N, 87.710^0 W). *A. gerardii* and to a lesser extent *Sorghastrum nutans* dominated the site. The remnant can be described as a black soil prairie with a mixture of clay, silt, and carbonate material. Soil type at the site is Beecher silt loam (USDA 2001).

Soil samples were collected directly from the rhizosphere of study species clumped in monocultures with other study species at least 1 m away. Soil cores were taken to a depth of 10 cm with a total volume of 331.83 cm^3**.** Sixteen additional soil samples were collected from random locations in the site. Samples

were labeled and then placed in soil collection bags on ice for transport back to the lab. Soils were refrigerated for two days at 3^0 C before seedlings were transplanted.

Plant Preparation and Harvest

Seeds from all four species were cold stratified at 3^0 C in moist vermiculite for 60 days and seeds were germinated in autoclaved sand. Roughly double the number of seedlings needed for the experiment was grown in sand under fluorescent grow lights to ensure that at least 16 plants of each species would survive for transplantation. After three weeks of germination the most physically similar seedlings of each species were transplanted into individual 656 ml Deepots (Stuewe and Sons, Tangent, OR) filled with a 6:1 mixture of collected field soil (from one sample) and autoclaved sand. Control soils were made by autoclaving randomly collected soils for one hour at 151^0 C. Samples (0.5 g) were also taken and counts made on petri films to ensure microbes were killed

The four species of plants were arranged randomly with rotation of plants done weekly. Tops of plants were kept at a minimum of 5 cm below the fluorescent tubes to ensure maximum light exposure without burning plants with heat. Lights were placed on a 14:10 hr light/dark cycle to simulate approximate spring photoperiod. After 60 days the light/dark cycle was changed to 16:8 hr to simulate summer photoperiod. Plants were watered to saturation weekly. Plants were harvested after 100 days. Aboveground portions of all plants were dried at 60^0C for 24 hr and weighed.

Root Physiology and Mycorrhizal Data

Roots were washed free of soil and separated into < 1 mm diameter (fibrous roots), > 1 mm diameter and rhizomes. Roots >1mm and rhizomes were dried at 60^{0}C for 24 hr and weighed.

Fibrous roots from each sample were optically scanned using an Epson Perfection 4990 Photo scanner. After scanning, WinRhizo software (Regent Instruments Inc., Quebec, Canada) was used to calculate total fibrous root length, mean root diameter and number of forks/cm.

After analysis by WinRhizo software, each fibrous root sample was weighed to determine fresh weight. Then two subsamples were collected from each sample to determine fibrous root dry weight and proportion AMF colonization of roots. The first set of subsamples was dried at 60^{0}C for 24 hr, and dry weight of each fibrous root sample was estimated from the ratio of the fresh to dry weights of the subsamples.

The second set of subsamples was used to determine proportion mycorrhizal colonization. Fibrous roots were first cleared in 10% w/v KOH, rinsed in deionized water, acidified with HCl and stained with 0.06% trypan blue in lactoglycerol (Bougher et al. 1996). Samples of stained roots were then mounted on microscope slides (Koske and Tessier, 1983).

Mean proportion of root length colonized per sample was determined by examining three slides per fibrous root sample (McGonigle et al. 1990). For each microscope slide, 12 complete passes across the slide perpendicular to its long axis were made to ensure a minimum of 36 root intersections per slide.

Proportion mycorrhizal colonization (MC) was calculated as total hyphal hits (hyphae, vesicle, or arbuscular) divided by total root segments intersected.

Mycorrhizal Dependence (MD)

The following equation was used to calculate mycorrhizal dependence (MD) (Mahadaven and Raman 1996).

$$\frac{\text{Total biomass of mycorrhizal plant} - \text{mean total biomass of control plants}}{\text{mean total biomass of control plants}} \times 100$$

Net Feedback (I)

Pairwise net plant-soil feedback coefficients (net feedback = I) were calculated to predict each plant species' growth in competition with one other species, through plant-soil-plant interactions. Net feedback (I) was calculated by summing total biomasses of each of two plant species grown in their own (conspecific) soil (A and B) and subtracting total biomasses of the two plant species, each grown in other species' (heterospecific) soil (B and A), using the following equation (Bever et al. 1997, Kulmatiski and Kardol 2008, Fitzsimons and Miller 2010):

$$I = A + B - B - A$$

Net feedback coefficients were calculated for AL vs. PI, AL vs. AG and PI vs. AG pairwise species combinations, with four replicate calculations per pairwise

combination. AN was not included in net feedback calculations because there was not an AN soil type in the study.

Microbial Analysis of Soils

Soil functional diversity was analyzed from 0.5g subsamples collected from soil samples soon after collection from the field and 0.5 g samples collected after the experiment. After collection from the field, soil samples were refrigerated for 48 hrs at 3^0C before being processed. A subsample (0.5 g) was randomly collected from each soil sample, and placed into a bottle containing 49.5 ml of distilled water. A sequential series of three 10-fold dilutions was made.

Biolog EcoPlates (Biolog Inc., Hayward CA, USA) were inoculated with 10^{-2} dilutions as suggested by Biolog. Each EcoPlate contains 31 carbon sources for soil community analysis. The 31 carbon sources as well as a water control are repeated three times within one EcoPlate. The metabolic use of these carbon sources was analyzed for each of the 48 (control soils were not run) samples and a metabolic pattern based on positive, negative, or borderline carbon metabolism for the microbial community was used for data analysis. EcoPlates were incubated in the dark for 5 days at room temperature. At 5 days each plate was analyzed by the Biolog reader.

To prepare EcoPlate results for statistical analysis, a composite of each triplicate run for each sample was compiled. Ecoplate reader output assigned a positive, negative, or borderline value for each carbon source based on strength of color change due to metabolism of carbon sources. Within each triplicate run,

a “positive” for carbon source use was assigned a value of 1, a “negative” a value of 0, and a “borderline” result was assigned a value of 0.5. To create a composite of the three repeated tests, two out of three “positives” were defined as a composite “positive.” Two “negatives” out of a possible three were defined as a composite “negative,” and two “borderlines” out of three were defined as a composite “borderline.” In the case of an EcoPlate result of “positive,” “negative” and “borderline” for a carbon source, a “positive” composite value was assigned.

Serial dilutions were plated in triplicate on aerobic petri films and yeast/mold petri films in triplicate for each treatment. For the pre-experiment counts 10^{-5} dilutions were used for the aerobic count plates for the *P. integrifolium* and *A. laevis soils.* A 10^{-6} dilution was used for the *A. gerardii* aerobic count films. For all three pre-study soil types 10^{-2} dilutions were used for the yeast/mold films. After the plants were harvested the above procedure was repeated with soil that had grown the test plants (excluding control soils). For all post-experiment soils 10^{-6} dilutions were used for aerobic count plates and 10^{-2} dilutions for yeast/mold films. Pre-experiment and post-experiment aerobic bacterial and yeast/mold films were read after 2 days of incubation at room temperature.

Statistical Analysis

With the 4 x 4 factorial design (4 plant species x 4 soil treatments), five response variables were analyzed: mean root diameter (RD), number of forks/cm fibrous (<1 mm diameter) roots, specific root length (SRL; SRL = fibrous root length/fibrous root mass), proportion mycorrhizal colonization (MC), and total biomass (TB). All response variables met normality assumptions.

Pairwise Pearson correlations were calculated among the response variables, with significance level adjusted using a sequential Bonferroni correction (Dunn, 1961). To test the hypotheses that proportion mycorrhizal colonization (MC) is higher with decreasing SRL, and total biomass is higher with larger proportion MC, two linear regressions were conducted: MC vs. SRL (excluding roots from control soils) and MC vs. total biomass. A MANOVA was conducted to determine overall effects of plant species, soil treatment and plant species x soil treatment on all response variables. If the MANOVAS were significant ANOVAs, with Tukey tests for significant detected differences, were conducted on individual response variables to interpret statistically significant main treatment and interaction effects.

A separate 4 x 4 factorial ANOVA was conducted to determine effects of plant species, soil treatment and plant species x soil treatment on mean mycorrhizal dependence (MD). A single factor ANOVA was conducted to detect differences in mean net feedback among the three pairwise species combinations (AL vs. PI, AL vs. AG and PI vs. AG); and a one-sample, one-tailed t-test was conducted to determine if overall net feedback (across all three species combinations) was > 0.

Biolog data was used to conduct two principal component analyses (PCAs) using PC-ORD version 6.08. The first PCA was conducted on data from soil before the experiment, and the second PCA was conducted on combined data from soil before and after the experiment.

Separate one-way ANOVAs were conducted on pre-experiment aerobic bacterial and fungal counts to detect differences due to soil type. Post-experiment soil counts were used to conduct a 4 x 4 factorial ANOVAs to detect differences due to plant species, soil type and plant species x soil type.

Results

Pre-experiment Soil Microbial Analysis

Based on PCA results of 48 soil samples axis 1 explained 20.017% of the variance among samples ($p=0.001$). Axis 2 explained 11.935% of the variance ($p=0.001$). Axis 3 also was statistically significant ($p<0.05$) but was not interpreted (Figure 1). Axis 1 most likely represents microbial functional diversity, as number of carbon sources utilized increases to the right along the axis. Axis 2 and partial axis 1 appear to be linked to specific carbon sources metabolized.

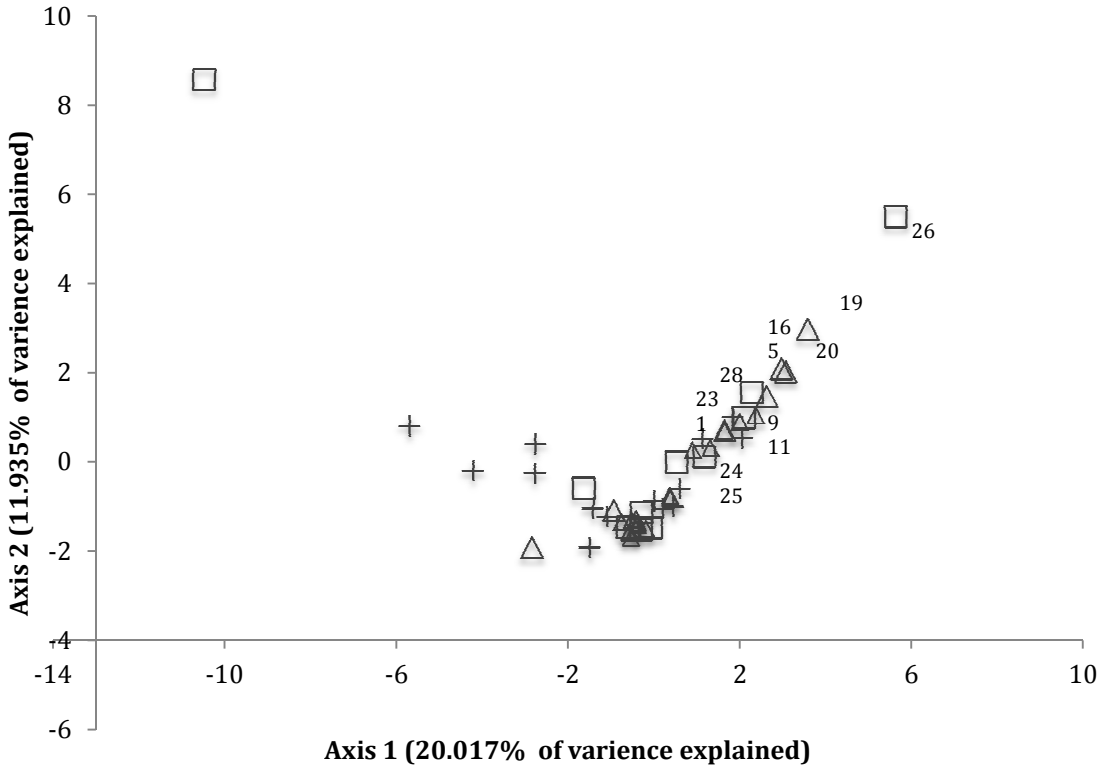

Fig. 1. PCA of 48 before-experiment soil samples (both axes statistically significant). Cross = *A. laevis* Square= *A. gerardii* Triangle = *P. integrifolium.* Numbers represent specific metabolized carbon sources. 1= -Methyl-D-Glucoside, 5=D-xylose, 8 =Tween 40, 9= i.Erythritol, 11=L-Phenyalinine, 16= -Cyclodextrin, 19 = L-Threonine, 20=Glycogen, 23= Glycyl-L-Glutamic Acid, 24= D-Cellobiose, 25= Glucose-1-Phosphate, 26= -Ketobutyric Acid, 28= -D-Lactose.

Mean aerobic bacterial counts and fungal counts differed among soil types ($F_{2,47}$ = 8.76, $p<0.05$ for aerobic bacteria; $F_{2,47}$ = 9.54, $p<0.05$ for fungi). Post-hoc Tukey tests showed differences in mean values among all three soil types for both bacterial and yeast/mold plates, with AG soil having the highest mean bacterial and fungal counts and AL soil having the lowest bacterial and fungal counts. (Table 1). PI soil was in the middle for both counts. Autoclaved soil counts were not high enough to give counts (Table 2).

Table 1. Pre-experiment Tukey test p-values for differences among soil types(AG=*A. gerardii*, PI= *P. integrifolium*, AL = *A. laevis*) in mean aerobic bacterial counts and mean fungal counts.

Soil	p-value Bacteria	p-value Fungi
AG vs PI	0.00025	0.006
AG vs AL	<0.00001	<0.00001
PI vs AL	<0.00001	<0.00001

Table 2. Pre-experiment mean aerobic bacteria and yeast/mold petri film counts (CFU/g) for AG, AL, and PI soil core samples (with standard deviation).

	Bacterial	Yeast/Mold
A. gerardii (AG)	2.78×10^7 +/- 1.9×10^7	4.40×10^3 +/- 2.6×10^3
P. Integrifolium (PI)	1.25×10^7 +/- 1.15×10^7	3.38×10^3 +/- 2.07×10^3
A. laevis (AL)	3.90×10^6 +/- 1.67×10^6	2.52×10^3 +/- 1.39×10^3
Control Soil	Uncountable*	Uncountable

* Uncountable signifies less than 10 CFU formed from the lowest dilution

Post-experiment Soil Microbial Analysis

The PCA results of post-experiment soils axis 1 explained 11.935% of the variance among samples (p=0.0001). Axis 2 explained 12.439% of the variance (p=0.001). Axis 3 also had a p-value < 0.05 but was not interpreted (Figure 2.). As in the pre-experiment results, axis 1 seems to be linked to decreasing number of carbons metabolized to the right. Axis 2 most likely represents specific carbon sources metabolized.

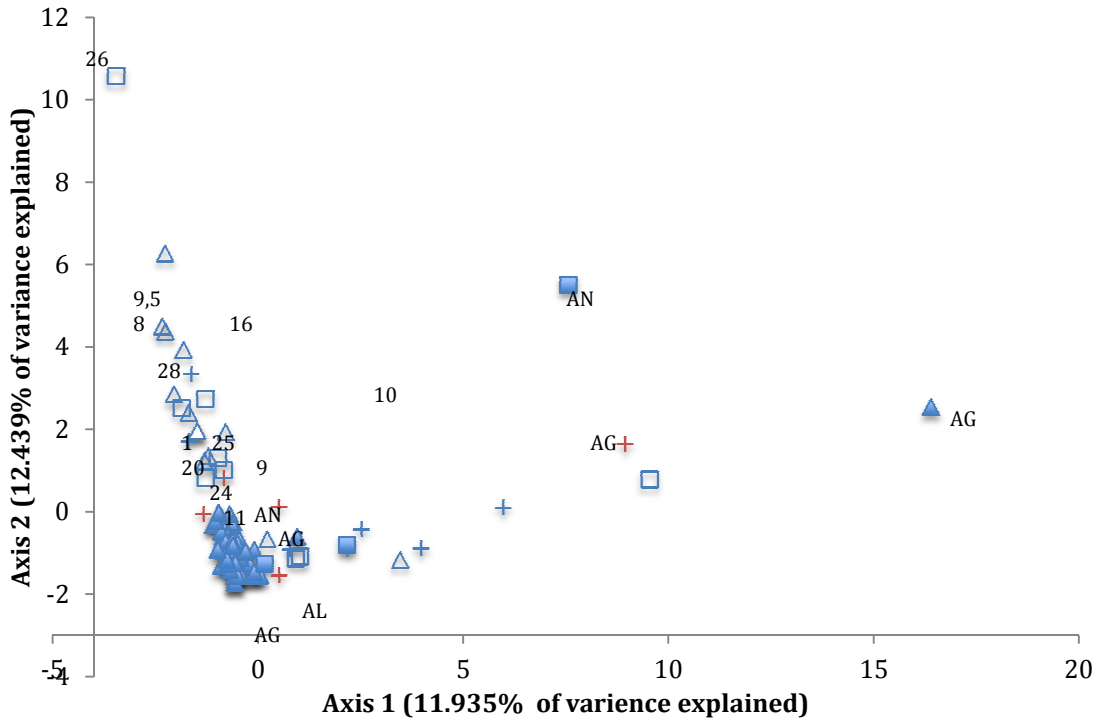

Fig. 2. PCA of 96 pre-experiment and post-experiment soil samples (both axes statistically significant). Symbol shapes represent soil types: Cross = *A. laevis* Square= *A. gerardii* Triangle = *P. integrifolium (before non-filled)*. Red Cross= *A. laevis* after Filled square= *A. gerardii* after Filled Triangle = *P. integrifolium* after. Letter codes denote plant species grown in soil. Numbers represent specific carbon sources metabolized. 1= -Methyl-D-Glucoside, 5=D-xylose, 8 =Tween 40, 9= i.Erythritol, 10 = 2- Hydroxy Benzoic Acid, 11=L-Phenyalinine, 16= -Cyclodextrin, 20=Glycogen, 23= Glycyl-L-Glutamic Acid, 24= D-Cellobiose, 25= Glucose-1-Phosphate, 26= -Ketobutyric Acid, 28= -D-Lactose.

Post-experiment differences in yeast/mold mean counts were found between PI and AL ($F_{2,47}$= 11.45, p<0.001) and AL and AG ($F_{2,47}$ = 8.78, p=0.018).

Table 3. Post-experiment mean aerobic bacteria and yeast/mold petri film counts (CFU/g) for AG, AL, and PI soil core samples (with standard deviation).

	Bacterial	Yeast/Mold
A. gerardii (AG)	4.63×10^7 +/- 4.1×10^7	3.96×10^3 +/- 1.07×10^3
P. Integrifolium (PI)	3.43×10^7 +/- 1.34×10^7	4.36×10^3 +/- 1.17×10^3
A. laevis (AL)	3.52×10^7 +/- 0.89×10^7	3.17×10^3 +/- 1.18×10^3
Control Soil	Uncountable*	Uncountable

* Uncountable signifies less than 10 CFU formed from the lowest dilution

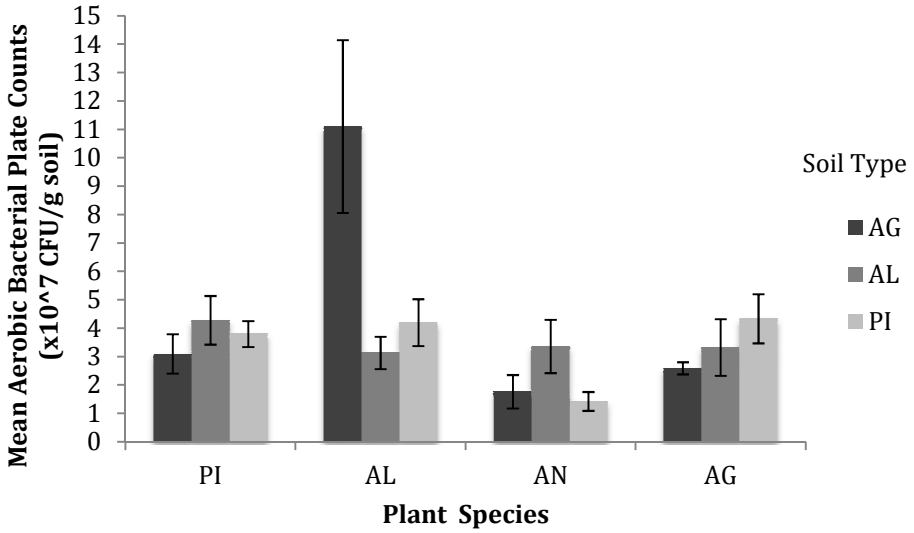

Fig. 3. Mean aerobic bacterial petri film counts/ g soil for plant and soil treatments (n=4 for each treatment combination; bars represent standard errors). PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii.*

Differences in mean aerobic bacterial counts were detected due to plant species ($F_{3,36}$= 26.76, p <0.001), soil type ($F_{2,36}$=5.82, p=0.0065) and plant species x soil type ($F_{6,36}$=20.69, p<0.001). AL plants in AG soil had higher mean aerobic bacterial counts than any other plant species x soil type combination (Figure 3).

Mean fungal counts differed due to plant species ($F_{3,36}$=22.53, p<0.001) and soil type ($F_{2,36}$=11.36, p=0.0001). Soil in containers with AG and PI plants had higher mean fungal counts than soil in containers with AL or AN plants, and AL soil type had lower mean fungal counts than the other soil types (Figure 4).

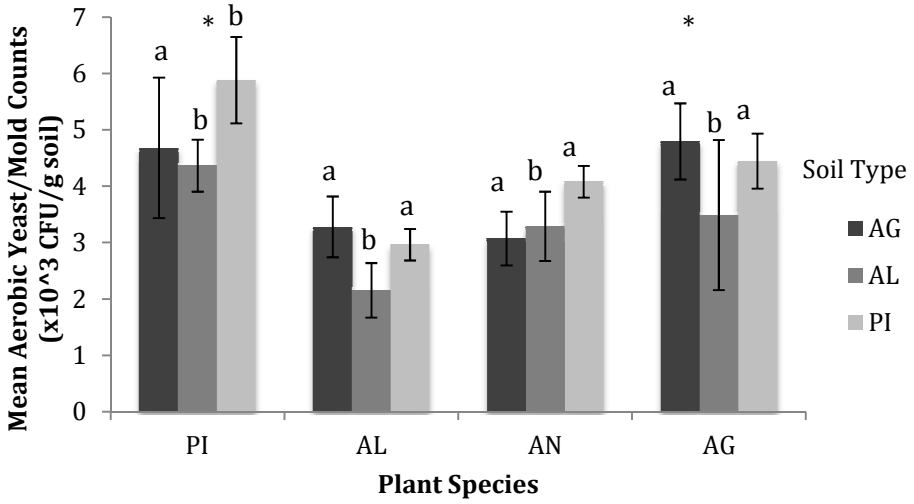

Fig. 4. Mean yeast/mold petri film counts/g soil for plant and soil treatments (n=4 for each treatment combination; error bars represent standard errors). Soil types with different letter superscripts are statistically significantly different (p < 0.05). Asterisks indicate plant species with higher means (p < 0.05). PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii.*

Control Soil Root Analysis

Plant species grown in control soils differed in mean average root diameter ($F_{3,12}$=5.52, p=0.0129), with PI having the largest mean average diameter and AN having the smallest mean average diameter (Figure 5).

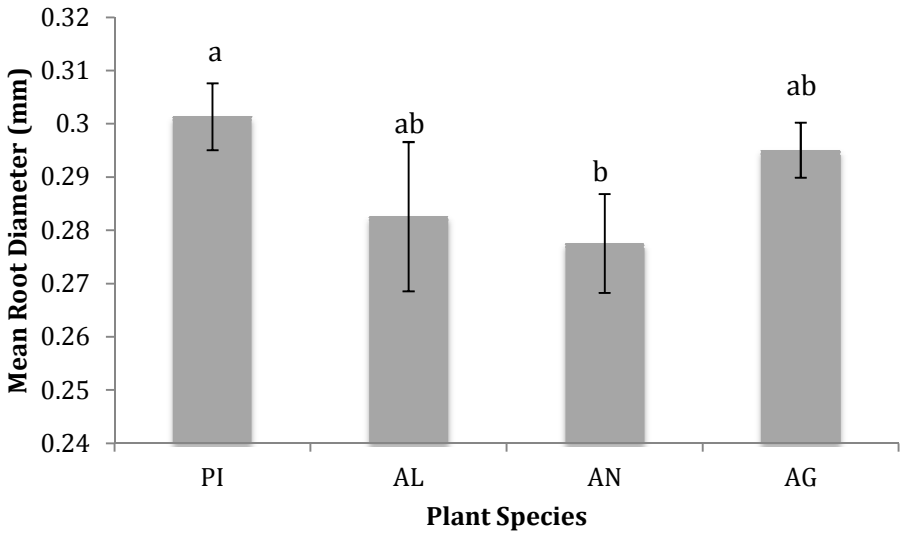

Fig. 5. Mean average root diameter (mm) of plants species in control (autoclaved) soils (n = 4; error bars represent standard errors). Different letter superscripts indicate statistically significant ($p < 0.05$) differences among plant species. PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii.*

Differences also were found among plant species in mean number of forks/cm fibrous roots ($F_{3,12}$=6.39, p=0.0078), with AG having the highest mean number of forks/cm and AN having the lowest mean number of forks/cm (Figure 6).

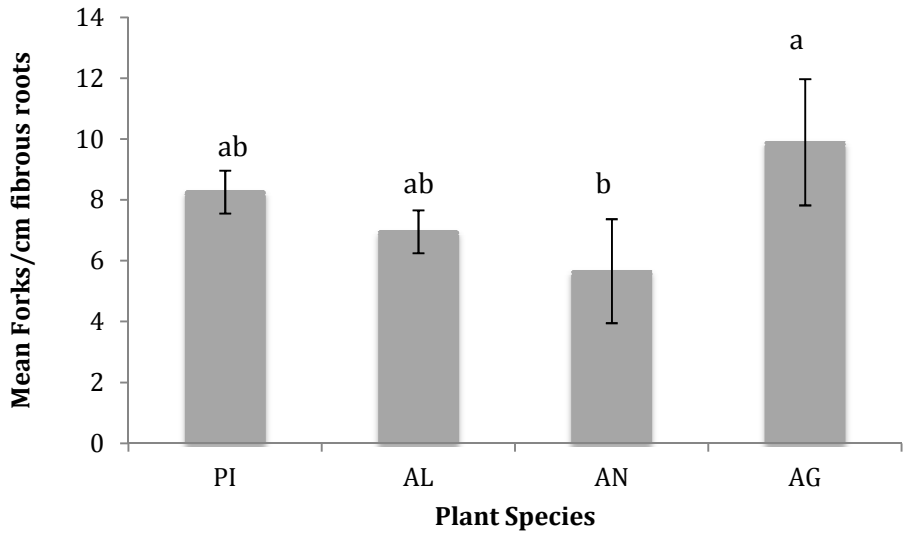

Fig. 6. Mean number of forks/cm fibrous roots of plant species in control (autoclaved) soils (n = 4; error bars represent standard errors). Different letter superscripts indicate statistically significant ($p < 0.05$) differences among plant species. PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii.*

Differences were detected among plant species in mean SRL ($F_{3,12}$=14.68, p=0.0003), with AL having higher mean SRL than the other three species (Figure 7).

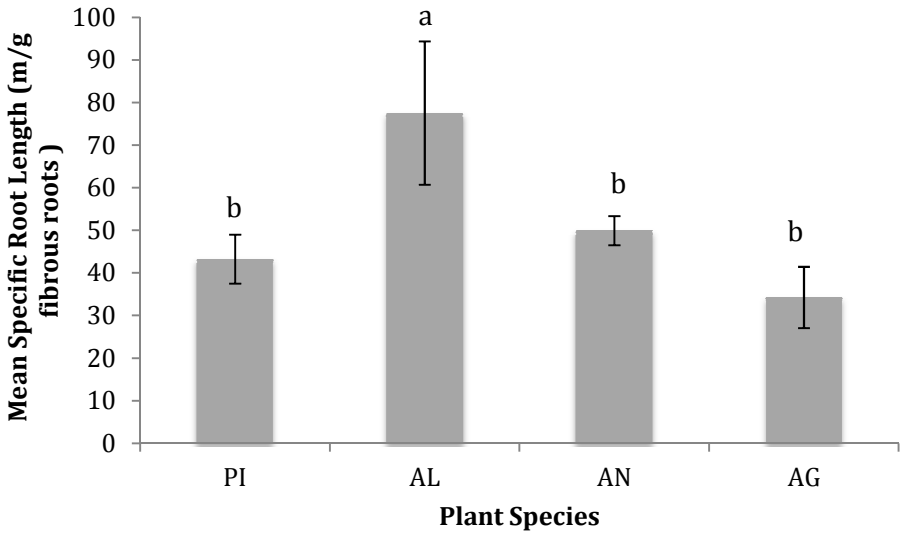

Fig. 7. Mean specific root length (m/g fibrous roots) in control soil (n = 4; error bars represent standard errors). Different letter superscripts indicate statistically significant ($p < 0.05$) differences among plant species. PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii.*

Plant Death

Only plants grown in control soils did not survive the entirety of the experiment. Two *P. integrifolium*, two *A. laevis*, one *Aster novae-angliae*, and three *Andropogon gerardii* died before final harvesting.

Correlations

Pearson correlation analysis demonstrated significant ($P<0.002$) negative correlations between average root diameter (RD) and tips/cm. Significant positive relationships were found between root length (RL) and proportion mycorrhizal colonization (MC), MC and total biomass, RL and RD, RL and total biomass, RD and tips/cm, and RD and total biomass (Table 4).

Table 4. Pearson correlation coefficients (statistically significant in bold) with (n=64)Prob > |r| under H0: Rho=0 for 15 response variables (Bonferroni correction = 0.002). SRL= specific root length MC= proportion mycorrhizal colonization RL = total length of roots RD = average root diameter.

	SRL	MC	RL	RD	Tips	Forks	Total Biomass
SRL	*1.0*	-0.288 0.021	0.068 0.591	-0.266 0.013	-0.308 0.013	-0.103 0.418	-0.289 0.021
MC		*1.0*	**0.529** **<0.001**	0.265 0.034	0.122 0.338	-0.024 0.851	**0.561** **<0.001**
RL			*1.0*	**0.469** **<0.001**	-0.258 0.039	0.238 0.058	**0.776** **<0.001**
RD				*1.0*	**-0.297** **<0.001**	0.172 0.173	**0.539** **<0.001**
Tips					*1.0*	0.20 0.113	-0.159 0.207
Forks						*1.0*	0.176 0.162
Total Biomass							*1.0*

The regression of MC vs SRL resulted in an r^2 value of 0.4148 ($F_{1,46}$ = 32.61, p<0.001), with MC increasing with decreasing SRL (Figure 8).

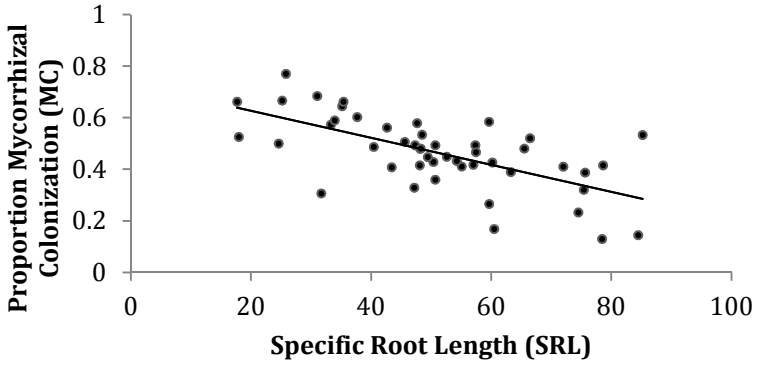

Fig. 8. Proportion mycorrhizal colonization vs specific root length without control treatment.

A positive correlative relationship was displayed through regression analyses of MC vs total biomass with $r^2 = 0.3203$. ($F_{1,62} = 29.21$, $p < 0.001$) (Figure 9).

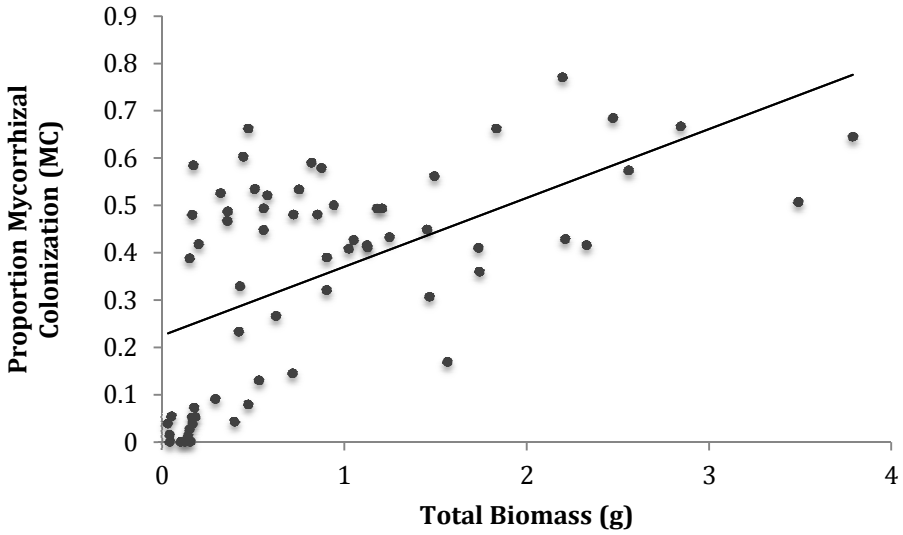

Fig. 9. Proportion mycorrhizal colonization (MC) vs total biomass.

Average Diameter (RD)

Plant species differed in mean average root diameter, ($F_{3,48}=4.33$, $p=0.0088$), with AN having lower mean average diameter than the other species (Figure 10).

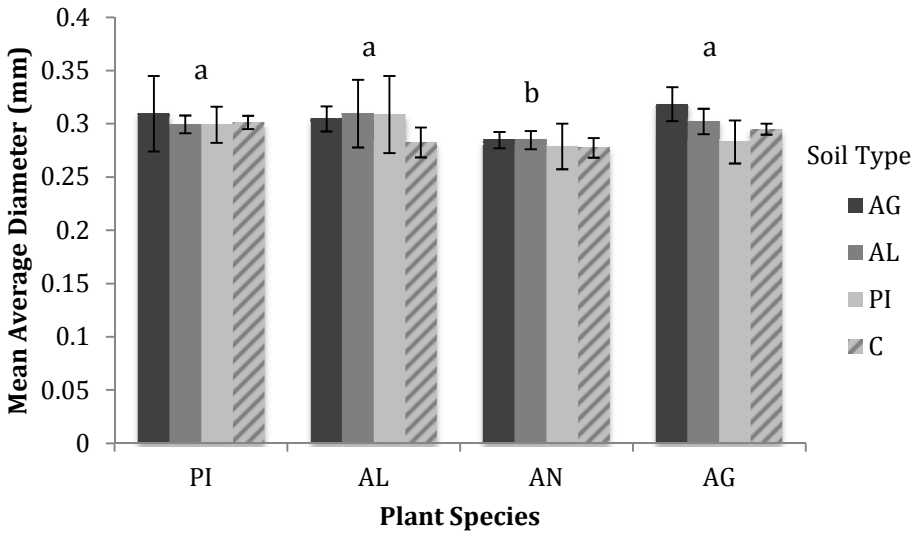

Fig. 10. Mean average root diameter for plant and soil treatments (n=4 for each treatment combination; error bars represent standard errors). Different letter superscripts indicated statistically significant ($p < 0.05$) differences among plant species. PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii* C= *Control.*

Specific Root Length (SRL)

For mean SRL, differences were found due to plant species ($F_{3,38}$=7.48, p=0.0003), soil type ($F_{3,38}$=3.17, p=0.0328) and plant species x soil type ($F_{9,48}$=2.25, p=0.0342). AL plants had higher mean SRL than PI, AN or AG plants ($p < 0.05$). Plants of all species in AG soil had the lowest mean SRL, and plants of all species in PI soil had the highest (AG soil vs. PI soil p = 0.0296; Figure 11).

Effect of plant species on SRL appeared to be stronger for AG and PI plants than for AL plants. AG was the only species for which mean SRL was lowest in control soil and highest in AL and PI soil; and PI mean SRL was uniformly low in all soil types (Figure 11).

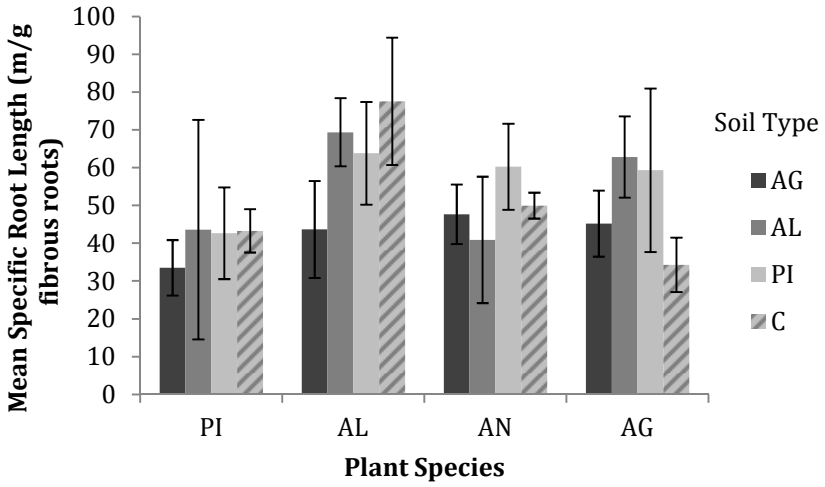

Fig. 11. Mean specific root length (m/g fibrous roots) for plant and soil treatments (n=4 for each treatment combination; error bars represent standard errors). PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii* C= *Control.*

Proportion Mycorrhizal Colonization (MC)

Mean proportion mycorrhizal colonization of roots differed due to plant species ($F_{3,48}$=8.53, p=0.0001), soil type ($F_{3,48}$=167.84, p<0.0001) and plant species x soil type ($F_{9,48}$=2.34, p=0.0278). There was a strong "inoculum" effect, with mean MC < 0.03 for all plant species in control soils. Highest mean MC occurred in AG and AL soils. Mean MC in PI soil was intermediate between control and AG and AL soils. AL plants in all soil types had lower mean MC than other plant species, and mean MC was lowest in PI soil for AL and AG plants (Figure 12).

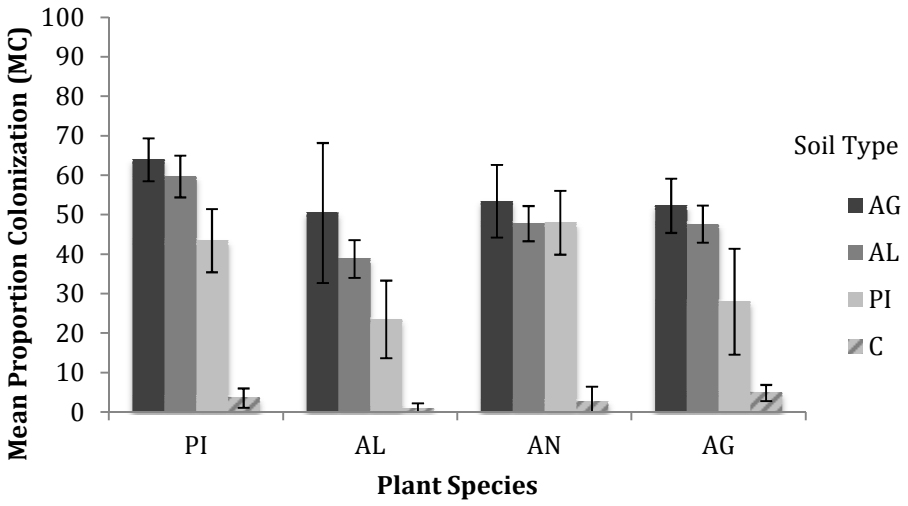

Fig. 12. Mean proportion mycorrhizal colonization of fibrous roots for plant and soil treatments (n=4 for each treatment combination; error bars represent standard errors). PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii* C= *Control.*

Total Biomass

Differences in mean total biomass were detected due to plant species ($F_{3,48}$=9.57, p<0.0001) and soil type ($F_{3,48}$=21.4 , p<0.0001). Plants in AG soil had the highest mean total biomass, and plants in control soil had the lowest mean total biomass (Figure 13).

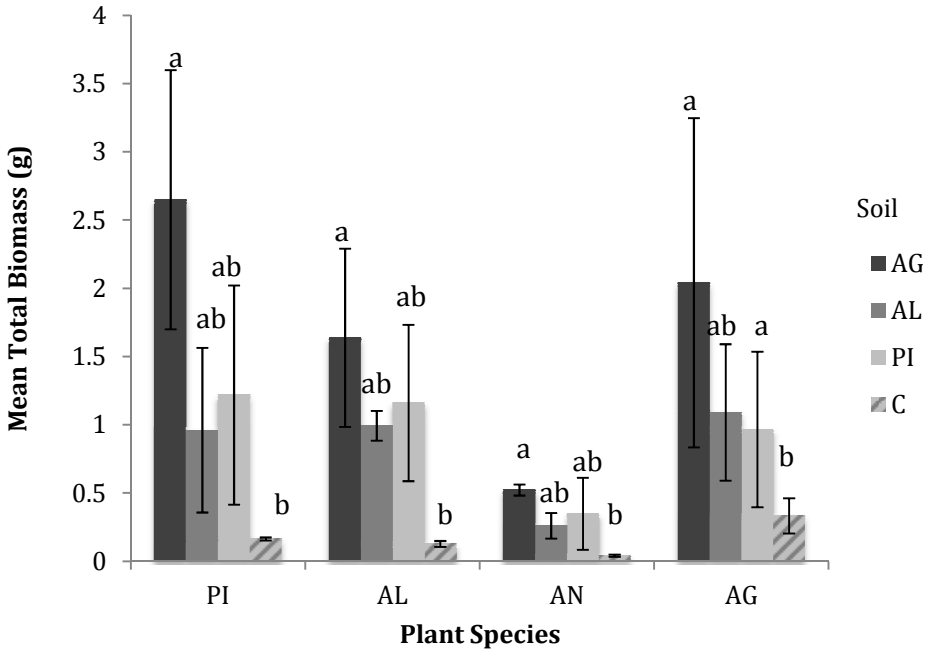

Fig. 13. Mean total biomass for plant and soil treatments (n=4 for each treatment combination; error bars represent standard errors). Different letter superscripts represent statistically significant ($p < 0.05$) differences among soil types. PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii* C= *Control.*

Mycorrhizal Dependence (MD)

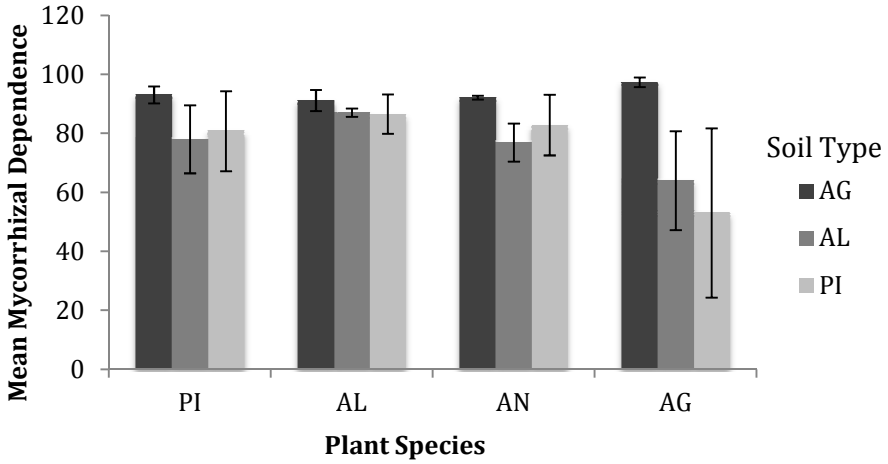

Fig. 14. Mycorrhizal dependence for plant and soil treatments (n=4 for each treatment combination; error bars represent standard errors). PI = *Parthenium integrifolium* AL = *Aster laevis* AN = *Aster novae-angliae* AG = *Andropogon gerardii.*

Differences in mean MD were detected due to plant species ($F_{3,36}$=4.93, p=0.0057), soil type ($F_{2,36}$=10.86 , p=0.0002) and plant species x soil type ($F_{6,36}$ = 9.86, p=0.0034). AG plants had lower overall mean MD than AL or AN plants; however, mean MD for AG plants was higher when grown in conspecific (AG) soil than in soil collected beneath the other two species. Moreover, all plant species had highest mean MD in AG soil (Figure 14).

Net Feedback (I)

No significant difference among pairwise species combinations was detected for net feedback ($F_{2,9}$ = 0.07, p =0.93; Figure 15). A one-sample t-test revealed overall net feedback was not significantly different from zero (t_{11} = 0.895, p>0.1)

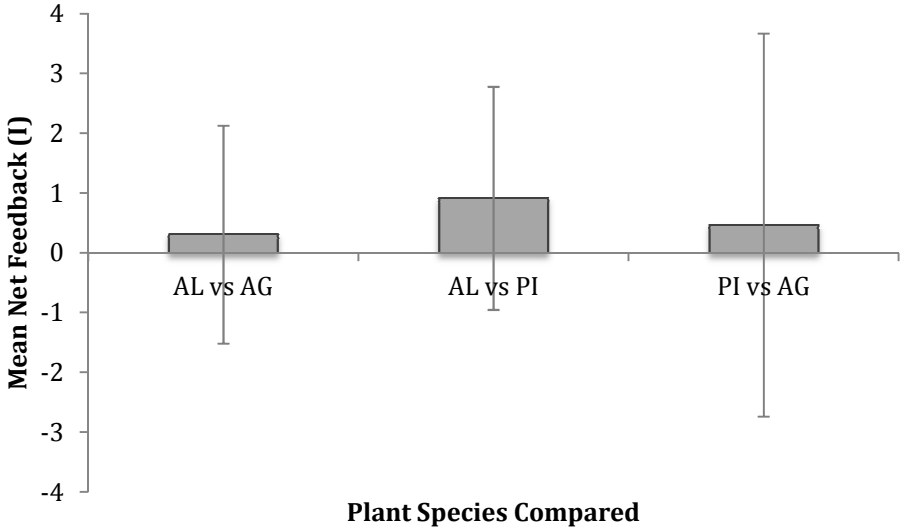

Fig. 15. Mean net feedback (I) for three pairwise species combinations (n=4 for each species combination; error bars represent standard errors). PI = *Parthenium integrifolium* AL = *Aster laevis* AG = *Andropogon gerardii.*

Discussion

Contrary to expectation, this study did not demonstrate negative plant-soil feedback. Instead, all plant species had increased biomass in AG soil, and overall net feedback was not different from zero. Although study species differed in root characteristics, all species had high mycorrhizal dependence. In addition, results showed that microbial community structure of soils collected beneath different species differed before the study, and changed in response to species grown during the study.

Soils collected beneath different species differed markedly in bacterial and fungal counts, and in microbial functional diversity before the experiment. Although plants have been shown to influence soil microbial communities primarily through root exudates in the rhizosphere (Achouak et al. 2008) and a shift from bacterial-dominated to fungal-dominated soil communities has been shown across a chronosequence of tallgrass prairie restorations (Allison et al. 2005), the extent to which individual plant species may influence bacterial vs. fungal dominance in soil microbiota is not known.

Before the experiment AG soil had the highest mean counts for both fungi and aerobic bacteria, and AL soil had the lowest mean counts for both. The PCA of EcoPlate data also showed differences among pre-experiment soils. AG soil samples were most dissimilar, and AL soil samples were most similar in terms of functional diversity. After the experiment mean fungal counts were still lowest in AL soils, but as expected in response to root exudates from plants (Bais et al. 2006) mean bacterial counts increased in AL soils compared to pre-experimental

counts. Highest post-experimental mean bacterial counts, however, occurred with AL plants growing in AG soil. PCA results from post-experimental EcoPlate data showed that functionally, most post experiment samples converged around the metabolism of: D-Galactonic Acid -Lactone**,** L-Arginine, Pyruvic Acid, Methyl Ester, D-Galacturonic Acid, L-Asparagine, D-Mannitol, 4-Hydroxy Benzoic Acid, L-Serine, N-Acetyl-D-Glucosamine, -Hydroxy Benxoic Acid, L-Threonine, D-Glucosaminic Acid, Itaconic Acid, Phenylethyl-amine, D-Malic acid and Putrescine, and did not differ with plant species or soil type. These carbon sources likely originated as root exudates from study plants. It is known that mycorrhizal helper bacteria (MHB) influence AMF populations (Garbaye 1994), and they may have been present in soil samples. MHBs facilitate in both assisting in the formation of plant-AMF symbiosis and positively enhancing the symbiotic relationship. MHB's may also be responsible for increased mycorrhizal colonization and/or root branching that may have occurred during this study (Frey-Klett et al 2007). Although MHB were not identified in this study, they would be an interesting area of future research for tallgrass prairie systems.

This study showed differences among plant study species in root characteristics. In control soils mean average fine root diameter was highest for PI, followed by AG and then the asters. Number of forks, a characteristic of root branching, was highest in AG plants in control soils, but no correlation was found between this variable and AMF colonization. Mean SRL in control soil was higher in AL than in the other three species.

Strength of main treatment and interaction effects varied when root diameter, SRL, MC and total biomass were analyzed across all plant species and soil types. Root diameter was influenced by plant species but not soil type, with AN having smaller mean average root diameter than the other three species. For SRL, although plant species still had the strongest influence (AL had highest mean SRL), soil type and interaction between plant species and soil type also had an influence. Plants of all species had lower SRL in AG soil and higher SRL in PI soil.

As expected, mean MC generally increased with decreasing SRL. It is yet unknown the degree to which AMF influences SRL and root architecture among plant species. A new approach of classifying roots based on orders (similar to stream classifications) may help to better understand how AMF colonize host plants (Eissenstat et al. 2008). Dreyer et al. (2014) also suggest root classification by order. This more integrative approach to describing which orders of roots are colonized may be a next step in assessing the relationship between AMF and root morphology. They observed that in the palm species *Phoenix canariensis* only certain root orders were colonized by AMF.

In this study root diameter did not play a significant role in AMF colonization and Barrow et al. (1995) results were similar. They did find, however, that benefit due to AMF was negatively correlated with root hair length. My results showed that number of root forks was not correlated with AMF colonization. Reinhart et al. (2012) also found that for many prairie species

physiological characteristics of roots were not as strong of a predictor for mycorrhizal colonization as previously thought.

In this study, plant species and soil type exerted equally strong influence on mean MC. And soil type continued to exert a strong influence on total biomass of plants. Although plants in AG and AL soils had highest mean MC, only plants in AG soil had highest mean biomass. MC differed between soil treatments for study species; these results suggest soil type may have substantial influence on MC. Some component of the microbial community in AL soils, such as pathogens, may have resulted in lower biomass in this soil type.

Plant mortality occurred only in control soil that was mostly free of AMF (<1% mean MC), aerobic bacteria and fungi. The death of non-inoculated C_4 grasses and forbs has been observed before (Hetrick et al.1988). AMF and microbial communities may play an important role as seedlings establish themselves under competitive conditions. Without AMF and most likely other soil biota, establishment dynamics may be altered.

Although plant species differed in mean MC, their mycorrhizal dependence was very high. AG, however, differed the most in mean mycorrhizal dependence among soil types. For this plant species, mean mycorrhizal dependence was highest when grown in conspecific (AG) soil, and lower when grown in heterospecific (AL or PI) soil.

Overall net feedback (I) was not different from zero, and did not differ among pairwise species combinations. Another soil-feedback study including AG and four additional tallgrass prairie species not in this study also found no

difference in net feedback among pairwise species combinations; however, they found an overall negative net feedback (Fitzsimons and Miller 2010). They grew plants in containers "trained" by growth of study species in 2 L pots for six months, whereas I grew plants in soil collected from beneath study species in the field for one hundred days; these differences in methods could have influenced results. In the same previous study, plants grown in trained "whole soil" demonstrated negative net feedback, while plants grown in sterilized soil with only AMF added had positive net feedback.

Net feedback results from my study are more similar to feedback results from Fitzsimons and Miller's "AMF only" treatment. Although overall mean net feedback was not different from zero, the individual means for each pairwise species combination were positive. The negative feedback demonstrated in the "whole soil" treatments in the previous study was absent in my study, perhaps due to low numbers of soil pathogens, or to mediation of negative feedback by AMF (Bever 2002).

No overall net feedback in this study also resulted from the pattern of biomass responses to AG soil across study species. While AG benefitted from growing in its own soil, other species also benefitted from growing in AG soil. As a result, other species often benefitted as much or more from growing in AG soil than AG benefitted from growing in its own soil. This nature of plant-soil-plant interaction can result in zero or negative net feedback, with stable coexistence of species possible in either case (Bever et al. 1997, Kulmatiski and Kardol 2008).

Because AG is a dominant species in tallgrass prairie, “AG soil” is likely to be a quite common substrate for establishment of other plant species. If biomass responses of the species in this study are similar in the field, both AG and the other species should have higher biomass when growing in “AG soil.” Blair et al. (2011) addressed this scenario in their study by predicting that positive AMF-caused growth effects for some species would be offset by negative competitive effects from more strongly mycorrhizal species. Although AG was shown to be strongly mycorrhizal (MD) in this study, so were the other three species. Although the no net feedback results in this study would predict that no species would have a competitive advantage in the field, AG might still have a competitive advantage over other species in this study through other mechanisms (e.g., competition for light due to tall stature, high biomass).

It is, of course, entirely likely that responses of these species in the field would be different in magnitude or direction from responses should in this study in the laboratory, as suggested and shown in other studies (Hartnett and Wilson 1997, Hartnett and Wilson 2002, Blair et al. 2011). In addition, plant responses may change over time from seedling to mature plant, or over numerous growing seasons. A combination of greenhouse and field research, including growth of plants in AMF–only vs. whole soil inoculations, and pairwise plant-soil feedback field trials, should increase understanding of responses of these species to soil microbiota and to other plant species.

This research is an early step in investigating how soil and the microenvironment may influence plant development of different species.

Discovering in which direction the cues are strongest is an important next step. Does AMF colonization of roots respond to engrained genetic root architecture or is root architecture of seedlings more influenced by soil conditions and the presence of microbial species? With more genetic work on both hosts and symbionts the answer may become clearer.

The relationship between AMF and host plants may even extend to the level of consumers. Christensen et al. (2003) observed that insect herbivore increased below-ground carbon transfer in young pea (*Pisium sativum*) plants and affected AMF colonization. AMF colonization strategies are complex and it is not known to what degree these strategies may differ among the hundreds of thousands of species that form AMF relationships.

Recently it has been learned that mycorrhizal networks are able to facilitate plant communication warning neighboring plants of aphid attacks. These communications warn neighboring plants and stimulate the production of compounds that repel aphids and attract aphid predators (Babikova et al. 2013). The common mycorrhizal network (CMN) is yet another component of soil biota that works to structure and influence plant communities. The role of CMNs in tallgrass prairie is an interesting area yet to be fully explored.

Some research has suggested that inoculation of soil with AMF before tallgrass prairie restoration begins can be beneficial, while other research suggests that suppression of AMF can increase plant species diversity in restorations. These studies, however, were not able to measure the long term effects of inoculation on tallgrass prairie restoration sites. AMF inoculation is

expected to beneficial in restoration sites that are low in soil phosphorus or that have sparse remnant AMF communities (Charvat et al. 2008). AMF suppression may be more important in prairie restoration that is done in a site with many dominant C_4 grasses. AMF suppression was shown to reduce the dominance of C_4 grasses and allow forb species to establish, thus increasing plant species diversity, but changing community structure (Blair et al. 2011). AMF, as well as plant-soil feedback, influences on tallgrass prairie systems are complex and should be better understood in order to produce restorations of the highest quality. Responses of individual species to AMF colonization are an important component of understanding fungal ecology in tallgrass prairies.

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