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Plant Interactions Across Common Mycorrhizal Networks

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PLANT INTERACTIONS ACROSS COMMON MYCORRHIZAL NETWORKS

By

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Arbuscular mycorrhizal fungi associate with roots of the majority of land plants and supply up to 80% and 25% of their P and N requirements, respectively. These fungi do not form mycorrhizas on individual plants in isolation, the way they often have been investigated. Instead, arbuscular mycorrhizas may form when hyphae connected to one root system branch throughout the soil while foraging for mineral nutrients, encounter, and colonize the roots of another plant, thereby forming a common mycorrhizal network (CMN). The importance of CMNs is their potential influence on the distribution of limiting mineral nutrients among plants. Although it is likely that most research on plant interactions may have incorporated CMNs unwittingly, until recently, few investigators have attempted to distinguish the effects of CMNs.

Throughout my dissertation research I have found that CMNs mediate belowground plant interactions. In Chapter One, I introduce arbuscular mycorrhizal fungi, their relationship with host plants, and the processes involved in the formation of CMNs. In Chapter Two, I investigate whether effects of CMNs can be detected on populations of *Andropogon gerardii*, a dominant grass species of tallgrass prairies. I found that CMNs intensify intraspecific competition among *A. gerardii*, and the competitive effects of CMNs increase size inequality within populations. In Chapter Three, I experimentally test the mechanisms behind the intensified competition found in

Chapter Two. I learned that CMNs amplify competition through reciprocal rewards between large, abundantly carbon-fixing plants and AM fungi, thereby suppressing the growth of small, neighbor plants. Chapter Three reaffirmed the results of Chapter Two by again finding that CMNs intensified competition and increased size inequality among plants with intact CMNs versus those with severed CMNs.

Chapter Four includes a second plant species by contextualizing the dependence upon mycorrhizas and responsiveness to mycorrhizas of *A. gerardii* and *Elymus canadensis*, a prairie sub-dominant grass, in preparation for the research of Chapter Five. I also consider how these mycorrhiza-related attributes influence plant functional traits. I found that *A. gerardii* is more dependent on AM fungi for mineral nutrient uptake and growth than is *E. canadensis*, although when fertilized, both species can grow without AM fungi. Then, using a phosphorus amount that I had determined to favor strong mycorrhiza responses by both species, in Chapter Five I went on to investigate how these two contrasting tallgrass prairie plant species interact via CMNs. I discovered that intact CMNs increased survival and growth of both species in monocultures and in mixture. For *E. canadensis*, intact CMNs improved plant water uptake, likely by increasing access beyond cone-tainers. For *A. gerardii*, intraspecific interactions were more intense than interspecific interactions with *E. canadensis*, and intact CMNs resulted in substantial overyielding by *A. gerardii* when in mixture with *E. canadensis*.

In Chapter Six, I synthesize the four data chapters and suggest that it is not just the presence of AM fungi that has implications for grasslands, but it is the interconnecting hyphae of CMNs that mediate plant interactions. Notably, my work found that CMNs improved Mn acquisition, enhanced the mycorrhizal colonization of

putatively carbon-limited plants, and increased stomatal conductance of *E. canadensis*. The extent to which such effects of CMNs can be realized, however, likely depends upon conditions of soil fertility, plant density, and whether or not root systems overlap. Hence in nature, CMNs may be a "mixed blessing," either benefitting plants through improved mineral nutrition and hydration that enhance survival and growth, or disadvantaging them by intensifying competition especially to the detriment of small individuals.

Dedication

I would like to dedicate this dissertation to my family – Mama, Tata, and Kasia.

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I would like to thank my committee members, David P. Janos, Leonel da Silveira Lobo O'Reilly Sternberg, Barbara A. Whitlock, Donald L. DeAngelis, and James D. Bever for their guidance and encouragement over the years. I would like to especially thank my mentor, David Janos, for taking a chance and bringing me into the fascinating and complicated world of mycorrhizas. He has taught me a great deal, but his most important lesson taught me to be patient and detail-minded in order to be a respectable scientist. I am also grateful for Leo Sternberg's stable isotope course, and for his advice and assistance in running the stable isotope analyses in Chapter Two. I'd like to thank Bruce McCune for his discussion of target-neighbor analyses, Michael Amaranthus at Mycorrhizal Application for providing the inoculum that has helped to reveal much about mycorrhizal colonization in this dissertation, and for the Fairchild Tropical Botanical Garden for providing screen house space. The completion of each of my experiments would not have been possible without the help of several undergraduate students, but I'd like to thank Kotaro Seto in particular, because he assisted me in three massive experiments. Chapter Five would not have been possible without the assistance from several classrooms of Richmond Heights Middle students in the Agriscience Program, so for that I'd like to thank Alison Lincoln for her enthusiasm, scientific curiosity, and support.

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Chapter One

Introduction

Summary

Over the past fifty years, the effects of arbuscular mycorrhizal (AM) fungi on plant growth have been investigated mostly by using plants grown individually in pots without or with AM fungi, often just a single AM fungus species. In nature, however, plants do not grow in isolation, and they typically are colonized by suites of AM fungi. While we have learned a tremendous amount from previous experiments, there is a growing acknowledgement that in nature, plants may be interconnected belowground by common mycorrhizal networks (CMNs). CMNs form when the hyphae of AM fungi colonizing one root system encounter and colonize a neighboring root system, thereby interconnecting plants within a community. CMNs may have the potential to profoundly influence plant interactions by influencing the direction of the flow of mineral nutrients and carbon among interconnected plants. The aim of my dissertation work was to investigate how CMNs affect intraspecific and interspecific plant interactions by providing all plants with AM fungi, but severing CMNs or keeping them intact.

Background

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi have symbiotically associated with plants for 460 million years, (Remy *et al.*, 1994) and today, they associate with the majority of plant species across almost all habitats (Wang & Qiu, 2006). AM fungi provide many benefits to their hosts, such as increased water uptake (Auge, 2001), pathogen protection

(Newsham *et al.*, 1995), plant communication signaling pathways (Babikova *et al.*, 2013), and increased uptake of growth-limiting mineral nutrients. In particular, AM fungi are known for their enhanced uptake of relatively immobile and unavailable phosphorus (Brady & Weil, 2008; Smith & Read, 2008), although they also have been shown to increase uptake of nitrogen, zinc, and copper (Ames *et al.*, 1983; Smith & Read, 2008).

Arbuscular mycorrhizal fungi are obligate symbiotic partners from the phylum Glomeromycota (Schüßler *et al.*, 2001), named after their short-lived, highly-branched structures called arbuscules. Because this group of fungi is able to penetrate the walls of root cortical cells, the plant and fungal plasma membrane lie side-by-side at the arbuscule (which lacks fungus cell wall at its finest branches), both expressing phosphorus, carbon, and other transporters (Harrison *et al.*, 2002; Javot *et al.*, 2007). Colonization by AM fungi upregulates photosynthesis of host plants (Wright *et al.*, 1998; Miller *et al.*, 2002) and carbon from the plant is supplied to AM fungi in the form of hexoses. The hexoses are converted to lipids by the fungi and ultimately are used in construction of fungal structures or stored in spherical structures called vesicles. Phosphorus also is exchanged at the periarbuscular membrane, in the form of phosphate (Harrison *et al.*, 2002; Javot *et al.*, 2007). The stoichiometry of phosphorus and carbon exchange, however, remains largely unknown. Nevertheless, because AM fungi can store phosphorus in vesicles and plants are not often carbon limited, the carbon-phosphorus exchange is likely coupled and bi-directionally controlled by both the fungus and plant (Jakobsen & Hammer, 2015).

The formation of common mycorrhizal networks

Arbuscular mycorrhizal fungi exist outside of plant root cells in the form of hyphae and spores. Short, branching hyphae temporarily form around rich nutrient patches (Bago *et al.*, 1998) and branching distributive hyphae forage and spread throughout the soil. Often, hyphae encounter the root systems of neighboring plants and colonize them, thereby interconnecting plants in a common mycorrhizal network.

In nature, fungal germlings can plug into already established networks of AM fungi (Giovannetti *et al.*, 2015). AM fungus species differ in the extensiveness of their hyphal networks, however, largely because of differences in hyphal branching, ability to spread distributive hyphae, and ability to anastomose, or fuse, hyphae (Giovannetti & Sbrana, 2001; Giovannetti *et al.*, 2004; Avio *et al.*, 2006; Giovannetti *et al.*, 2006). Anastomosis allows for increased branching by fusing fungal cells wells and protoplasm, allowing for continuity across the hyphae (Avio *et al.*, 2006; Giovannetti *et al.*, 2015). Species in the genera *Acaulospora* and *Rhizophagus* are able to anastomose frequently and spread beyond phosphorus-depletion zones around roots (Avio *et al.*, 2006; Giovannetti *et al.*, 2006). In contrast, species in *Gigaspora* and *Scutellospora* often take up phosphorus in competition with root surfaces (Schnepf *et al.*, 2008). Although AM fungus species are not able to anastomose with other species, in some genera anastomosis likely occurs frequently among hyphae of the same species and more so for the same fungus isolate (Giovannetti *et al.*, 2015). Anastomoses can create fungal hyphal densities that range up to 20.5 meters of hyphae per gram of soil (Mikkelsen *et al.*, 2008). Thus, neighboring plants may be interconnected by common mycorrhizal networks formed by many different AM fungal species, and nutrient exchange may be dynamic depending

upon environmental conditions such as sunlight (Zheng *et al.*, 2015) and AM fungus species identities (Kiers *et al.*, 2011; Werner *et al.*, 2014).

Nutrient movement within common mycorrhizal networks

Host plants may provide up to 20 % of their total fixed carbon to AM fungus associates (Jakobsen & Rosendahl, 1990), and receive from the fungi up to 80 % and 25 % of their phosphorus and nitrogen requirements, respectively. Therefore, how mineral nutrients are distributed in CMNs may influence the outcome of competitive plant interactions. It once was thought that CMNs distribute mineral nutrients equally among interconnected plants regardless of species, size, or distance between them (Chiariello *et al.*, 1982). Transport of phosphorus in extraradical mycelium can occur at distances of 20 cm in soil (Jakobsen *et al.*, 1992; Schweiger & Jakobsen, 1999). Recent *in vitro* root organ culture work, however, has found that CMNs may preferentially distribute mineral nutrients to abundant carbon-supplying root systems (Lekberg *et al.*, 2010; Hammer *et al.*, 2011). Different species of AM fungi also have been found to differ in their exchange rates of phosphorus for carbon (Kiers *et al.*, 2011). Exchange rates, particularly preferential mineral nutrient supply to host plants that provide the most carbon, are referred to as "reciprocal rewards." The consequences of reciprocal rewards for plant interactions across CMNs, however, are largely unknown.

Direct and Indirect P uptake pathways

Plants have direct and indirect pathways to take up important mineral nutrients such as phosphorus in the form of orthophosphate (Pi). High affinity Pi transporters are located on the root epidermis and root hairs and serve as a direct pathway for plant mineral

nutrient uptake (Harrison & Vanbuuren, 1995; Javot *et al.*, 2007; Smith *et al.*, 2011; Yang *et al.*, 2012). The expression of high affinity Pi transporters is influenced by soil P concentration and host P status (Maldonado-Mendoza *et al.*, 2001), but is independent of C status (Olsson *et al.*, 2006). When roots become colonized by AM fungi, however, plants can suppress root Pi transporters and an indirect pathway through AM fungus hyphae becomes an important source of phosphorus to the plant (Smith *et al.*, 2004; Yang *et al.*, 2012).

Different plant species differ in the degree of suppression of their direct pathway after colonization by AM fungi. For example, tomato's (*Lycopersicon esculentum* Mill.cv Riogrande 76R) direct pathway became completely inoperative when it was colonized by *Glomus intraradices* Schenck and Smith [now *Rhizophagus intraradices* (Krüger *et al.*, 2012)] despite the ineffectiveness of the indirect pathway in nutrient uptake that resulted in a negative effect on tomato's growth (Smith *et al.*, 2004). In contrast, the direct pathway of *Medicago truncatula* L. cv Jemalong was not completely suppressed and the plant responded positively to AM fungi when it was colonized by *G. intraradices* and *G. caledonium* (Nicol. and Gerd.) Trappe and Gerdemann (Smith *et al.*, 2004). When soil phosphorus increases, the AM contribution to mineral nutrient uptake can decrease, leading to an increase in direct uptake by roots (Nagy, 2009). This dynamic system may be an indication that the roles of AM CMNs and roots in competition for mineral nutrients might change depending on the surrounding soil fertility and individual plant species' responses to AM colonization.

Although AM fungi are mostly associated with beneficial effects on plant growth, changes in environmental conditions can cause the association to range from beneficial,

to neutral, to disadvantageous (Peng *et al.*, 1993; Johnson *et al.*, 1997; Janos, 2007). Increased soil fertility levels, for example, produce an increased cost of mycorrhizas to host plants because sustaining mycorrhizal fungi outweighs the benefits of mineral nutrient uptake. The differences in growth for plants with and without mycorrhizas, or mycorrhiza responsiveness, is dynamic across a gradient of soil fertilities (Janos, 2007). Plant dependence upon mycorrhizas, or the lowest level of phosphorus availability at which a plant can grow without mycorrhizas, also lies along a continuum, from facultative to obligate mycotrophy (Janos, 2007). Obligately dependent plant species are unable to grow without the presence of AM fungi, even at high soil fertilities, while fertilization may compensate for the lack of mycorrhizas for facultatively mycotrophic species.

Grasslands

Grassland species are good models for understanding competition across common mycorrhizal networks because they rely on mycorrhizas for growth, they have different degrees of mycotrophy, and they typically grow at high densities. Also, grasses have a graminoid growth form that minimizes aboveground competition, generally thought to be asymmetric. Consequently, treatment effects are likely to be attributable especially to belowground interactions. Grasslands are characterized by low diversity at the local scale because of high dominance by certain species. Dominant species tend to be warm-season, highly-mycotrophic, C4 grasses such as *Andropogon gerardii* Vitman and *Sorghastrum nutans* L. (Smith *et al.*, 1999; Reinhart *et al.*, 2012). Subordinate species within grasslands tend to be cool-season, C3 grasses such as *Elymus canadensis* L. and forbs, both of which may be less mycorrhiza-responsive than dominants (Grime, 1977).

A novel methodology: rotatable containers

Three out of four chapters in this dissertation report the use of rotatable cone-tainers to investigate the roles of CMNs in plant interactions (Figure 1.1). Rotatable cone-tainers are modeled after the rotated in-growth cores first used by Johnson *et al.* (2001), that were used to study ^{33}P movement from soil to plants by either severing or keeping AM mycelial networks intact. I studied the effects of severing CMNs by growing individual plants in modified Ray Leach Cone-tainers with two slots in their opposite sides covered by silk screen, nylon mesh cloth to confine roots but allow mycorrhizal fungus hyphae to grow freely through the mesh. In chapter two, I additionally covered the silk screen mesh with a layer of Gore-tex™ that prevented the movement of water and water-soluble mineral nutrients but allowed AM hyphae to cross over (Mader *et al.*, 1993). Rotatable cone-tainers, therefore, allowed AM fungus interconnections to form among neighboring individuals, forming CMNs. To reveal CMN effects on plant interactions, I repeatedly severed CMNs by rotation of containers and will compare this treatment to non-rotated containers among which CMNs remain intact. Thus, I avoided extreme and quite unnatural comparisons between plants with mycorrhizas versus those entirely without them.

Research questions

I addressed the following questions in my research:

1. What are the consequences of CMNs for the population structure of a grassland dominant, *A. gerardii*?
2. How is nitrogen distributed among *A. gerardii* host plants by CMNs*?*

3. How do two grass species (*A. gerardii* and *E. canadensis*) with different dependencies on AM fungi compete via CMNs?

Chapter Two, the foundation for the remainder of the dissertation, investigates if the effects of CMNs can be detected in populations of *A. gerardii.* This experiment demonstrates the feasibility of using rotatable cone-tainers in investigating the effects of CMNs. In this experiment, I grew *A. gerardii* seedlings in monocultures with intact, severed or no CMNs. Using principal components analyses, I detect competitive effects mediated by CMNs among individual plants and their neighbors in microcosms. I then examine the consequences of CMN-mediated plant competition for population structure though analyses of changes in size hierarchies. Nutrient analyses then assist in interpreting whether CMNs benefitted the largest individuals over their neighbors, leading to positive feedback between mycorrhiza formation, nutrient supply, and host growth.

Chapter Three builds upon the findings of Chapter Two, but experimentally determines if CMNs preferentially supply mineral nutrients to large plants that are able to fix and provide more carbon than small plants. Chapter Three essentially repeats parts of the Chapter Two experiment, but utilizes ^{15}N tracing as well as shading of plants to manipulate carbon supply to CMNs. Several of the findings of Chapter Two are confirmed in Chapter Three. The use of ^{15}N, however, allows me to explain the findings mechanistically through tracing mineral nutrient movement in CMNs in response to fixed-carbon provision. In Chapter Three, I explore the possibility of reciprocal rewards and the potential consequences this hypothesis has for the population structure of *A. gerardii* seedlings.

Both Chapters Four and Five explore how differences in the degrees to which plants depend on mycorrhizal fungi may influence the outcomes of competition mediated by CMNs. Chapter Four investigates how *A. gerardii* and *E. canadensis* associate with AM fungi. I examine how AM fungi affect various plant functional traits such as root-to-shoot ratios and nitrogen and phosphorus shoot concentrations across a gradient of phosphorus availabilities. This experimental design also allows me to quantify relatively each plant species' reliance on AM fungi for mineral nutrient uptake – its dependence on mycorrhizas – and the change in growth when colonized by mycorrhizas – its responsiveness to mycorrhizas. I then relate both dependence and responsiveness to how plant functional traits were affected, and I use the information provided by this experiment to determine an appropriate soil P supply at which AM fungi benefit both *A. gerardii* and *E. canadensis* for an interspecific competition experiment that I report in Chapter Five.

In Chapter Five, I investigate the roles of CMNs in the growth and survival of *A. gerardii* and *E. canadensis* seedlings in monocultures and mixed cultures with intact and severed CMNs. I examine if CMNs amplify intraspecific competition for both species, resulting in skewed size hierarchies with increased dominance and suppression. Because I found *A. gerardii* to be more dependent on AM fungi than *E. canadensis* in Chapter Four, Chapter Five also investigates if CMNs contribute to an increased competitive ability of *A. gerardii* over *E. canadensis* in mixture.

Figure 1.1. Rotatable container that is either rotated to sever CMN hyphae or not rotated to keep the CMN intact. Opening into container is covered with silk screen mesh (yellow) that allows CMN hyphae to grow into and out of the container while roots remain confined.

Chapter Two

Common mycorrhizal networks amplify size inequality in *Andropogon gerardii* monocultures

Summary

- Arbuscular mycorrhizal fungi can interconnect plant root systems through hyphal common mycorrhizal networks (CMNs) which may influence the distribution of limiting mineral nutrients among interconnected individuals, potentially affecting competition and consequent size inequality. Using a microcosm model system, we investigated whether the members of *Andropogon gerardii* monocultures compete via CMNs.
- We grew *A. gerardii* seedlings with isolated root systems in individual, adjacent containers while preventing, disrupting or allowing CMNs among them. Fertile soil was placed within the containers, which were embedded within infertile sand. We assessed mycorrhizas, leaf tissue mineral nutrient concentrations, size hierarchies, and the growth of nearest neighbors.
- Plants interconnected by CMNs had 8% greater colonized root length, 12% higher phosphorus and 35% higher manganese concentrations than plants severed from CMNs. Interconnected plants were 15% larger on average and had 32% greater size inequality as reflected by Gini coefficients than those with severed connections. Only with intact CMNs were whole plant dry weights negatively associated with those of their neighbors.

- In the absence of root system overlap, CMNs likely promote asymmetric competition belowground, thereby exaggerating size inequality within *A. gerardii* populations.

Background

Arbuscular mycorrhizal (AM) fungi can influence plant community composition. They may do so by increasing plant productivity (van der Heijden *et al.*, 1998) through improved host acquisition of limiting mineral nutrients, such as relatively immobile phosphorus. Mineral nutrient acquisition is enhanced by means of extensively branched networks of AM fungal hyphae that forage throughout the soil. Because AM fungi have little host specificity, such hyphal networks can interconnect and distribute mineral nutrients among many plant individuals regardless of species (Chiariello *et al.*, 1982); hence the networks are called 'common mycorrhizal networks' (CMNs). Interconnections via CMNs can influence seedling establishment (Eissenstat & Newman, 1990; Kytöviita *et al.*, 2003; Janouskova *et al.*, 2011), potentially by influencing competition for mineral nutrients (e.g., Hartnett *et al.*, 1993; West, 1996). For example, seedlings connected to large plants by CMNs may grow less than those not connected, which suggests resource pre-emption across CMNs (Allsopp & Stock, 1992; Hartnett *et al.*, 1993; Janouskova *et al.*, 2011). Little is known, however, about whether CMN interconnections influence competition among individuals that are similar in age and size.

CMNs can influence the distribution of mineral nutrients among networked plants (Chiariello *et al.*, 1982; Wilson *et al.*, 2006; He *et al.*, 2009), but whether CMNs confer similar access to mineral nutrients upon all interconnected plants or intensify competition among them is poorly understood. In an early field experiment that employed radioactive

phosphorus as a tracer, Chiariello *et al.* (1982) found that CMN interconnected individuals obtained phosphorus from a donor with shoot excised regardless of their species, size, or proximity to the donor. Chiariello *et al.* (1982) suggested that mineral nutrients taken up by CMNs are 'shared' among interconnected plants with no clear pattern as to which individuals obtain the most mineral nutrients. In contrast, in an *in vitro* culture experiment, roots that provided carbon to CMNs had twice as many arbuscules and received up to ten times more phosphorus than did carbon-limited roots (Lekberg *et al.*, 2010). This latter experiment suggests that CMNs might make competition for phosphorus among interconnected individuals asymmetric, with strong carbon-donor root systems disproportionately obtaining phosphorus from CMNs.

It once was thought that carbon flow across AM fungus CMNs might facilitate the growth of shaded, suppressed plants (Francis & Read, 1984; Grime *et al.*, 1987), but it now appears that with the exceptions of mycoheterotrophic and mixotrophic plant species, AM fungi simply may shunt carbon acquired from carbon-rich individuals to storage locations within the roots of suppressed individuals. While labeling studies have shown that carbon from one plant frequently can be detected in the root system of another (Fitter *et al.*, 1998), recent *in vitro* research suggests that the labeled carbon remains within intraradical fungus tissue (Pfeffer *et al.*, 2004). Moreover, AM fungi preferentially may transfer mineral nutrients to host individuals that are strong carbon suppliers, and hosts reciprocally may reward AM fungi that are strong phosphorus suppliers (Lekberg *et al.*, 2010; Hammer *et al.*, 2011). Hammer *et al.* (2011) showed that the AM fungus *Glomus intraradices* accumulated phosphorus when connected to a carbon-limited host, but did not when its host provided sufficient carbon to the fungus.

The fungus accumulated up to seven times more phosphorus in its spores and nine times more phosphorus in its hyphae under reduced host carbon than under carbon sufficient conditions (Hammer *et al.*, 2011). Another *in vitro* study suggested that carbon-limited plants attached to CMNs may serve as protected storage sites for AM fungi, because the fungi within their roots accumulate labeled carbon in the form of storage lipids (Lekberg *et al.*, 2010). Although the lack of shoots in these *in vitro* experiments precludes transpiration, shoot phosphorus sinks, and diurnal changes in carbon supply, such functional differences between carbon suppliers and carbon-poor whole plants interconnected by AM CMNs might exacerbate competition between large and small plants and accelerate the development of a size hierarchy.

Plants that tend to recruit near members of their own species (Harper, 1977) are likely to compete intraspecifically, and within such cohorts, competition can lead to the development of a size hierarchy. Dense seedling cohort size distributions change through time in a typical manner from symmetric, normal distributions shortly after germination to right skewed, asymmetric ones of many small and a few large plants as they age. Dissimilar germination times (Weiner, 1990), intrinsic natural variation in individuals' exponential growth rates (Koyama & Kira, 1956), and mortality as populations self-thin (Mohler *et al.*, 1978) can contribute to size inequality. Dominance and suppression, which are recognized as reflecting asymmetric competition, also can contribute to size differences within cohorts (Ford & Diggle, 1981; Weiner & Thomas, 1986; Weiner, 1990). Asymmetric competition is thought primarily to take place aboveground because large individuals preempt light acquisition by shading small individuals, thereby disproportionately obtaining the resource (Weiner, 1990). Alternatively, belowground

competition is considered predominantly symmetric (Weiner, 1990) with resources obtained in proportion to root system size. If CMNs functionally associate phosphorus supply with photosynthate provision by hosts, however, then CMNs might translate aboveground asymmetric competition for light into belowground asymmetric competition for phosphorus, thereby amplifying size inequality within a cohort.

Size inequality arising in consequence of competition usually increases with elevated plant density, because competitive interactions begin quickly at high density (Weiner & Thomas, 1986). Competitive interactions also can be accelerated by inoculation with AM fungi which may result in greater size inequalities in mycorrhizal populations than in those without mycorrhizas (Allsopp & Stock, 1992; Shumway & Koide, 1995; Facelli & Facelli, 2002). For example, Facelli *et al.* (2002) found mycorrhizas to increase size inequality of *Trifolium subterraneum*, and they attributed size inequality to asymmetric competition for P through mycorrhizas. Allsopp and Stock (1992) investigated density-dependent intraspecific competitive interactions among separate populations of *Otholobium hirtum* and *Aspalathus linearis* and attributed greater size inequality after mycorrhizal inoculation to early germinants experiencing rapid mycorrhiza formation and consequent growth that resulted in the suppression of other individuals. Although Shumway and Koide (1995) did not find mycorrhizas to contribute to size inequalities of whole plant dry weight, they did find that mycorrhizas contributed to inequality in fecundity.

In contrast to the preceding studies, others (Turner & Rabinowitz, 1983; Ayres *et al.*, 2006) have found neither elevated plant density nor mycorrhizal inoculation to increase size inequality within populations. Turner and Rabinowitz (1983) observed that

size distributions of crowded *Festuca paradoxa* did not differ from those of isolated plants. They attributed a lack of right skew at high density to minimal aboveground competition because of *F. paradoxa*'s erect, graminoid growth form, and they contended that competition principally was belowground and symmetric. Ayres *et al.* (2006) investigated the effects of both density and AM fungi on size distributions of *Plantago lanceolata* and found high density populations to have more equitable size distributions than those experiencing little competition, with mycorrhizas having no effect on size inequality. Ayres *et al.* (2006) speculated that belowground competition among dense, mycorrhizal *P. lanceolata* was symmetric and the distribution of resources among them was relatively uniform, similar to the conclusions of Turner and Rabinowitz (1983).

None of the previously cited studies directly investigated CMNs. Instead, when involving AM, they compared inoculated plants to those without mycorrhizas. Alternatively, we decided to inoculate all plants with AM fungi and to isolate root systems while preventing or allowing CMNs to form, and also repeatedly severing CMNs. We thereby assessed the effects of CMNs on size inequality of high density seedling populations of *Andropogon gerardii*, a strongly mycotrophic (Hartnett *et al.*, 1993; Hartnett & Wilson, 1999), dominant mid-western prairie grass. By extrapolating *in vitro* findings which hint that CMNs might unequally distribute mineral nutrients among networked plants (Pfeffer *et al.*, 2004; Lekberg *et al.*, 2010; Hammer *et al.*, 2011; Kiers *et al.*, 2011), we hypothesized that CMNs would mediate intraspecific competition and consequently amplify plant size inequality.

Materials and Methods

We constructed wooden box microcosms as model systems in which to examine intraspecific interactions among young *Andropogon gerardii* Vitman seedlings. We imposed three treatments upon different microcosms with three replicate microcosms for each treatment: intact CMNs, severed CMNs, and no CMNs (designated 'controls'). Every seedling was grown individually in a Ray Leach Cone-tainer (2.5 cm diameter x 12.1 cm length; 49 ml volume) which confined roots and prevented direct interactions among neighboring root systems. The control treatment comprised cone-tainers without modification, but cone-tainers that were intended to allow CMNs to form were modified by cutting two 2 x 5 cm slots in their opposite sides. We glued silk screen, nylon mesh cloth (40 μm pores) over both slots. Mycorrhizal fungus hyphae could grow freely through openings in the mesh, but roots could not. For the severed CMNs treatment, we manually rotated each cone-tainer through two complete revolutions twice a week, watering immediately after rotating to reestablish hydrological continuity. Neither were intact CMN cone-tainers nor controls rotated.

Microcosm establishment

The microcosms were 52 cm × 52 cm × 10 cm deep with plank sides and plywood bottoms. In each microcosm, we arranged cone-tainers in a twelve row by twelve column, square array (Fig. 2.1). To precisely position each cone-tainer, the plywood bottom was drilled (1.9 cm diameter holes) to accept the conical bottoms of the cone-tainers which thereby could drain externally. Surrounding the central one hundred (ten rows by ten columns) treatment plants were forty-four non-modified cone-tainers

intended to mitigate aboveground edge effects. Each cone-tainer was 2.5 cm away from each of its four nearest neighbors.

We filled cone-tainers with a homogenized soil mixture of two parts relatively infertile sandy flatwoods soil from Archbold Biological Station (27°11'2.41"N, 81°20'55.66"W) and one part University of Miami Gifford Arboretum (25°43'26.03"N, 80°16'47.48"W) fertile soil (Table 2.1). This soil mixture ensured relatively low mineral nutrient availability in order to encourage competition among seedlings. The soil mixture had pH 7.3, cation exchange capacity 0.039 meq g^{-1}, and bulk density 1.4 g ml^{-1}. In order to limit seedlings' ability to acquire mineral nutrients elsewhere than within cone-tainers, we filled the interstices between them with infertile silica sand (Table 2.1). The sand consisted of a 2:1 mixture of 30-65 grade medium sand and 6-20 grade fine sand from Surface Prep Supply Co, Miami, FL. The interstitial sand had pH 8.1, cation exchange capacity 0.009 meq g^{-1}, and bulk density 1.6 g ml^{-1}. The sand mixture in interstices tightly conformed to the cone-tainer sides and nylon mesh.

We inoculated every cone-tainer with AM fungi by collecting fine roots of *Stenotaphrum secundatum* (Walt.) Kuntze from a lawn in the Gifford Arboretum and then cutting the roots into 1–2 cm pieces by hand. We mixed these root pieces uniformly throughout the soil with which we filled the cone-tainers. This inoculum predominantly comprised *Sclerocystis rubiformis, Glomus clarum* and several unidentified AM fungal species of the genus *Glomus sensu lato*.

We fostered potential CMN formation through a pre-treatment during which we grew transplanted *A. gerardii* in all cone-tainers within the microcosms for eight weeks after inoculation. At that time, *A. gerardii* seeds (Easy Wild Flowers Nursery, Willow

Springs, MO) were sown directly into the cone-tainers which contained the predecessor, pre-treatment plants. We began counting 'days after germination' (DAG) when at least one germinant had appeared in every cone-tainer. Fourteen DAG, we clipped the pre-treatment plants below their basal meristems to eliminate them, and by similar clipping, left only one most vigorous germinant in each cone-tainer. Thus, the microcosms had a total of ten weeks of pre-treatment plant growth during which to establish interconnecting CMNs, similar to the time allowed for CMNs to establish in other studies (e.g., Johnson *et al.*, 2001; Walder *et al.*, 2012).

All nine microcosms were randomized on benches in a glasshouse at the University of Miami. We re-randomized them 42 DAG. Microcosms were watered daily by hand. A preliminary experiment suggested that *A. gerardii* might become nitrogen deficient in the soil mixture, so at 42 DAG, we began to add 10 ml of a 30 ppm KNO_3 solution to every individual cone-tainer once a week until harvest. Final ammonium and nitrate concentrations of the soil mixture are shown in Table 2.1.

Measurements and harvest

Beginning 14 DAG, we weekly measured the length of the longest leaf of each experimental seedling (excluding those in the buffer rows) from the leaf sheath to the leaf tip. We harvested at 94 DAG to prevent plants from becoming root-bound in the cone-tainers. We clipped shoots directly above the basal meristem and dried them to constant weight at 60 °C. We weighed the dried tissues for each individual before compositing them into eight groups, or samples, in order to ensure that sufficient tissue was available within each sample for element analysis by the Kansas State Agronomy Soil Testing Laboratory, Manhattan, KS. Rather than randomly assigning individuals to each of the

eight groups, we composited individuals according to whole plant dry weight. Each sample was compiled by rank ordering whole plant dry weights of all surviving plants from the three replicate microcosms per treatment, then dividing the rank ordered plants into eight nearly equal groups (called 'octiles') by number of individuals. When the number of surviving plants could not be divided evenly by eight, extra plants were distributed as uniformly as possible among the groups (e.g., four extra plants were distributed as one to every second group) which resulted in 31-35 plants per octile. Thus, for example, the upper (eighth) octile contained the largest 12.5 % of all plants within a treatment.

We removed root systems from the cone-tainers, rinsed them in gently running water over a 1 mm sieve, and preserved them in 50 % ethanol until we finished the harvest. Then, we blotted the root systems dry and weighed each root system to determine its total moist weight. After randomly removing a subsample of fine roots from each root system and preserving them in 50 % ethanol for later assessment of mycorrhizal colonization, we again weighed the remaining roots before placing them in an oven at 60 °C to dry to constant weight. We weighed the dried roots and used the dry weight to moist weight ratio to calculate the dry weights of entire root systems.

For assessment of percentage colonized root length by AM fungi, we composited root systems into the identical eight octiles per treatment as for shoot tissue. We cleared the roots in 10 % KOH at room temperature for 5 days, acidified them in 5 % HCl for 30 minutes and then placed them in 0.05 % trypan blue in lactoglycerol for 15 hours at room temperature to stain AM fungi. For each octile, we mounted 25 1–2 cm root segments on

microscope slides and scored mycorrhizal colonization by using the magnified gridline intersection method (McGonigle et al, 1990), examining 250 intersections per octile.

Statistical analyses

We analyzed differences in percentage colonized root length by AM fungi among treatments with a one-way analysis of covariance (ANCOVA) using octile position (first through eighth) as the covariate after examining heteroscedasticity with Levene's test. To compare the relationships between mean whole plant dry weight per octile and percent colonized root length among treatments, we used least squares linear regression. We also used ANCOVAs to detect treatment differences in mean leaf tissue element concentrations and contents (= [concentration × total whole leaf dry weight of an octile]/number of individuals in the octile). We used least squares linear regression to compare the relationships between whole plant dry weight per octile and concentrations of phosphorus and manganese. For both elements, because the control and severed CMNs treatments did not differ from one another, we combined their data for comparison to the intact CMNs treatment.

To compare plant growth throughout the experiment, we used a one-way, repeated measures analysis of variance (ANOVA) of longest leaf length per microcosm ($n = 9$) followed by a least significant difference (LSD) post-hoc test at $\alpha = 0.05$. We used Levene's test to examine heteroscedasticity. We similarly used one-way, repeated measures ANOVAs to investigate longest leaf length size hierarchy differences among treatments over time after establishing that the distributions of whole plant dry weights at harvest did differ among treatments (all replicates combined within treatments) with three pairwise Kolmogorov-Smirnov tests that we Bonferroni-corrected ($\alpha = 0.0166$). We

examined the following size hierarchy descriptors: standard deviation, Gini mean of differences, Gini coefficient and Lorenz coefficient of asymmetry for longest leaf lengths. We also calculated all these descriptors for above ground and whole plant dry weights, and tested them for differences among treatments by one-way ANOVAs and LSD post-hoc tests after using corrected Kolmogorov-Smirnov tests to establish that distributions differed. All the aforementioned statistical analyses were conducted with Statistix v. 9.0 (Analytical Software, Tallahassee, FL).

We calculated the size hierarchy descriptors Gini mean of differences, Gini coefficient and Lorenz coefficient of asymmetry for each microcosm with a Wolfram Mathmatica v. 8.0 (Champaign, IL) notebook (Damgaard 2000, http://mathworld.wolfram.com/GiniCoefficient.html). The Gini mean of differences is a measure of dispersion. It is the arithmetic average of the differences between all possible pairs of individuals within a population (Weiner & Solbrig, 1984). Gini coefficients represent the inequality of a distribution, with a minimum value of 0 indicating that all plants within a population are uniform in size and a maximum value of 1 indicating maximum inequality (Damgaard & Weiner, 2000). The Gini coefficient is based on the Lorenz curve that graphically represents the distribution of a population by ranking individuals from smallest to largest and then plotting the cumulative percentage of a size parameter against the cumulative percentage of individuals (Weiner, 1985). A population of uniformly sized individuals would produce a straight line, while inequality in the population causes the line to curve below the line of equality. Inequality within a population is reflected by the Lorenz asymmetry coefficient with values below or above 1.0 indicating right or left skew, respectively (Damgaard & Weiner, 2000).

We wished to examine our data for evidence of asymmetric competition such as dominance by large plants being associated with suppression of their neighbors (i.e., a negative correlation between plant and neighbor size). In order to summarize the patterns of co-variation among whole plant dry weights within our treatments, we performed separate, but procedurally identical, principal components analyses (PCA) for each treatment using the software PC-ORD v. 6.07 (McCune & Mefford, 2011). For each treatment, we examined associations between all surviving individuals (= 'targets') and PCA first axes derived from variance/covariance cross-products matrices. We used covariances in order to center variables while giving weight to divergent values. We rank ordered by dry weight the four nearest neighbors of each target and separately rank ordered the four diagonal neighbors of each target (see Fig. 2.1). We then used these rank categories for both distances as eight 'neighbor' variables. For the lowest ranks at both distances, however, dead plants resulted in zeros, and we eliminated three variables with more than 15 % zeros (among the 900 experimental plants of all treatments) because zeros were not informative for our analyses (i.e., zeros did not capture time of death and hence, did not reflect how long a neighbor and target might have competed before the neighbor's death). Five neighbor variables were retained: three nearest neighbors excluding only the smallest and two diagonal neighbors excluding the two smallest. We tested the significance of PCA axes by randomization tests with 999 runs. After performing PCA, we rotated each ordination so that Axis 1 was positively associated with the second-largest, nearest neighbor (the middle of the three nearest-neighbor variables), and then calculated Pearson correlations between the targets and Axis 1.

Results

Mycorrhizal colonization and leaf tissue composition

Mycorrhizal colonization differed among treatments ($F_{2,20}$ = 60.27, P <0.0001). *A.gerardii* seedlings with intact CMNs had the greatest mean colonization (71.1 %). Severing CMNs significantly reduced mean colonized root length to 65.2 %. Control, non-modified cone-tainers further significantly reduced colonization to 47.9 %. When we regressed mean whole plant dry weight by octile against percent colonized root length (Fig. 2.2) relativized by treatment mean, the regression was significant ($F_{1,22}$ = 8.20, P = 0.009). Although the slopes for the severed CMNs and control treatments did not differ ($F_{1,12}$ = 0.05, P = 0.827), the slope for the intact CMNs treatment differed from those of both the control and severed treatments ($F_{1,12}$ =9.67, P = 0.009, $F_{1,12}$ =12.29, P = 0.004, respectively).

Among the elements that we assessed (Fig. 2.3), only phosphorus and manganese concentrations differed significantly among treatments (P: $F_{2,20}$ = 18.05, P <0.0001; Mn: $F_{2,20}$ = 18.94, P <0.0001). All three treatments differed from one another by LSD post hoc test for both P and Mn, and seedlings with intact CMNs had the highest mean concentrations followed successively by the controls and those with severed CMNs (Fig. 2.3). All treatments combined showed a decrease in plant size with increasing P concentration ($F_{1, 22}$ = 5.90, P = 0.024), but showed an increase in plant size with increased Mn concentration ($F_{1, 22}$ = 13.10, P = 0.002). For P, severed CMNs and control treatments' slopes did not differ ($F_{1, 12}$ = 0.67, P = 0.430; Fig. 2.4a). When those two treatments were combined, however, their slope differed significantly from that of the intact CMNs treatment ($F_{1, 20}$ = 27.45, P = 0.0001). For Mn also, the severed CMNs and

control treatments' slopes did not differ ($F_{1, 12} = 0.48$, $P = 0.501$), but when combined, their slope was significantly exceeded by that of the intact CMNs treatment ($F_{1, 20} = 10.58$, $P = 0.004$). Mean element contents for intact CMNs shoots significantly exceeded those with severed CMNs for all elements except iron and zinc (Table 2.2) when not Bonferroni corrected.

Longest leaf lengths

At the first measurement 14 DAG, mean longest leaf length within microcosms did not differ significantly ($F_{2, 6} = 4.19$, $P = 0.073$) among treatments, but during the entire 94 day experiment, there was a treatment main effect on mean longest leaf length (Table 2.3; Fig. 2.5). Mean longest leaf lengths of seedlings with intact CMNs exceeded those of seedlings in the severed CMNs and control treatments. There also was a treatment × time interaction (Table 2.3, Fig. 2.5).

Treatment produced significant main effects on size hierarchy descriptors based on longest leaf lengths for standard deviation and Gini mean difference (Table 2.3). The intact CMNs standard deviation and Gini mean difference were greater than those of the severed CMNs and control treatments. Treatment interacted significantly with time for the Gini ratio and coefficient of variation (Table 2.3). The Lorenz asymmetry coefficient did not differ among treatments either as main effects or interaction with time. Figure 2.6 illustrates that while size hierarchy distributions did not differ among treatments 21 DAG, by 94 DAG the distribution of longest leaf lengths for plants with intact CMNs differed from those with severed CMNs (Kolmogorov-Smirnov two tailed test statistic = 0.21, $P = 0.0001$ and from the controls (Kolmogorov-Smirnov two tailed test statistic =

0.18, *P*=0.0002), but the latter two treatments' distributions did not differ from one another (Kolmogorov-Smirnov two tailed test statistic = 0.04, *P* = 1.00; Fig. 2.6).

Dry weights

At harvest, mean shoot dry weights differed significantly among treatments (Table 2.3) with the intact CMNs treatment exceeding the other two treatments which were identical. Size distributions of shoot dry weight for the intact CMNs treatment also differed from the severed CMNs (Kolmogorov-Smirnov two tailed test statistic = 0.31, *P*= 0.0001) and control treatments (Kolmogorov-Smirnov two-tailed test statistics = 0.25, *P* = 0.0001). Severed CMNs and control treatments size distributions did not differ from one another (Kolmogorov-Smirnov two tailed test statistic = 0.06, *P*= 0.717). Shoot dry weight standard deviations, Gini mean differences and Gini ratios all differed significantly among treatments (Table 2.3). The intact CMNs treatment had greater mean standard deviation and Gini mean differences than the severed CMNs and control treatments which did not differ from one another. For the Gini ratio of shoot dry weight, the intact CMNs treatment differed significantly from the severed CMNs treatment, but neither differed significantly from the controls which were intermediate (Table 2.3).

Whole plant dry weight size hierarchies differed among treatments similarly to shoot dry weights. The intact CMNs treatment differed significantly from both severed CMNs (Two-tailed Kolmogorov-Smirnov statistic = 0.31, *P* = 0.0001) and controls (Two-tailed Kolmogorov-Smirnov statistic = 0.24, *P*= 0.0001) which did not differ from one another after Bonferroni correction (Two-tailed Kolmogorov-Smirnov statistic = 0.13, *P* = 0.0270). Whole plant dry weight means, standard deviations, Gini mean differences and Gini ratios (Table 2.3) were affected significantly by treatments. For

those descriptors, the intact CMNs treatment exceeded the severed CMNs and control treatments which did not differ from one another (Table 2.3).

The PCA results for each treatment are summarized in Table 2.4. Randomization tests showed first axes to represent more variation than expected by chance for all three PCAs, but the second axis was significant only for the intact CMNs treatment. Rotation of ordinations maintained 90.4 %, 90.5 % and 99.0 % orthogonality between Axes 1 and 2 for control, severed CMNs and intact CMNs treatments, respectively. Rotation improved the percentage of variance represented by Axis 1 for the control and severed CMNs treatments, but resulted in Axis 2 representing more variance than Axis 1 for the intact CMNs treatment. Nevertheless, Axis 1 continued to represent 41.7 % to 59.3 % of the variance among neighbors for all treatments. As intended, rotation caused the second-largest, nearest neighbor to be strongly associated with Axis 1 in all three PCAs (Pearson's r ranged from 0.725 to 0.834). For the severed CMNs and intact CMNs treatments, however, the largest nearest-neighbors were the most strongly associated with Axis 1. For the controls, the third-largest, nearest neighbor was most strongly associated with Axis 1. Neighbors on the diagonals always had the weakest associations with Axis 1. Target plant correlation with the first axes of the three PCAs was positive for the control (r = 0.045) and severed CMNs (r = 0.171) treatments, but was negative (r = -0.220) for the intact CMNs treatment.

Discussion

Our study suggests that size hierarchy development within *A.gerardii* monocultures was influenced by hyphal interconnections in the form of CMNs. Although we lack direct evidence for CMNs, three results strongly support that CMNs formed in our microcosms:

(1) in spite of similar initial inoculation of all treatments, plants in the 'intact CMNs' treatment had the greatest colonized root length; (2) the most likely source of the potentially limiting mineral nutrient, Mn, was cone-tainer soil; and (3) competitive interactions suggested by a negative correlation between plant dry weights and those of their nearest neighbors only could be found under conditions in which CMNs, if formed, were likely to have remained intact.

Plants grown in the intact CMNs treatment had greater colonized root length than those with severed CMNs or controls, and of the latter two treatments, colonization of severed CMNs roots exceeded that of the controls (Fig. 2.2) even though the sand between cone-tainers was never inoculated. The elevated mycorrhiza formation by plants in both slotted cone-tainer treatments probably was a consequence of hypha spread among cone-tainers. Nevertheless, repeated severing somewhat retarded hypha spread among cone-tainers, resulting in less root colonization on average than when hyphae were not severed.

CMN influence on mineral nutrition

High root colonization of plants with intact CMNs by itself is unlikely to have accounted for that treatment's significantly highest mean plant dry weight because there was substantial overlap in both mean percent colonized root length and whole plant dry weights of small individuals in both of the slotted cone-tainers treatments (Fig. 2.2). Instead, the steep increase in dry weight of intact CMNs plants with small increases in percentage colonized root length suggests that hyphae interconnecting cone-tainers improved mineral nutrition and plant growth. Hyphal networks of the intact CMNs treatment likely extended into neighboring cone-tainers where they could obtain

disproportionate Mn for large host individuals. Although both cone-tainer soil and interstitial sand had low concentrations of available Mn, the concentration of Mn in the soil was seventeen times higher than that of the sand, and the total content of Mn in all cone-tainer soil potentially accessible to a CMN was approximately four times the total content of Mn in all of the sand within a microcosm (Table 2.1).

Plants in the intact CMNs treatment had significantly higher Mn and P concentrations than those in the other two treatments. As phosphorus concentration increased, however, mean whole plant dry weight per octile decreased (Fig. 2.4a), suggesting a dilution effect of plant dry weight (Johnson *et al.*, 1980; Estrada-Luna *et al.*, 2000). In contrast, as manganese concentration increased, intact CMNs mean whole plant dry weight per octile increased markedly (Fig. 2.4b), suggesting that manganese and not phosphorus was the primary growth-limiting mineral nutrient. Other studies also have found AM to increase host Mn concentrations (Ratti *et al.*, 2010; Baslam *et al.*, 2011). Ratti *et al.* (2010) grew *Catharanthus roseus* in treatments inoculated with different *Glomus* species versus controls lacking AM and found inoculation to increase plant Mn concentration, chlorophyll content and total plant dry weight. Baslam *et al* (2011) similarly found that AM increased the Mn concentration and improved the growth of *Lactuca sativa*. Manganese plays a key role in electron transport in photosynthesis (Raven *et al.*, 2005), so increased Mn might positively feedback to AM fungi by enhancing host provision of fixed carbon which could increase both root colonization and extraradical mycelium spread. In return, that might enhance the supply of mineral nutrients to hosts (Lekberg *et al.*, 2010; Kiers *et al.*, 2011). Such positive feedbacks

likely contributed to the greater inequality in size distributions that we found for plants with intact CMNs versus those lacking persistent AM fungus interconnections.

Size hierarchy development and competition

Our model system separated the effects of CMNs from those of root interactions and demonstrated that intact CMNs contribute to skewing of *A.gerardii* seedling size distributions. Every size hierarchy descriptor that we found to differ significantly among treatments whether derived from leaf lengths or dry weights, distinguished plants with intact CMNs from those of the other two treatments which consistently did not differ from one another. Although severing CMNs may have disrupted competition for water and mineral nutrients acquired via mass flow along hypha surfaces, the lack of statistically significant differences between the controls in non-modified cone-tainers and the severed CMNs treatment suggests that mass flow into modified cone-tainers after hypha severing did not substantially influence our results. It also suggests that even though glomeromycotan fungi can exhibit hyphal anastomoses (Giovannetti *et al.*, 1999) and wound healing (Gerdemann, 1955), rotation twice weekly was sufficient to disrupt CMN function.

Both longest leaf length distributions and dry weight distributions (Fig. 2.6) revealed size hierarchy differences, although leaf lengths were less sensitive in revealing those differences than were dry weights. Nevertheless, leaf length measurements revealed size hierarchy shifts through time, and in spite of not reflecting numbers of leaves, differences in longest leaf length distributions agreed well with both shoot and whole plant dry weight distribution differences. For example, among the size hierarchy descriptors based upon the Lorenz curve, the Gini mean of differences and Gini

coefficient revealed significant treatment main effects or interactions with time for both leaf lengths and dry weights. In contrast, Ayres *et al.* (2006) found no effect of mycorrhizas on whole plant dry weight Gini coefficients for dense *P. lanceolata*, but their dense monocultures comprised only 25 % as many individuals in total as ours, which might have made an advantage to just a few individuals hard to detect.

The greater inequality of size distributions that we found with intact CMNs reflected more large plants in the presence of intact CMNs than in the other two treatments. Across all three treatments, the smallest plants remained similar in size (Fig. 2.6) even as they continued to grow throughout the experiment (data not shown). Nevertheless, our target-neighbor PCAs indicate that intact CMNs did mediate a negative association between plants and their neighbors that did not appear when belowground interactions across CMNs either were prevented (controls) or disrupted (severed CMNs). Such suppression of their neighbors by dominant, large plants likely amplified the size inequality within the intact CMNs treatment, and furthermore, it suggests that across intact CMNs belowground competition was asymmetric.

Conclusions

We conclude that CMNs contributed to seedling population size inequality through positive feedbacks between mycorrhiza formation, mineral nutrient uptake and host growth. Although our model system was less controlled than recent root organ culture work, in contrast it provided physiological and environmental realism to common mycorrhizal network function. Our results are consistent with studies that have found AM fungi to contribute to size inequality as a result of differences in mycorrhizal colonization (Allsopp & Stock, 1992) and rapid uptake of mineral nutrients by AM fungi

(Allsopp & Stock, 1992; Shumway & Koide, 1995; Facelli & Facelli, 2002). In our model system, even though the largest plants of the control and severed CMNs treatments had the highest mean colonized root lengths of their treatments, they neither attained the size of the largest plants with intact CMNs nor were they associated with small neighbors. Instead, with intact CMNs, the negative association between targets and neighbors suggests competitive dominance and suppression uniquely across CMNs (Allsopp & Stock, 1992; Janouskova *et al.*, 2011).

Our study prevented direct root system interactions among plants and thereby may have unmasked the contribution of CMNs to belowground competition. Both Turner and Rabinowitz (1983) and Ayres *et al.* (2006) failed to find differences in size hierarchies, which they attributed to little or no aboveground asymmetric competition and to symmetric competition belowground. In our study too, aboveground competition among *A. gerardii* seedlings was unlikely to have contributed to the observed differences in size hierarchies among treatments because of a graminoid growth form combined with a relatively small difference in mean longest leaf length among treatments (i.e., only about 3.5 cm at the time of maximum difference). In spite of this lack of strong, differential, aboveground effects, however, we found that size hierarchies did differ among treatments. It may be that direct, symmetric competitive interactions among root systems – which we prevented – tend to conceal the asymmetric effects of CMNs.

In nature, plants likely are interconnected by CMNs, but neighboring root systems often overlap. Although many factors may influence whether belowground or aboveground, symmetric or asymmetric competition predominates among plants, our study suggests that CMNs can promote asymmetric competition belowground. Thereby,

CMNs may have consequences for plant fitness (Weiner, 1990), natural selection and community assemblage for plant species that recruit in dense, monospecific seedling stands.

Table 2.1. Soil mineral nutrient concentrations and contents of the sand mixture surrounding cone-tainers in a microcosm and for the soil within the 100 cone-tainers in a microcosm

	Concentration (ppm)		Content (mg)	
Soil characteristic*	Interstitial sand	Soil (within cone-tainers)	Interstitial sand	Soil (within cone-tainers
Ammonium	2.8	4.9	89	34
Nitrate	1.0	15.6	32	107
Olsen phosphorus	1.2	6.5	38	45
Potassium	7.0	46.0	221	316
Calcium	155	1505	4,902	10,327
Magnesium	25.0	68.0	791	467
Manganese	0.1	1.7	3	12

* Samples were analyzed by the Kansas State University Soil Testing Laboratory, Manhattan, KS, U.S.A.

Table 2.2. ANCOVA main effects (F statistics and associated probabilities, P) based upon mean element content in shoot tissues of individuals in intact CMNs, severed CMNs and control treatments

Element	Mean Content (mg)	$F_{2,1}$	P
Manganese	Intact: $0.00476^{\dagger A}$	**7.25***	**0.0043**
	Severed 0.00210^{B}		
	Control 0.00288^{B}		
Phosphorus	Intact 0.00354^{A}	**10.84**	**0.0006**
	Severed 0.00230^{B}		
	Control 0.00263^{B}		
Nitrogen	Intact 0.0176^{A}	**3.65**	**0.0446**
	Severed 0.0131^{B}		
	Control 0.0145^{AB}		
Potassium	Intact 0.0215^{A}	**6.22**	**0.0080**
	Severed 0.0145^{B}		
	Control 0.0160^{B}		
Calcium	Intact 0.0155^{A}	**4.77**	**0.0202**
	Severed 0.0124^{B}		
	Control 0.0124^{B}		
Magnesium	Intact 0.00740^{A}	**6.45**	**0.0069**
	Severed 0.00545^{B}		
	Control 0.00559^{B}		
Iron	Intact 0.0197^{A}	0.73	0.4964
	Control 0.0163^{A}		
	Severed 0.0172^{A}		
Zinc	Intact 0.00796^{A}	2.96	0.0748
	Severed 0.00639^{A}		
	Control 0.00703^{A}		

* Significant differences are shown in bold

† Within each descriptor, treatment means (N=8) followed by the same letter do not differ significantly ($P \leq 0.05$) by least significant difference post-hoc test

Table 2.3. One-way, repeated measures ANOVA main effects and interactions with time (*F* statistics and associated probabilities, *P*) based upon *Andropogon gerardii* longest leaf length measurements 94 days after germination (DAG), and one-way ANOVA main effects based upon shoot and whole plant dry weights at harvest (94 DAG) for descriptors of size hierarchies

		Longest leaf length (cm)			Shoot dry weight (g)		Whole plant dry weight (g)	
Descriptor	Treatment	Value	Main effect ($F_{2,6}$; *P*)	Interaction with time ($F_{20,60}$; *P*)	Value	Main effect ($F_{2,6}$; *P*)	Value	Main effect ($F_{2,6}$; *P*)
Mean	Intact	$15.401^{A\dagger}$	**12.35*; 0.0075**	**1.97**; **0.0227**	0.122^{A}	**26.51**; **0.0011**	0.414^{A}	**10.93**; **0.0100**
	Severed	13.647^{B}			0.099^{B}		0.353^{B}	
	Control	13.614^{B}			0.099^{B}		0.369^{B}	
Standard Deviation	Intact	4.200^{A}	**8.63**; **0.0172**	1.38; 0.1706	0.044^{A}	**12.28**; **0.0076**	0.163^{A}	**18.06**; **0.0029**
	Severed	3.169^{B}			0.023^{B}		0.090^{B}	
	Control	3.157^{B}			0.025^{B}		0.089^{B}	
Gini mean difference	Intact	4.656^{A}	**8.36**; **0.0184**	1.58; 0.0871	0.046^{A}	**12.51**; **0.0072**	0.170^{A}	**27.03**; **0.0010**
	Severed	3.533^{B}			0.025^{B}		0.098^{B}	
	Control	3.553^{B}			0.028^{B}		0.097^{B}	
Gini coefficient	Intact	0.173	2.82; 0.1370	**2.02**; **0.0192**	0.191^{A}	**6.48**; **0.0316**	0.205^{A}	**17.99**; **0.0029**
	Severed	0.168			0.126^{B}		0.139^{B}	
	Control	0.146			0.141^{AB}		0.131^{B}	
Lorenz asymmetry	Intact	0.871	0.12; 0.8910	0.85; 0.6512	0.978	0.10; 0.9033	1.011	0.46; 0.6517
	Severed	0.866			0.956		0.967	

Control	0.881	0.970	1.005

* Significant differences are shown in bold

† Within each descriptor, treatment means (N = 3) followed by the same letter do not differ significantly ($P \leq 0.05$) by least significant difference post-hoc test

Table 2.4. Principle components analysis (PCA) proportion of variance represented by the first two axes (r^2 = coefficient of determination), correlations (Pearson's r) with Axis 1 for the five PCA summarized *Andropogon gerardii* neighbor variables, and the correlation between Axis 1 and target *A. gerardii* whole plant dry weights for control, severed common mycorrhizal networks (CMNs) and intact CMNs treatments

Treatment (N)	r^2 (*P**)		Pearson's correlation (r) with Axis 1†					
	Axis 1	Axis 2	Largest, nearest neighbor	2nd largest, nearest neighbor	3rd largest, nearest neighbor	Largest, diagonal neighbor	2nd largest, diagonal neighbor	Targets
Control (280)	0.473 (0.001)	0.387 (0.054)	0.639	0.834	0.866	0.211	0.421	0.045
Severed CMNs (249)	0.593 (0.001)	0.315 (0.876)	0.834	0.807	0.738	0.503	0.581	0.171
Intact CMNs (276)	0.417 (0.001)	0.505 (0.001)	0.965	0.725	0.384	-0.081	0.069	-0.220

* The probability is from a randomization test with 999 runs

† Axis 1 was rotated to maximize congruence with the second-largest, nearest neighbor for each PCA

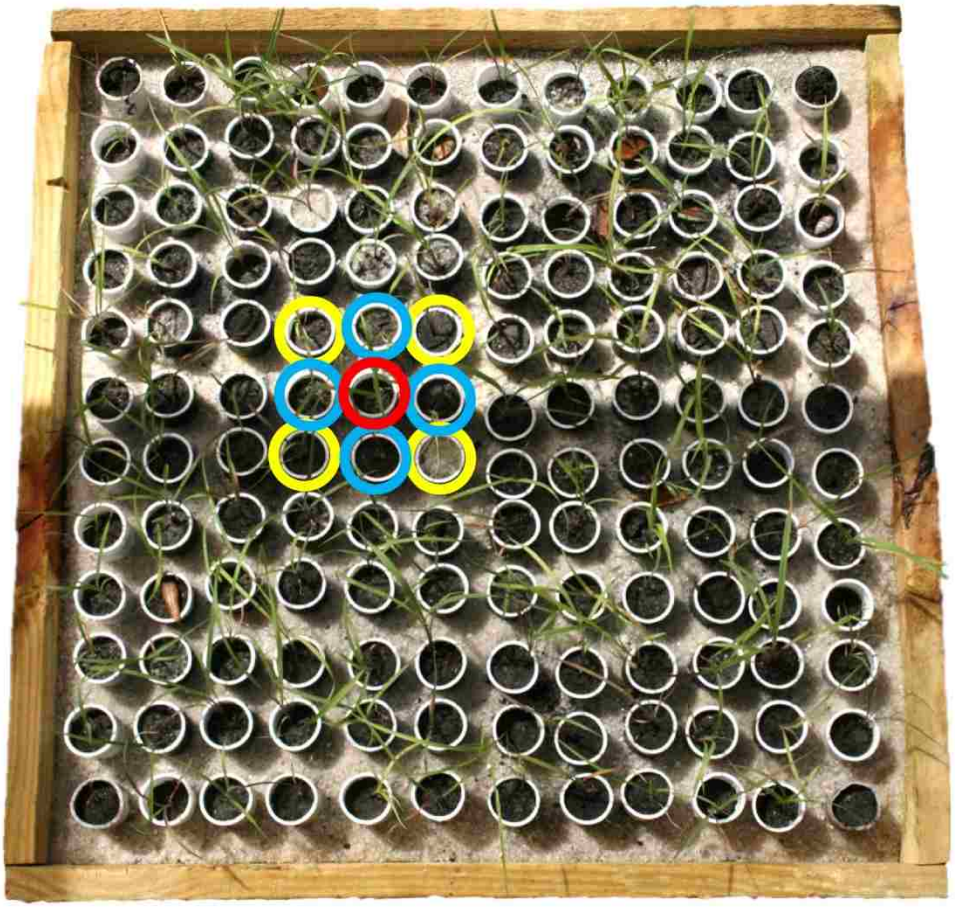

Figure 2.1. Microcosm (52 cm × 52 cm × 10 cm deep) with 144 *Andropogon gerardii* individuals growing individually in cone-tainers. The outermost 44 plants were intended to mitigate aboveground edge effects and were neither treated nor measured; only the central 100 cone-tainers (10 rows × 10 columns) were treated and assessed. All cone-tainers were filled with a soil mixture, but the interstices between cone-tainers were filled with infertile sand. For principal components analyses, each individual was considered to be a target plant (red circle) potentially associated with four nearest neighbors (blue circles) and four diagonal neighbors (yellow circles).

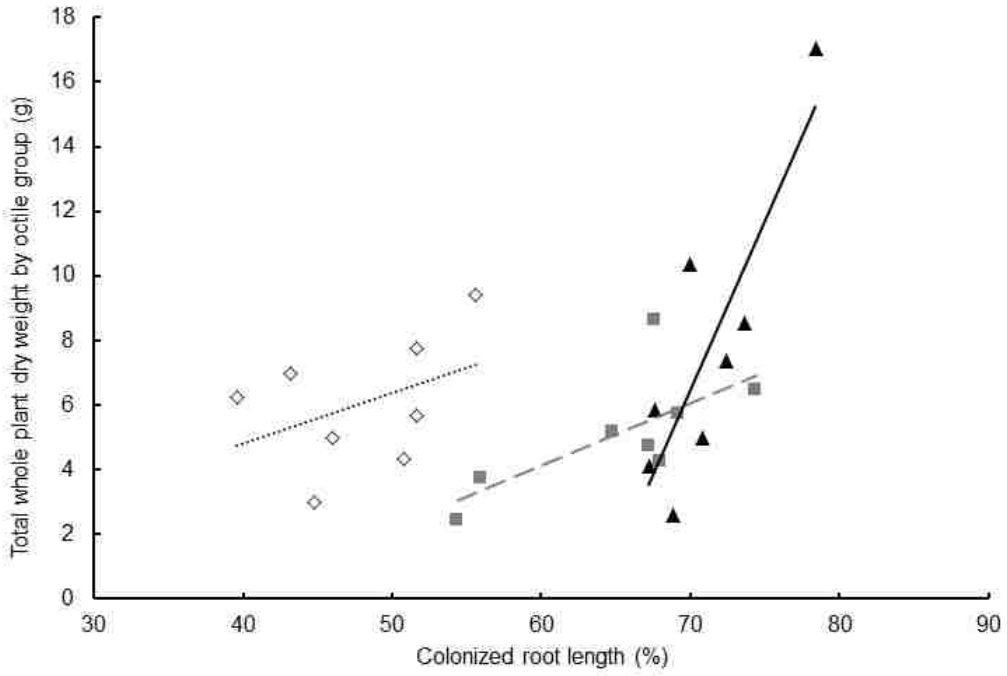

Figure 2.2. *Andropogon gerardii* mean whole plant dry weight (DW; g) per octile group versus percent colonized root length (%CRL) for control (open diamonds), severed common mycorrhizal networks (CMNs; shaded squares) and intact CMNs (solid triangles) treatments. The slopes of the linear regressions differed among treatments ($F_{1, 18} = 7.20$, $P = 0.005$) with the intact CMNs treatment (DW = 1.05 × %CRL - 66.9) differing significantly from both severed CMNs (DW = 0.19 × %CRL – 7.4) and control treatments (DW = 0.16 × %CRL – 1.5) which did not differ from one another ($F_{1,12} = 0.05$, P=0.083).

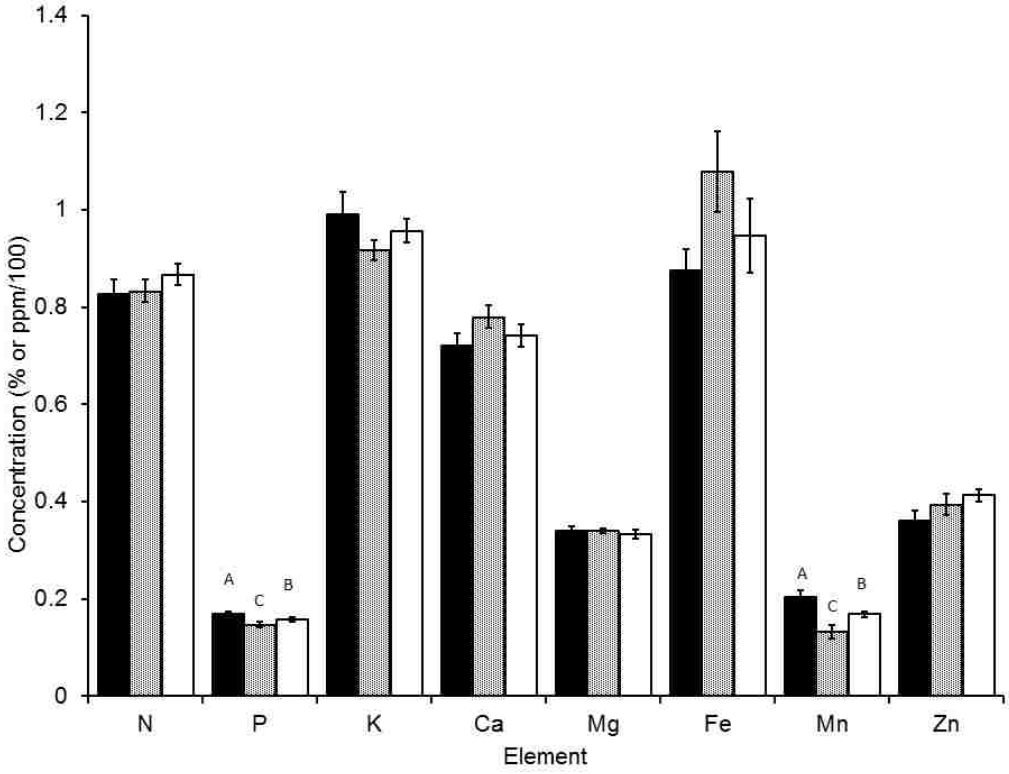

Figure 2.3. Mean (± SE) foliar element concentrations (% N, P, K, Ca, Mg and ppm/100 for Fe, Mn, Zn) of *Andropogon gerardii* individuals in intact common mycorrhizal networks (CMNs; solid bars), severed CMNs (shaded bars) and control (open bars) treatments. Bars topped by different letters within an element differ significantly by least significant difference post-hoc test at $P \leq 0.05$; bars within elements that are not topped by letters do not differ significantly by ANCOVA.

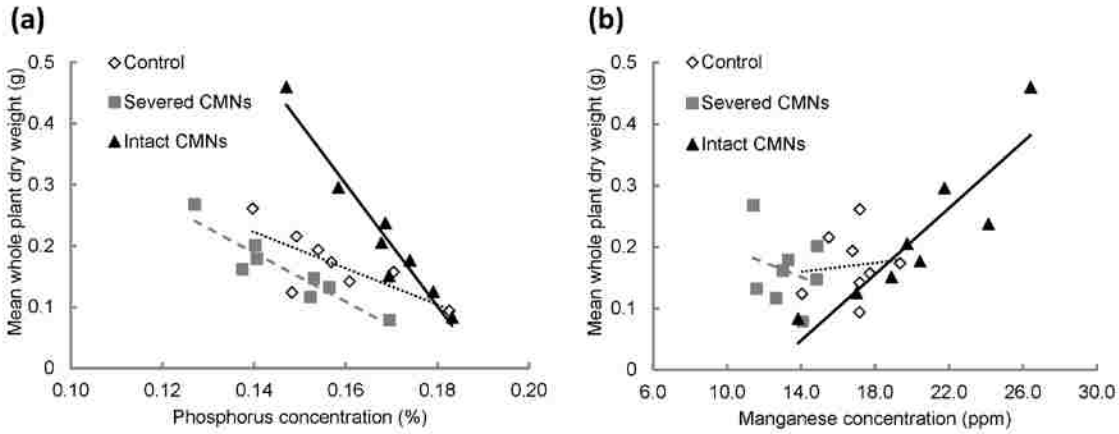

Figure 2.4. Mean whole plant dry weight (DW; g) per octile group versus phosphorus concentration (%; a) and manganese concentration (ppm; b) in shoot tissues of *Andropogon gerardii* for control (open diamonds), severed common mycorrhizal networks (CMNs; shaded squares) and intact CMNs (solid triangles) treatments. (a) The linear regression slopes of severed CMNs and control treatments did not differ ($F_{1, 12}$ = 0.67, P = 0.430). When those two treatments are combined, their slope (DW = -2.78 × [P] + 0.59) differed significantly ($F_{1, 20}$ = 27.45, P = 0.0001) from that of the intact CMNs treatment (DW = -9.95 × [P] + 1.89). (b) The linear regression slopes of severed CMNs and control treatments did not differ ($F_{1, 12}$ = 0.48, P = 0.501), but when combined their slope (DW = 0.0006 × [Mn] + 0.15) differed significantly ($F_{1, 20}$ = 10.58, P = 0.004) from that of the intact CMNs treatment (DW = 0.03 × [Mn] – 0.33).

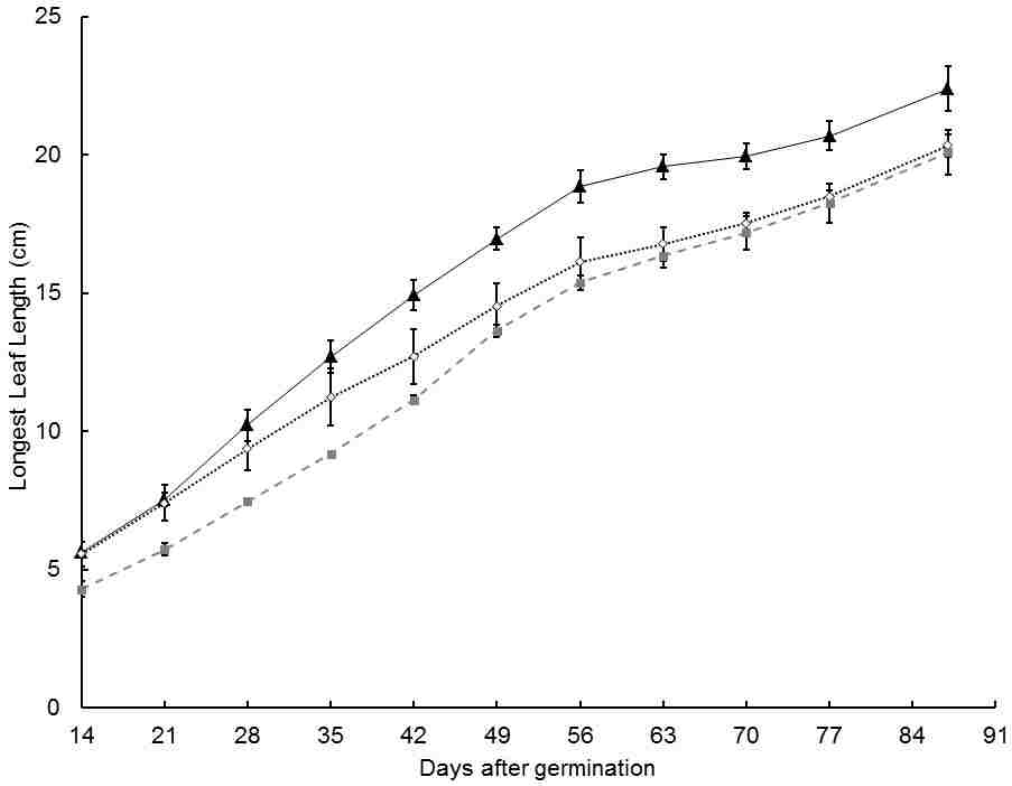

Figure 2.5. Mean (n = 3 for each treatment) longest leaf length (±SE) of *Andropogon gerardii* versus days after germination in and intact common mycorrhizal networks (CMNs; solid triangles), control (open diamonds) and severed CMNs (shaded squares) treatments. Mean longest leaf length did not differ significantly among treatments at the start of the experiment (One-way ANOVA, $F_{2,6} = 4.19$, $P = 0.073$).

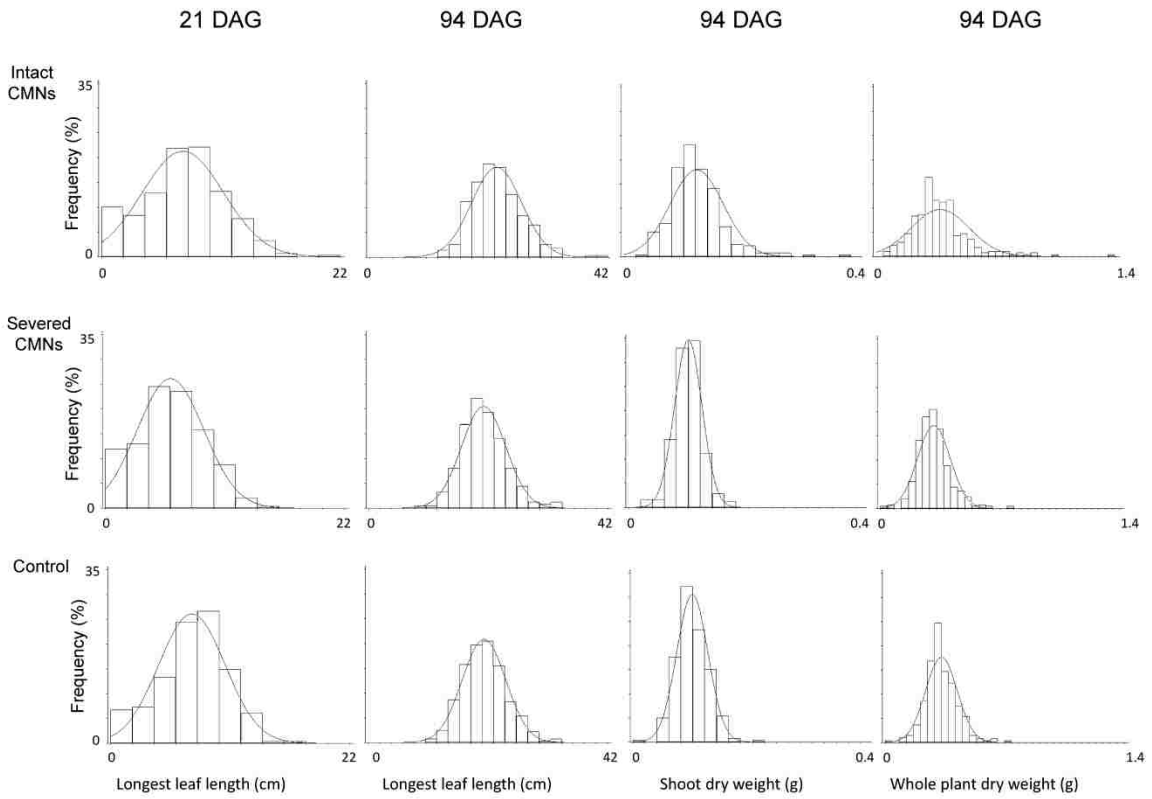

Figure 2.6. Size frequency distributions of *Andropogon gerardii* seedlings based on longest leaf lengths 21 and 94 days after germination (DAG), shoot dry weight and whole plant dry weight at harvest 94 DAG for control, severed and intact common mycorrhizal networks (CMNs) treatments. Superimposed normal curves facilitate visual comparison but do not imply that the distributions are normal. By 94 DAG, the distribution of longest leaf lengths for plants with intact CMNs differed from those with severed CMNs (Kolmogorov-Smirnov two tailed test statistic = 0.21, P = 0.0001) and from the controls (Kolmogorov-Smirnov two tailed test statistic = 0.18, P = 0.0002), but the latter two treatments' distributions did not differ from one another (Kolmogorov-Smirnov two tailed test statistic = 0.04, P = 1.00). At harvest, shoot and whole plant dry weight of plants

with intact CMNs significantly differed from both those with severed CMNs and controls (Shoot dry weight: Kolmogorov-Smirnov two-tailed test statistic = 0.31 $P = 0.0001$, and Kolmogorov-Smirnov two-tailed test statistic = 0.25, $P= 0.0001$ respectively; Whole plant dry weight: Kolmogorov-Smirnov two-tailed statistic = 0.31, $P = 0.0001$, Kolmogorov-Smirnov two-tailed statistic = 0.24, $P = 0.0001$, respectively) the latter two of which did not differ significantly from one another after Bonferroni correction (Shoot dry weight: Kolmogorov-Smirnov two tailed test statistic = 0.06, $P = 0.717$; Whole plant dry weight: Two-tailed Kolmogorov-Smirnov statistic = 0.13, $P = 0.0270$).

Chapter Three

Common mycorrhizal networks amplify competition by preferential mineral nutrient allocation to large host plants

Summary

- Arbuscular mycorrhizal (AM) fungi interconnect plants in common mycorrhizal networks (CMNs) which can amplify competition among neighbors. Amplified competition might result from the fungi supplying mineral nutrients preferentially to hosts that abundantly provide fixed carbon, as suggested by research with organ-cultured roots. We examined whether CMNs supplied ^{15}N preferentially to large, non-shaded, whole plants.
- We conducted an intraspecific target-neighbor pot experiment with *Andropogon gerardii* and a suite of AM fungi in intact, severed, or prevented CMNs. Neighbors were supplied with ^{15}N, and half of the target plants were shaded.
- Intact CMNs increased target plant dry weight, but also intensified competition and increased size inequality. Shading decreased target weight, but shaded plants in intact CMNs had mycorrhizal colonization similar to that of sunlit plants. AM fungi in intact CMNs preferentially allocated ^{15}N acquired from neighbors' substrate to sunlit, large target plants.
- Sunlit target plants with intact CMNs acquired as much as 27 % of their nitrogen from the vicinity of their neighbors, but shaded targets did not. These results suggest that multiple arbuscular mycorrhizal fungus species in CMNs preferentially provide mineral nutrients to those conspecific host individuals best able to provision them with fixed carbon, thereby potentially amplifying asymmetric competition belowground.

Background

In nature, individual plants of different species and sizes are interconnected belowground by arbuscular mycorrhizal (AM) fungus hyphal networks called "common mycorrhizal networks" (CMNs). Because AM fungi associate with most plant species (Smith & Read, 2008), CMNs can have large impacts on ecosystems by being sinks for carbon, by altering mineral nutrient movement (Treseder & Allen, 2000; Read & Perez-Moreno, 2003; Wilson *et al.*, 2009), by affecting soil structure (Rillig, 2004), and by influencing plant community composition (van der Heijden *et al.*, 1998; Rillig, 2004; Wilson *et al.*, 2009). While extending beyond root mineral nutrient depletion zones (Li *et al.*, 1991; Marschner & Dell, 1994), AM fungi can provide host plants with as much as 80 % and 25 % of their P and N requirements, respectively. In return, host plants provide 4–20 % of their net total fixed carbon to AM fungi (Jakobsen & Rosendahl, 1990; Douds *et al.*, 2000). The stoichiometry of exchange between host plants and AM fungi is influenced by soil fertility (Johnson *et al.*, 1997; Janos, 2007; Olsson *et al.*, 2010), AM fungus species (Kiers *et al.*, 2011), and neighboring host plants of different sizes (Janouskova *et al.*, 2011; Merrild *et al.*, 2013; Weremijewicz & Janos, 2013) or species (Walder *et al.*, 2012; Merrild *et al.*, 2013). How CMNs acquire carbon and partition mineral nutrients among neighboring plants likely influences both plant competition and facilitation (Connell, 1983).

CMNs have been demonstrated to affect plant population structure by influencing belowground interactions. Weremijewicz and Janos (2013) found *Andropogon gerardii* populations with intact CMNs had more skewed size distributions than populations with severed or no CMNs. Large plants were surrounded by small neighbors principally when

connected to them by CMNs, suggesting that CMNs preferentially may have benefited the large individuals at the expense of the small plants. Weremijewicz and Janos (2013) suggested that CMNs provided Mn, the probable growth-limiting mineral nutrient in their experiment, preferentially to large plants. In another experiment, Merrild *et al.* (2013) found that clipping the shoots of large plants reduced growth suppression and increased P uptake by interconnected small neighbors 6.5-fold. It is possible that CMNs amplify competition by promoting mineral nutrient transfer from the vicinity of small plants to large plants, thereby affecting the growth of neighbors and influencing community composition.

How CMNs affect plant interactions may depend upon the species of hosts and fungi involved. Walder *et al.* (2012) found that sorghum and flax compete under unequal "terms of trade" within CMNs formed by either *Glomus intradices* or *Glomus mosseae*. Using P, N, and carbon isotope tracing, Walder *et al.* (2012) showed that although sorghum provided AM fungi with large amounts of carbon, sorghum did not predominate in receiving P or N when competing with flax in a CMN. Instead, flax, which supplied less carbon than sorghum, received 94 % of ^{32}P and 80 % of ^{15}N from the CMN. These nutrient dynamics and plant interactions, however, might have been affected by Walder et al.'s (2012) use of single AM fungus species in their experiments. AM fungus species differ in their rates of mineral nutrient exchange with host plants (Kiers *et al.*, 2011), and hosts colonized by several species of AM fungi under carbon-stressed conditions can preferentially distribute carbon to the most beneficial fungus species (Zheng *et al.*, 2015). Consequently, CMN formation by a suite of AM fungus species, as most likely in nature, may be needed to fully elucidate the role of CMNs in plant interactions.

Although recently questioned (Walder & van der Heijden, 2015), *in vitro* research with root organ cultures has led to the hypothesis that within CMNs, exchange between AM fungi and host plants involves "reciprocal rewards" in which those roots supplying the most carbon to AM fungi garner the greatest mineral nutrient transfer from them (Lekberg *et al.*, 2010; Hammer *et al.*, 2011; Kiers *et al.*, 2011; Fellbaum *et al.*, 2014). Organ-cultured roots supplying abundant carbon have been demonstrated to receive up to ten times more P than those supplying little carbon to AM fungi (Lekberg *et al.*, 2010). Moreover, carbon-limited roots may serve AM fungi predominantly as storage sites (Lekberg *et al.*, 2010; Hammer *et al.*, 2011). If "rewards" indeed are reciprocal between AM fungi and intact host plants, such exchanges might intensify plant competition.

More mycorrhiza research has investigated the exchange of carbon for P than for N. Nevertheless, AM fungi can take up significant amounts of inorganic nitrogen and provide it to their host plants (e.g., Ames *et al.*, 1983; Johansen *et al.*, 1993; He *et al.*, 2003; Govindarajulu *et al.*, 2005; He *et al.*, 2009). Mycorrhizal fungi can transfer nitrogen supplied as either $^{15}NH_4^+$ or $^{15}NO_3^-$ to host plant tissues (Ames *et al.*, 1983; Johansen *et al.*, 1993), but they take up NH_4^+ preferentially to NO_3^- in order to minimize energy expenditure (Johansen *et al.*, 1996; Toussaint *et al.*, 2004; Fellbaum *et al.*, 2012). Ames *et al.* (1983) found the amount of ^{15}N transferred via extraradical mycelium to host plants was correlated with mycorrhizal colonization. Other studies have confirmed that CMNs can transfer ^{15}N between the same and different plant species (He *et al.*, 2003; He *et al.*, 2009). Nevertheless, whether N transfer to hosts from CMNs is governed by "reciprocal rewards" has not been determined.

Our objective was to investigate whether CMNs affect competition among similarly-aged *A. gerardii* seedlings by differential allocation of ^{15}N, thereby potentially elucidating a general mechanism that might underlie the findings of Weremijewicz and Janos (2013). To differentiate plant size *per se* from potential carbon supply to AM fungi, we included a partial-shade treatment. We hypothesized that: 1) intact CMNs would maximize competition between target plants and their neighbors, 2) shading of target plants would reduce root colonization by AM fungi, thereby potentially limiting the provision of ^{15}N by CMNs, and 3) intact CMNs would supply the most ^{15}N, and possibly other mineral nutrients, to full-sun, large target plants that likely had the greatest capacity to provide fixed carbon to AM fungi.

Materials and Methods

We grew *Andropogon gerardii* Vitman seedlings in a fully-factorial, target plant experiment, with CMN treatment and shading as factors. In each pot, a central, target plant was surrounded by six conspecific, equally-spaced neighboring plants. All plants in a pot received one of three CMN treatments: intact CMNs, severed CMNs, or no CMN. In half of the pots, the target plants alone were shaded, while the rest received ambient sun. Each treatment had 20 replicate pots, for a total of 120 pots.

Similar to Weremijewicz and Janos (2013), all plants were grown in Ray Leach Cone-tainers (2.5 cm diameter × 12.1 cm length; 49 ml volume; Tangent, Oregon, USA). Plants in the intact and severed CMNs treatments were grown in modified cone-tainers (Fig. 3.1a), which had two 2 × 5 cm slots opened in their opposite sides over which two layers of fabric were glued externally. The layers of fabric consisted of a nylon silk screen cloth (40 μm pores) root barrier beneath water-proof Gore-tex (Newark, DE,

USA) cloth. Gore-tex prevents the movement of liquid water but allows mycorrhizal hyphae to grow freely through the cloth (Mader *et al.*, 1993). Plants in the "no CMN" treatment were grown in solid cone-tainers which completely precluded CMN establishment. We manually rotated each cone-tainer in the severed CMNs treatment through a complete revolution once a week, watering immediately after rotating to eliminate any gaps between the cone-tainer and surrounding sand. Intact and no CMN cone-tainers were not rotated.

Pot set-up

Plastic pots (15.5 cm diameter × 13.5 cm height) were modified to keep cone-tainers equally spaced while allowing external drainage. We removed the bottoms of pots and replaced them with circular pieces of 3.6 cm-thick Styrofoam completely penetrated by seven, 1.9 cm diameter holes that fit the conical bottoms of cone-tainers (Fig. 3.1b). Six holes were spaced 1.2 cm apart around the circumference of an 11 cm diameter circle, and the seventh, for the target plant, was centered. The space surrounding cone-tainers was filled with an acid-washed, nutrient-poor silica sand mixture (Table 3.1; Fig. 3.1b). This mixture was acid washed for 24 hrs in 50 % HCl and rinsed with 100 °C deionized water before being used to fill the pots. The sand comprised a 2:1 mixture of 30–65 grade medium sand and 6–20 grade fine sand from Florida Silica Sand (Miami, FL, USA). Cone-tainers were filled with a soil mixture of two parts infertile sandy flatwoods soil from Archbold Biological Station (Venus, FL, USA) and one part University of Miami Gifford Arboretum topsoil (Coral Gables, FL, USA; Table 3.1).

We inoculated the soil within cone-tainers with several species of AM fungi using pieces of fine roots of *Stenotaphrum secundatum* (Walt.) Kuntze lawn grass collected

from the Gifford Arboretum. The roots contained *Sclerocystis rubiformis* Gerd. & Trappe, *Glomus clarum* Nicholson & Schenck (now *Rhizophagus clarus* Walker & Schußler) and several species of *Glomus sensu lato* (Weremijewicz and Janos 2013). Freshly-collected roots were cut into 1–2 cm pieces by hand and mixed uniformly throughout the entire soil volume before the cone-tainers were filled. To avoid inoculum limitation, we additionally inoculated each cone-tainer with 1 mL (ca. 333 spores/mL) of a commercial inoculant (Mycorrhizal Applications, Grants Pass, Oregon, USA) by pipetting the slurry of spores and root fragments into a 4 cm deep planting hole. The slurry contained four "*Glomus*" species (*G. intraradices* Schenck & Smith [now *Rhizophagus intraradices* (Krüger *et al.*, 2012)], *G. etunicatum* Becker & Gerd. [now *Claroideoglomus etunicatum* Walker & Schußler]*, G. mosseae* Gerd. & Trappe [*now Funneliformis mosseae* Walker & Schußler], and *G. aggregatum* Schenck & Smith).

We grew *A. gerardii* (Everwilde Farms, Sandcreek, WI, USA) plants as a "pre-treatment" in all cone-tainers for eight weeks with none rotated to allow hyphae to spread and establish CMNs among slotted cone-tainers. Following pre-treatment, we again seeded cone-tainers with *A. gerardii*, and after all cone-tainers had a germinant, we clipped the pre-treatment plants below their basal meristems to eliminate them. We similarly clipped excess germinants to leave one healthy individual in each cone-tainer. Plants were grown in a shade house with 30 % shade at the Fairchild Tropical Botanic Garden Research Center (Coral Gables, Florida, USA) from May to July 2014. Over the course of the experiment, the average temperature was 27.7 °C (± 1.2 SD °C) and average monthly rainfall was 1.1 cm (± 1.8 SD cm).

Fertilization and target plant shading

Eleven days after germination, we began fertilizing all cone-tainers once per week. All plants received 5 mL of modified Hewitt's solution containing 1.5 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM Ferric Citrate, 0.03 mM H_3BO_3, 0.011 mM $MnSO_4$, 0.002 mM $ZnSO_4$, 0.0003 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_20$, and 0.1 mM $CuSO_4 \cdot 5H_20$. The solution contained 20 ppm P in the form of NaH_2PO_4 for the first two weeks of fertilization and then 10 ppm P thereafter. In an attempt to make N limiting, all cone-tainers received only the trace amount of ammonium in the molybdate salt and an additional 15 ppm N as KNO_3. We altered the latter to 7.5 ppm N as each of NH_4Cl and KNO_3 46 days after germination. In each pot, only "neighbor" cone-tainers received 0.5 % ^{15}N-enriched NH_4Cl and KNO_3 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) in solution together with unenriched NH_4Cl and KNO_3, while targets plants received only non-enriched fertilizer. We did not water plants for two days after each fertilization.

We began shading half of the target plants one month after germination. We made shade tubes (4 cm diameter × 23 cm height) to place over target plants by attaching a woven, beige polyester shade cloth that blocked 84–90% of UV rays (Coolaroo™, Altamonte Springs, FL, USA) around cylindrical, semi-rigid seedling protection tubes (Forestry Suppliers, Jackson, MS, USA; Fig. 3.1c). The top of each tube and its bottom 3 cm remained uncovered for ventilation. We placed seedling protection tubes without shade cloth onto cone-tainers of not-shaded plants, hereafter referred to as "plants in the sun" or "sun plants" (Fig 3.1c). PAR sensors (Apogee Instruments Inc., Model No. SQ-110) run simultaneously in one randomly selected shaded protection tube and one non-shaded tube with a 60 minute recording interval for one day (June 26, 2014) from 0900

until 2000 hr gave mean photosynthetic photon flux densities of 197.5 ± 302.2 SD μmol m^{-2} s^{-1} for shaded target plants and 582.5 ± 400 SD μmol m^{-2} s^{-1} for sun plants. These measurements suggested that shaded plants received about 35% of the photosynthetically active radiation received by sun plants.

Measurements and harvest

We began measuring the length of the longest leaf of each plant 11 days after germination, beginning May 14, 2014, and took subsequent measurements every two weeks for 80 days. Following each measurement, we re-randomized the positions of all pots. When growth began to slow, we harvested all plants by clipping their shoots directly above the basal meristem and drying them to a constant weight at 60 °C for assessment of shoot dry weight (DW).

We removed roots from all cone-tainers, rinsed them in water on a 1 mm sieve, and preserved them in 50 % ethanol. We then blotted dry and weighed each root system to determine the total fresh weight. We clipped subsamples of root systems randomly and preserved them in 50 % ethanol for later assessment of mycorrhizal colonization. Clipped root systems were weighed again and dried at 60 °C to constant weight. We weighed dried root systems and used the DW to fresh weight ratio to calculate the DW of the entire root system.

We assessed mycorrhizal colonization of target plants that were selected for isotopic analysis. We cleared root clippings in 10 % KOH at room temperature for 5 days, acidified them in 5 % HCl for 30 minutes and placed them into 0.05 % Trypan blue in laco-glycerol for 24 hours to stain AM fungi. We then mounted 34 1–2 cm root pieces per plant onto microscope slides and scored mycorrhizal colonization using the gridline

interaction method (McGonigle *et al.*, 1990) at three intersections per root piece for a total of 100 intersections.

Isotope ratio mass spectrometry was performed on target and composited neighbor shoots from all replicates in the sun, intact CMNs treatment and on ten pots per treatment for all other treatments. To ensure that we analyzed plants across a range of sizes from each treatment other than sun, intact CMNs, target plants were rank-ordered by DW into ten groups of two (= deciles) for each treatment. We then randomly picked one of the two target plants in each decile and its corresponding neighbor plants for isotopic analysis at the University of Miami Stable Isotope Facility (Coral Gables, FL, USA). We loaded ~5 mg of ground leaf tissue into tin cups (5 × 8 mm; Elementar America) which were combusted in an elemental analyzer (Eurovector) connected to an Isoprime stable isotope mass spectrometer (Elementar). We used the customary expression to describe isotopic abundance:

$$\delta^{15}\text{N (‰)} = \left[\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right] \times 1000$$

where R represents the $^{15}N/^{14}N$ ratio of a sample or of the standard which is atmospheric N. The precision of the analysis was ± 0.1 ‰. Mineral nutrient analyses of target plants required 0.2 g of dried tissue, so within each treatment we combined the remaining tissues of target plants of similar sizes to total that amount before sending them to Kansas State Agronomy Soil Testing Laboratory (Manhattan, KS, USA).

Statistical analyses

We analyzed aboveground and belowground DW of targets and neighbors by two-way analysis of variance (ANOVA) with shading and CMN treatment as factors. Before

ANOVA, we examined the data for heteroscedasticity with Levene's test. Aboveground and belowground DW of target plants had heteroscedastic variance, so we log transformed the data. To discern relationships between target aboveground DW and total neighbor aboveground DW, we used least-squares linear regression. Total neighbor DW was determined by summing the aboveground DW of all neighbor plants per pot. Because no neighbor plants were shaded, we used all aboveground DW within each CMN treatment, ignoring shading, to calculate Gini coefficients (Weiner & Solbrig, 1984) for assessment of size inequalities, and then used a one-way ANOVA to compare Gini coefficients of plants with intact CMNs to those without CMNs (combined severed or no CMNs). We assessed differences in percentage root length colonized by AM fungi by two-way ANOVA on arcsine square-root transformed data. After all ANOVAs, we made comparisons among treatments with Tukey's Honestly Significant Difference post-hoc test. We examined the relationship between colonization and aboveground DW with least-squares linear regressions.

Before statistically analyzing mineral nutrient concentrations, we excluded three pots from the analyses: one from the sun, no CMN treatment, one from the shade, intact CMNs treatment, and one from the shade, severed CMNs treatment. These pots were eliminated according to Chauvenet's Criterion (Taylor, 1982) which uses the probability of obtaining values as high as observed for each potential outlier pot relative to the standard deviation for its treatment. The three eliminated pots had less than a 5 % chance of having values as high as observed relative to all others in their treatment, and thus their ^{15}N values misrepresented their treatments.

We compared mineral nutrient concentrations using two-way ANOVAs, and examined the relationship between aboveground DW and mineral nutrient concentrations with least-squares linear regressions. To determine the percentage of their total nitrogen that target plants obtained from neighbor cone-tainers into which ^{15}N was added, we used the mass balance equation:

$$\delta^{15}N_{Target} = x \cdot \delta^{15}N_{Neighbors} + (1 - x) \times \delta^{15}N_{No\ CMN}$$

where $\delta^{15}N$ represents the isotopic abundance of targets, neighbors, and target plants in the no CMN treatment, and x represents (as a decimal fraction) the percent nitrogen obtained by the target plant from neighbor cone-tainers. Values for $\delta^{15}N_{Neighbors}$ were obtained from each target plant's composited neighbors. We calculated average values for $\delta^{15}N_{No\ CMN}$ separately for the sun and shade treatments. We then used a two-way ANOVA to assess differences among treatments, and used least-squares linear regressions to assess the relationship between aboveground DW and the percentage of nitrogen obtained from neighbor cone-tainers. All statistical analyses were performed with Statistix v. 10.0 (Analytical Software, Tallahassee, FL, USA).

Results

Andropogon gerardii seedling sizes were affected by both shading and CMN treatments. Aboveground DW of target plants was diminished by shading ($F_{1, 109} = 13.09$, $P = 0.0005$) and CMNs severing or prevention ($F_{2, 109} = 8.87$, $P = 0.0003$), but there was no significant interaction between the two factors ($F_{2, 109} = 0.35$, $P = 0.7066$; Fig. 3.2a). Belowground DW of target plants was affected by shading, ($F_{1, 109} = 14.80$, $P = 0.0002$), but neither by CMN treatments ($F_{2, 109} = 1.65$, $P = 0.1975$) nor by the interaction of the factors ($F_{2, 109} = 0.46$, $P = 0.6321$; Fig. 3.2a). Composited aboveground and belowground

neighbor sizes were not affected by shading of target plants (aboveground: $F_{1, 119} = 1.73$, $P = 0.1905$; belowground: $F_{1, 119} = 0.86$, $P = 0.3568$), by CMN treatments (aboveground: $F_{2, 119} = 1.78$, $P = 0.1734$; belowground: $F_{2, 119} = 0.87$, $P = 0.4231$), or by the interaction of shading and CMN treatments (aboveground: $F_{2, 119} = 2.83$, $P = 0.0635$; belowground: $F_{1, 109} = 2.85$, $P = 0.0621$; Fig. 3.2b). For all plants (both targets and neighbors), Gini coefficients of size inequality were marginally significant ($F_{1, 4} = 7.62$, $P = 0.0508$) between treatments with and without CMNs (Gini coefficient of the intact CMNs treatment = 0.2122 versus without CMNs treatments = 0.1930).

Aboveground DW of target plants in the sun was negatively related to their respective composited neighbor DW per pot (Fig. 3.3a). For plants in the sun, linear regressions for severed CMNs and no CMN treatments did not differ from one another ($F_{1, 34} = 0.54$, $P = 0.4683$). When combined, their slope ($DW_{Neighbor} = 0.53\ DW_{Target} + 0.97$) was not significantly different from zero ($F_{1, 36} = 2.06$, $P = 0.1600$), but did differ significantly ($F_{1, 53} = 5.76$, $P = 0.0199$) from the negative slope of the intact CMNs treatment ($DW_{Neighbor} = -0.62\ DW_{Target} + 1.24$). For shaded plants (Fig. 3.3b), we similarly combined severed and no CMN treatments because they did not differ ($F_{1, 29} = 1.65$, $P = 0.2094$). Their combined slope ($DW_{Neighbor} = -0.27\ DW_{Target} + 1.14$) did not differ from zero ($F_{1, 32} = 0.24$, $P = 0.6300$), but did differ significantly ($F_{1, 49} = 7.68$, $P = 0.0079$) from the negative slope of the shaded, intact CMNs treatment ($DW_{Neighbor} = -2.74\ DW_{Target} + 1.46$).

Colonization of target plant root systems by AM fungi was affected by shading ($F_{1, 54} = 43.24$, $P = 0.00001$), CMNs ($F_{2, 54} = 6.84$, $P = 0.0024$), and by their interaction ($F_{2, 54} = 4.48$, $P = 0.0163$). All treatments in the sun and the intact CMNs treatment in the

shade did not differ from one another, but all of those treatments differed significantly from the severed CMNs and no CMN treatments in the shade, which did not differ from one another (Fig. 3.4). We did not find a significant regression relationship between colonization and target aboveground dry weight for sun plants ($F_{1, 27} = 0.64$, $P = 0.4317$) or shaded plants ($F_{1, 26} = 0.33$, $P = 0.5712$).

Among the mineral nutrients that we assessed (N, P, K, Ca, Mg, Fe, Mn, Zn; Table 3.2), Mn was positively associated with target aboveground DW over all sun treatments ($F_{1, 40} = 4.95$, $P = 0.0319$) with no significant differences among slopes ($F_{2, 35} = 0.34$, $P = 0.7114$), but differences in elevation ($F_{2, 37} = 4.10$, $P = 0.0247$; Fig. 3.5a). Elevations did not differ between intact CMNs and severed CMNs ($F_{1, 25} = 3.39$, $P = 0.0776$) or between severed CMNs and no CMN treatments ($F_{1, 23} = 0.44$, $P = 0.5122$), but they did differ for intact CMNs versus no CMN ($F_{1, 26} = 7.15$, $P = 0.0128$). For shaded plants, Mn had only a marginally significant relationship with aboveground DW over all CMN treatments ($F_{1, 28} = 3.50$, $P = 0.0723$; Fig. 3.5b).

The only other element concentration that we assessed which was associated significantly with target aboveground DW over all sun treatments was N. Foliar N concentrations were significantly affected by shading ($F_{1, 51} = 79.78$, $P = 0.00001$), but not by CMN treatments ($F_{2, 51} = 3.05$, $P = 0.057$) or by the interaction between shading and CMN treatments ($F_{2, 51} = 1.81$, $P = 0.1746$). Nitrogen concentrations significantly decreased with aboveground DW for target plants in the sun ($F_{1, 26} = 9.15$, $P = 0.0057$; Fig. 3.5c). Slopes among CMN treatments did not differ ($F_{2, 21} = 1.20$, $P = 0.3196$), but elevations did differ ($F_{2, 23} = 4.11$, $P = 0.0298$). Intact CMNs had a significantly higher elevation than both severed CMNs ($F_{1, 18} = 4.82$, $P = 0.0415$) and no CMN ($F_{1, 15} = 5.18$,

$P = 0.0380$) which did not differ from one another ($F_{1, 12} = 0.09$, $P = 0.7705$). For shaded plants, target plants with intact CMNs (DW = 0.16 %N + 0.13) had a positive slope that significantly differed ($F_{2, 19} = 18.97$, $P = 0.0003$) from the negative slopes of the severed and no CMN treatments, which did not differ from one another ($F_{1, 6} = 0.02$, $P = 0.8908$; DW = -0.17 %N + 0.42; Fig. 3.5d).

Both shading ($F_{1, 68} = 10.80$, $P = 0.0017$) and CMN treatments ($F_{2, 68} = 9.85$, $P = 0.002$) had a significant effect on $\delta^{15}N$ values, with a significant interaction ($F_{2, 68} = 6.83$, $P = 0.0021$). Plants with intact CMNs in the sun had a significantly higher ^{15}N concentration than all other groups, which did not differ from one another (Fig. 3.6a). Similarly, shading ($F_{1, 68} = 10.93$, $P = 0.0016$), CMN treatments ($F_{2, 68} = 9.62$, $P = 0.0002$) and their interaction ($F_{2, 68} = 6.57$, $P = 0.0026$) affected the percentage ^{15}N obtained from neighbors' cone-tainers (Fig. 3.6b).

Target aboveground DW was associated with the amount of nitrogen obtained from neighbors' cone-tainers only for plants in the sun ($F_{1, 32} = 40.68$, $P = 0.00001$) over all CMN treatments. Regressions for severed CMNs and no CMN treatments did not differ ($F_{1, 18} = 0.03$, $P = 0.8550$), but when combined (DW = 0.009 $\%^{15}N$ + 0.01004), they differed significantly ($F_{1, 29} = 24.36$, $P = 0.00001$) from that for intact CMNs (DW = 0.55 $\%^{15}N - 0.04$). For shaded plants, there was no significant relationship between target aboveground DW and the amount of nitrogen obtained from neighbors' cone-tainers ($F_{1, 32} = 2.42$, $P = 0.1298$). When we compared target plants with intact CMNs grown in the sun versus those in the shade, their regressions differed significantly ($F_{1, 25} = 13.37$, $P = 0.0012$) because intact CMNs target plants in the sun had a positive slope that

differed significantly from zero ($F_{1, 13} = 36.33$, $P = 0.0001$) while the slope for intact CMNs, shaded target plants did not differ from zero ($F_{1, 13} = 1.02$, $P = 0.3321$; Fig. 3.7).

Discussion

Whether CMNs have a positive, negative, or neutral effect on plant growth has been debated (Van Der Heijden & Horton, 2009), but our work and that of others (Merrild *et al.*, 2013; Weremijewicz & Janos, 2013) has found that even as arbuscular mycorrhizas increase mean plant size, CMNs can intensify competition for mineral nutrients among interconnected individuals. In our previous study (Weremijewicz & Janos, 2013), a negative relationship between target and neighbor size existed only for plants with intact CMNs, consistent with the growth of small plants having been suppressed by large neighbors. The absence of such a relationship for plants with severed CMNs or no CMNs suggests these plants were released from belowground competitive interactions with neighbors. Aboveground interactions likely were minimal but relatively consistent among CMN treatments, and direct root system overlap was prevented entirely, so we concluded that intact CMNs mediated belowground competition.

In this study, similar to our previous results (Weremijewicz & Janos, 2013), we found negative relationships between total neighbor DW and target plant DW only in the presence of intact CMNs. Shaded target plants with intact CMNs, however, had a 4.4-times steeper negative slope than plants with intact CMNs in the sun. Although total neighbor DW was similar for the sun and shade treatments because neighbors were not shaded, shading considerably reduced the DW of target plants. This target plant growth reduction likely was a consequence of both competition with large, non-shaded neighbors mediated by intact CMNs and limitation of target plant photosynthesis by shading.

Limitation of target plant photosynthesis might have elevated the proportional carbon cost of mycorrhizas (Olsson *et al.*, 2010).

Although we expected shading to decrease root colonization by AM fungi, we found that shaded target plants with intact CMNs had similar colonization to that of plants in the sun. Carbon supplied to AM fungi in intact CMNs by carbon-fixing, sunlit neighbors apparently allowed the fungi to abundantly colonize the roots of likely carbon-stressed, shaded targets. Severing or preventing CMNs significantly reduced the colonization of shaded target plants. That diminished colonization was unlikely to have been a consequence of reduced inoculum potential because target plants with severed or no CMNs in the sun had similar colonization to plants with intact CMNs. Several studies have suggested that shading diminishes colonization because of decreased carbon fixation and diminished allocation belowground (Hayman, 1974; Tester *et al.*, 1986), but those studies grew individual plants in pots without possible CMN connections to sunlit plants. In contrast, in a field study in which plants probably were interconnected by CMNs, Francis and Read (1984) found root colonization did not differ among shaded and not-shaded plants, similar to our findings. Furthermore, *in vitro* root organ-culture work that has mimicked shaded conditions by growing roots on a low-carbon medium also has found that when low- and high-carbon roots are interconnected via CMNs, colonization rates of the low-carbon roots are similar to those of high-carbon roots (Lekberg *et al.*, 2010; Hammer *et al.*, 2011).

In our experiment, even though fixed carbon supplied by neighbors to AM fungi in intact CMNs may have sustained AM fungi in shaded target plant roots, it is not likely to have been transferred to plant tissues or to have supported plant growth (Pfeffer *et al.*,

2004). Robinson and Fitter (1999) suggested that carbon transfer to hyphae within the roots of carbon-stressed plants likely is a strategy of AM fungi for their own growth, with minor consequences for plant communities. AM fungi might colonize carbon-stressed roots for protection of fungal structures and material storage, as a way of fostering the spread extraradical hyphae, or to be well positioned to take advantage of a host's fixed carbon in case its shading is temporary. Nevertheless, we have shown that such a fungus strategy is not without potential liability to shaded host plants because mineral nutrients might be removed from their vicinity.

As in our previous research (Weremijewicz & Janos, 2013) which used an identical soil mixture within cone-tainers, we found that Mn was most likely to be the growth-limiting mineral nutrient because among the elements for which we assessed foliar concentrations, only Mn concentrations uniformly were positively associated with plant DW. Nitrogen concentrations also were positively associated with plant DW, but only for shaded targets with intact CMNs. For all other treatments, N concentrations were negatively associated with plant DW, suggesting that nitrogen was diluted by plant growth and was not growth-limiting (Johnson *et al.*, 1980; Estrada-Luna *et al.*, 2000). For shaded target plants with intact CMNs, however, nitrogen preemption by large, fully insolated, carbon-fixing neighbors, may have lowered N availability to the point at which it became growth-limiting. In order to obtain plant-growth-limiting Mn and N, AM fungi probably had to access the soil of adjacent cone-tainers because the interstitial, acid-washed sand between them contained relatively limited mineral nutrients. Mineral nutrient preemption may have intensified asymmetric competition among interconnected individuals, resulting in more unequal size hierarchies for populations with intact CMNs

than those without CMNs as demonstrated by an elevated Gini coefficient, similar to the findings of Weremijewicz and Janos (2013).

Using ^{15}N tracing, we found that intact CMNs which potentially provided hyphae access to adjacent cone-tainers, intensified N acquisition from them. In our experiment, intact CMN hyphae probably were the only path for mineral nutrient movement among cone-tainers because root systems could not spread beyond cone-tainers and the Gore-tex fabric likely prevented mass flow. Consequently, sunlit target plants with intact CMNs obtained on average at least 2.8-fold more N from neighbors' cone-tainers than target plants in any other treatment. The largest sunlit target plant with intact CMNs obtained 27 % of its nitrogen from neighbor cone-tainers. Despite similar mycorrhizal colonization to sunlit targets with intact CMNs, severing CMNs of sunlit target plants and also shading targets with intact CMNs greatly reduced the percentage of N obtained from neighbors' cone-tainers. That shading target plants with intact CMNs reduced N acquisition from neighbors' cone-tainers suggests that carbon supply to AM fungi influenced ^{15}N distribution, consistent with the "reciprocal rewards" hypothesis. Shaded, intact CMNs target plants had ^{15}N concentrations similar to plants with severed or no CMN. Although plants with severed and no CMN had ^{15}N in their leaf tissues, we found that our non-enriched KNO_3 fertilizer had a $\delta^{15}N$ of approximately16 ‰, which potentially led to traces of ^{15}N in those plants after weeks of fertilization.

Our findings contrast with those of Ames *et al.* (1983), who suggested that colonization is positively correlated with mineral nutrient transfer from mycorrhizal fungi. We found that in spite of there being no statistically significant differences in colonization among all sun plants and shaded, intact CMNs plants, nitrogen uptake from

neighboring plants and its relationship with target aboveground DW differed among these treatments. Although colonization might set an upper limit on the rate of mineral nutrient supply to host plants by AM fungi, the realized supply is likely to be governed by host ability to provision fixed carbon to mycorrhizal fungus associates. Olsson *et al.* (2010) found that shaded plants may continue to supply fixed carbon to AM fungi even with reduced colonization. In our experiment, if fixed-carbon supply to AM fungi by shaded target plants continued, the net consequence may have been that the plants effectively were parasitized by the AM fungi (Johnson *et al.*, 1997) because of not being recompensed by adequate fungus-proffered mineral nutrients.

In combination with Weremijewicz and Janos (2013), our findings show that differences in mineral nutrient allocation by CMNs to large versus small plants ultimately can affect plant competition and size hierarchies. Using intact plants and ^{15}N, we detected "reciprocal rewards" as found originally in root organ-culture work. Our research realistically represented the mineral nutrition benefit of CMNs because our inoculum consisted of several species of AM fungi while most previous studies have investigated CMNs formed by single fungus species. In nature, host plants typically are colonized by three to ten different AM fungi on a single root (Vandenkoornhuyse *et al.*, 2003; Scheublin *et al.*, 2007). Even if some of those fungi do not benefit the host plant, our findings together with those of Weremijewicz and Janos (2013), suggest that a mixture of AM fungi forming CMNs may have a mutualistic effect on those individual plants best able to supply fixed carbon to the fungus networks. Thereby, CMNs likely influence plant fitness, community structure, and community composition.

Table 3.1. Mineral nutrient concentrations and contents of the interstitial sand surrounding cone-tainers within pots and of the soil mixture within cone-tainers

	Concentration		Content (mg)	
Soil characteristic* (units)	Interstitial sand	Soil mixture	Interstitial sand	Soil mixture
Nitrate (ppm)	0.5	3	1.6	1.4
Mehlich 3 phosphorus (ppm)	2.1	12	6.8	5.8
Potassium (ppm)	11.0	29	35.8	13.9
Calcium (ppm)	9.2	4576	29.9	2198
Manganese (ppm)	0.1	14	0.3	6.7
pH	7.0	8.0	–	–

* Samples were analyzed by the Kansas State University Soil Testing Laboratory, Manhattan, KS, U.S.A

Table 3.2. Mean foliar mineral nutrient concentrations for sun and shade treatments with intact CMNs, severed CMNs, and no CMN

	Sun			Shade		
Mineral nutrient (units *)	Intact CMNs	Severed CMNs	No CMN	Intact CMNs	Severed CMNs	No CMN
P (%)	0.359	0.369	0.346	0.359	0.398	0.370
K (%)	1.753	1.863	1.902	1.853	2.030	1.895
Ca (%)	0.743	0.744	0.672	0.735	0.758	0.681
Mg (%)	0.323	0.319	0.319	0.325	0.347	0.345
Fe (ppm)	83.267	102.640	100.890	88.671	113.320	99.225
Mn (ppm)	29.393	26.062	29.300	32.214	32.200	26.925
Zn (ppm)	47.320	41.677	42.179	38.107	39.517	47.387

* Samples were analyzed by the Kansas State University Soil Testing Laboratory, Manhattan, KS, U.S.A

Figure 3.1. Experiment set-up involved Ray Leach cone-tainers with slots opened in both sides covered with a silkscreen mesh (not visible) and Gore-Tex (Newark, DE, USA) cloth (a), target plant pot set up with acid-washed interstitial sand and a soil mix in cone-tainers (b), and shading tubes (c) placed on half of the targets to diminish photosynthesis.

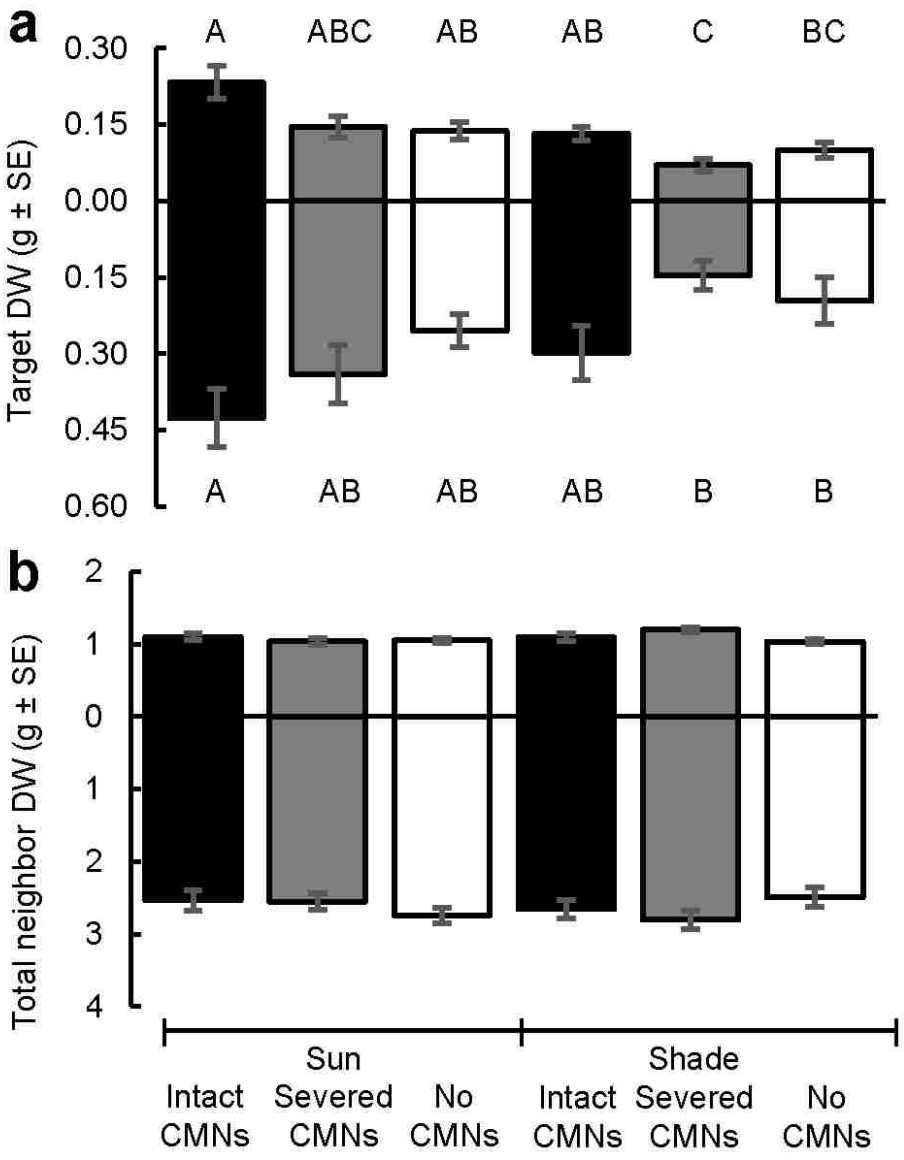

Figure 3.2. Mean dry weights (DW) ± SE (g) aboveground and belowground (shown as positive values below the x-axis) of target (a) and neighbor (b) *Andropogon gerardii* plants with intact common mycorrhizal networks (CMNs; dark bars), severed CMNs (gray bars), and no CMN (white bars). Target plants in each CMN treatment were shaded or not. Bars topped by the same letters in (a) do not differ significantly by Tukey's Honestly Significant Difference post-hoc test at $P \leq 0.05$. Total neighbor DW was not significantly affected by treatment, so post-hoc tests were not conducted.

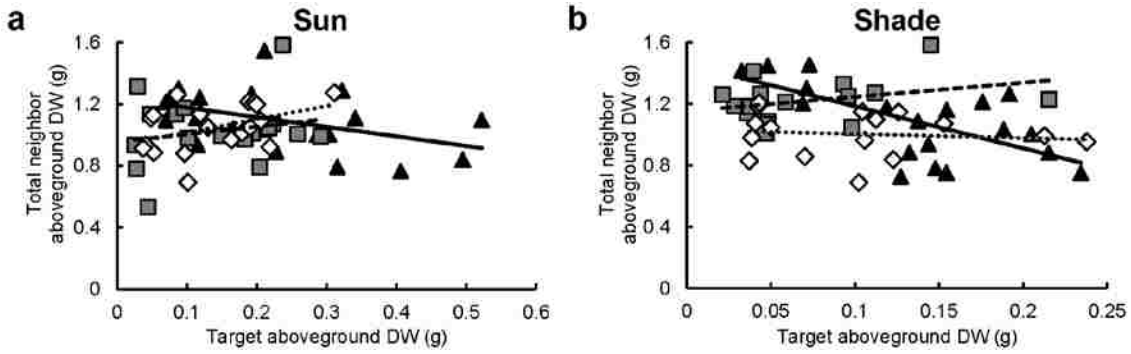

Figure 3.3. Total neighbor aboveground dry weight (DW; g) versus target plant aboveground DW (g) for *Andropogon gerardii* plants with intact common mycorrhizal networks (CMNs; dark triangles; solid line), severed CMNs (gray squares; dashed line), and no CMN (white diamonds; dotted line) with target plants grown in ambient sun (a) or shaded (b).

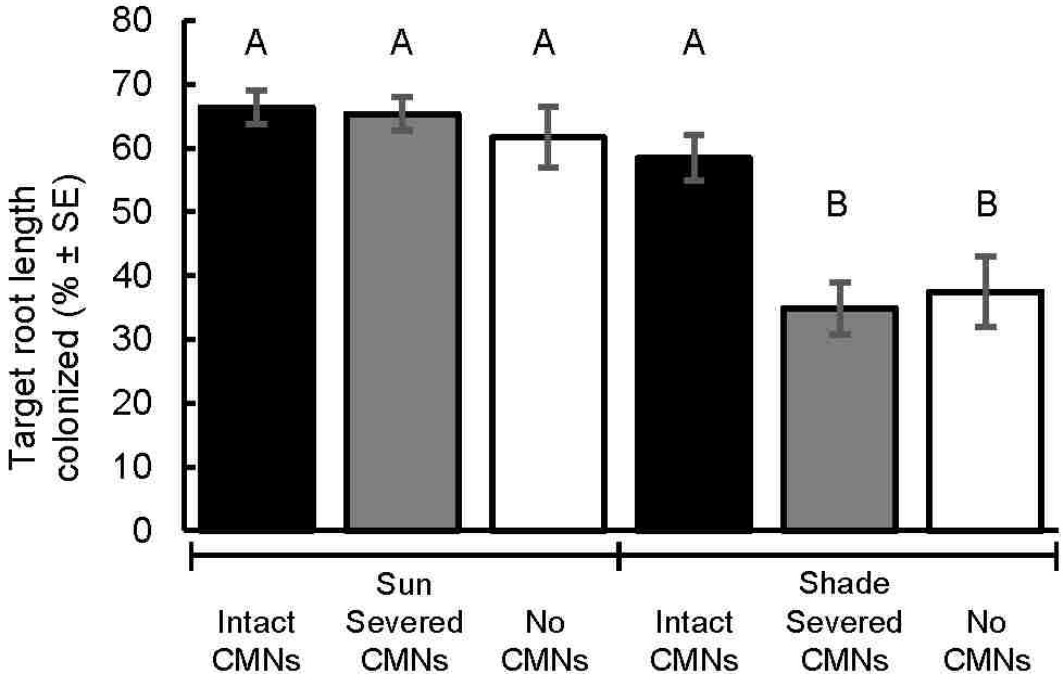

Figure 3.4. Percent root length colonized ± SE of target *Andropogon gerardii* plants with intact common mycorrhizal networks (CMNs; black bars), severed CMNs (gray bars), and no CMN (white bars) grown in ambient sun or shaded. Bars topped by the same letters do not differ significantly by Tukey's Honestly Significant Difference post-hoc test at $P \leq 0.05$.

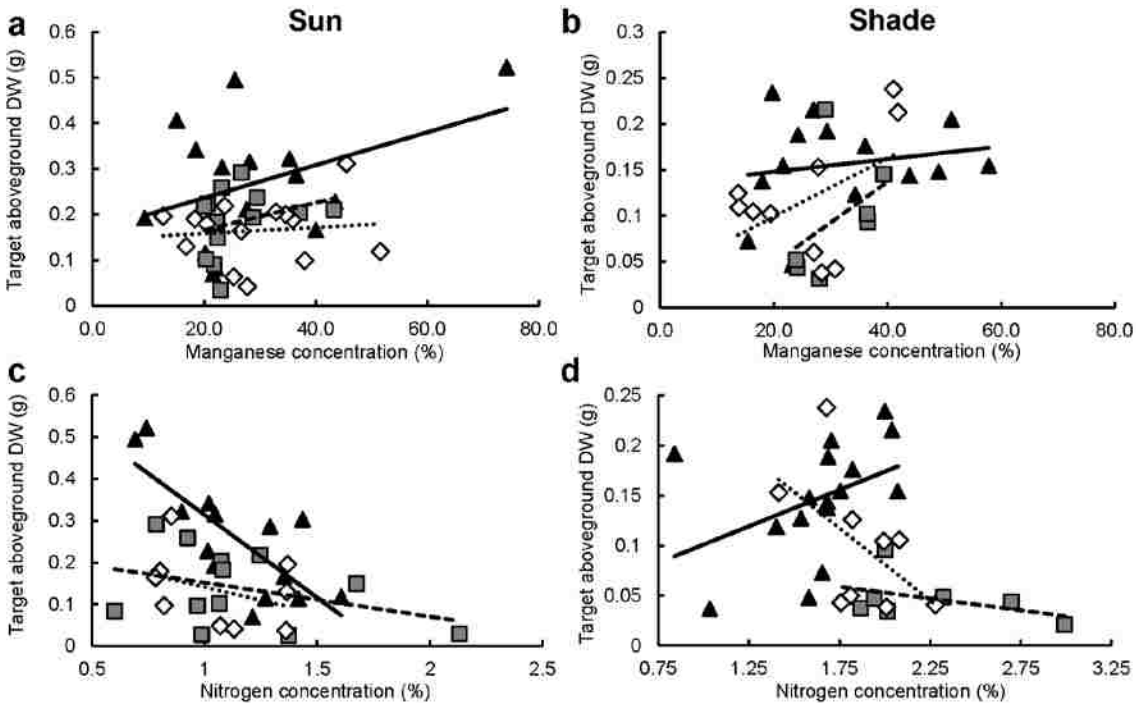

Figure 3.5. Target aboveground dry weights (DW; g) versus manganese (%; a, b) or nitrogen (%; c, d) for *Andropogon gerardii* target plants in ambient sun (a, c) or shaded (b, d) with intact common mycorrhizal networks (CMNs; black triangles, solid lines), severed CMNs (gray squares, dashed lines), or no CMN (white diamonds, dotted lines).

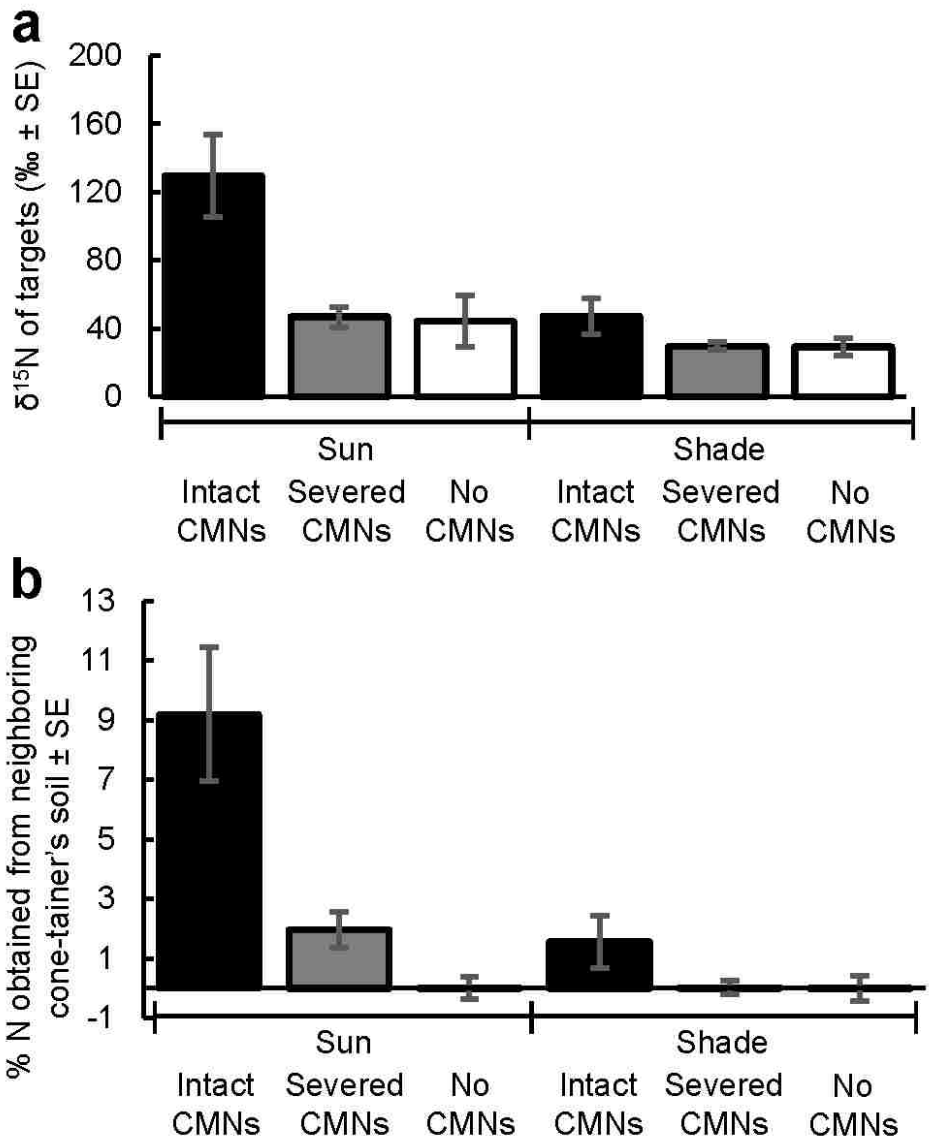

Figure 3.6. $\delta^{15}N$ (‰) ± SE of target plants (a) and percent nitrogen obtained by target plants from neighbor cone-tainer soil (b) with intact common mycorrhizal networks (CMNs; black bars), severed CMNs (gray bars), and no CMN (white bars) grown in ambient sun or shaded. Percent nitrogen obtained from neighbors' cone-tainers was calculated using ^{15}N as a proxy and the mass balance equation in which $\delta^{15}N_{Target} = x \cdot \delta^{15}N_{Neighbors} + (1 - x) \times \delta^{15}N_{NoCMN}$.

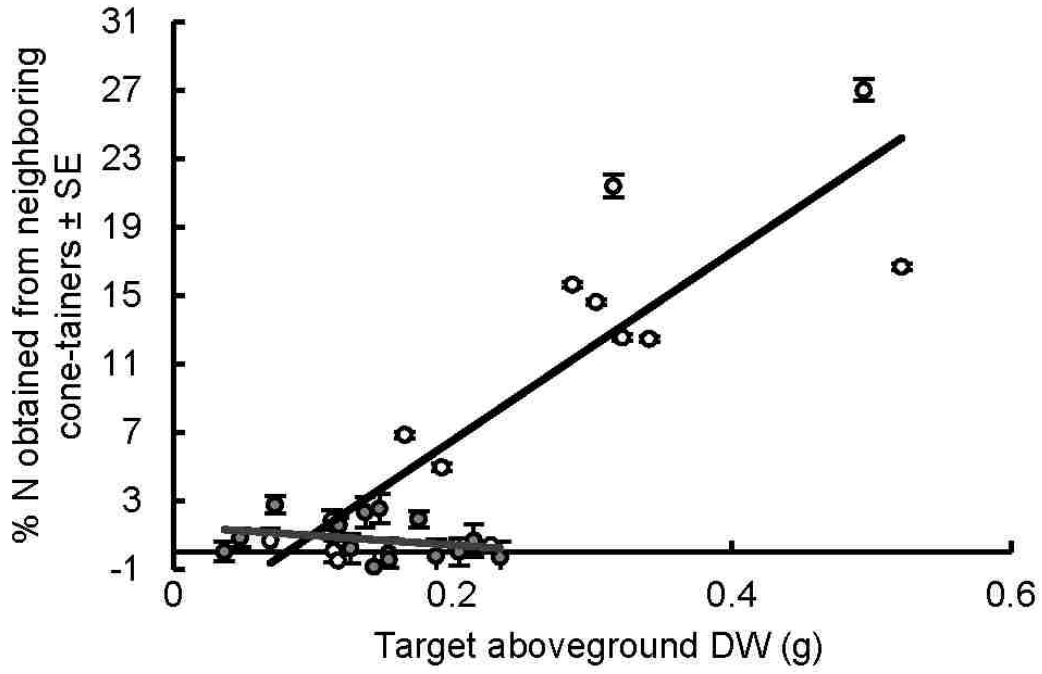

Figure 3.7. $\delta^{15}N$ (‰) ± SE of target plants (a) and percent nitrogen obtained by target plants from neighbor cone-tainer soil (b) with intact common mycorrhizal networks (CMNs; black bars), severed CMNs (gray bars), and no CMN (white bars) grown in ambient sun or shaded. Percent nitrogen obtained from neighbors' cone-tainers was calculated using ^{15}N as a proxy and the mass balance equation in which $\delta^{15}N_{Target} = x \cdot \delta^{15}N_{Neighbors} + (1 - x) \times \delta^{15}N_{NoCMN}$.

Chapter Four

Mycorrhizas influence functional traits of two tallgrass prairie species

Summary

- Over the past decade, functional traits that influence plant performance and thus, population, community, and ecosystem biology have garnered increasing attention. Generally lacking, however, has been consideration of how ubiquitous arbuscular mycorrhizas influence plant allometric and stoichiometric functional traits.
- We assessed how plant dependence on and responsiveness to mycorrhizas influence plant functional traits of a warm-season, C4 grass, *Andropogon gerardii* Vitman, and the contrasting, cool-season, C3 grass, *Elymus canadensis* L. We grew both host species with and without inoculation with mycorrhizal fungi, across a broad gradient of soil phosphorus availabilities.
- Both host species were facultatively mycotrophic, able to grow without mycorrhizas at high soil phosphorus availability. *A. gerardii* was most dependent upon mycorrhizas and *E. canadensis* was weakly dependent, but highly responsive to mycorrhizas.
- The high dependence of *A. gerardii* on mycorrhizas resulted in higher tissue P and N concentrations of inoculated than non-inoculated plants. When not inoculated, *E. canadensis* was able to take up both P and N in similar amounts to inoculated plants because of its weak dependence on mycorrhizas for nutrient uptake and its pronounced ability to change root to shoot ratios.

- Unlike other highly dependent species, *A. gerardii* had a high root-to-shoot ratio and was able to suppress colonization by mycorrhizal fungi at high soil fertilities. *E. canadensis*, however, was unable to suppress colonization and had a lower root-to shoot ratio than *A. gerardii*.
- *Synthesis*: The mycorrhiza-related functional traits of both host species likely influence their performance in nature: both species attained the maximum responsiveness from mycorrhizas at soil phosphorus availabilities similar to those of tallgrass prairies. Dependence upon mycorrhizas affects performance in the absence of mycorrhizas. Responsiveness to mycorrhizal fungi is also a function of the environment and can be influenced by both mycorrhizal fungus species and soil fertility.

Background

Arbuscular mycorrhizal (AM) fungi associate with the vast majority of plant species (Wang & Qiu 2006) and provide their host plants with many benefits, such as pathogen protection (Newsham *et al*. 1995), improved water relations (Auge 2001) and especially uptake of mineral nutrients such as nitrogen and phosphorus (Smith & Read 2008); consequently, the degree to which a host species associated with AM fungi may influence its functional traits. Plant functional traits have been defined as measurable morphological, physiological or phenological properties that affect plant performance (McGill *et al.* 2006; Westoby & Wright 2006; Friesen *et al.* 2011). Thus, functional traits can determine where a species establishes, how it interacts with neighboring individuals, and its overall productivity, all of which affect population, community, and ecosystem functioning (McGill *et al.* 2006). Because AM fungi can increase or decrease

plant biomass (Johnson, Graham & Smith 1997), change root architecture (Hetrick 1991), and increase both nitrogen and phosphorus concentrations within tissues (Smith, Grace & Smith 2009), they can strongly influence plant functional traits.

Root-to-shoot ratio, a well-known plant functional trait, is a measure of plant belowground versus aboveground carbon allocation. Root-to-shoot ratios differ across species and can alter ecosystem carbon dynamics through differences in root metabolism and root turnover (Westoby & Wright 2006). These ratios may evolve in consort with dependence on mycorrhizas such that plant species highly dependent upon mycorrhizas allocate less carbon to root production than those potentially independent of mycorrhizas for nutrient uptake (Hetrick 1991). Phenotypically, mycorrhizal colonization can diminish root-to-shoot ratios (Sanders *et al.* 1977; Veresoglou *et al*. 2011), potentially in response to elevated plant tissue P concentration (Smith 1980; Ceasar *et al.* 2014).

Stoichiometric plant functional traits, such as N and P concentrations, may have implications for competition between plant species (Koerselman & Meuleman 1996). Furthermore, plant N:P ratios may indicate potential nutrient limitation within a community, with N:P ratios greater than 16 (Koerselman & Meuleman 1996) or 20 (Güsewell 2004) suggesting P limitation, those less than 10 (Güsewell 2004) or 14 (Koerselman and Meuleman 1996) suggesting N limitation, and those between 14 and 16 (or 10 and 20) suggesting co-limitation. Mycorrhizas can increase both N (Hodge & Fitter 2010) and P (Smith & Read 2008) concentrations in plants by adding hyphae to the surface area across which mineral nutrients are absorbed and by hyphae extending beyond zones of nutrient depletion that develop around roots (Smith & Read 2008). Increased mineral nutrient concentrations within plant tissues can affect photosynthesis

rate, thereby influencing biomass production (Koerselman & Meuleman 1996; Johnson 2010).

Plant species' reliance on AM fungi for mineral nutrient uptake – their dependence on mycorrhizas – and the change in growth when colonized by mycorrhizas – their responsiveness to mycorrhizas – may explicate mycorrhizas' influence on plant functional traits. The terms 'dependence' and 'responsiveness' have been used interchangeably to denote a positive effect of mycorrhizas on plant growth (e.g., Hetrick, Kitt & Wilson 1986; Hetrick, Kitt & Wilson 1988; Hetrick, Wilson & Hartnett 1989; Baon, Smith & Alston 1993; Wilson & Hartnett 1998; Van Der Heijden & Horton 2009), but Janos (2007) drew a distinction between the terms. Janos (2007) defined mycorrhiza dependence as strictly a property of plant genotype because it is assessed as the inability of a plant species to grow without mycorrhizas. Dependence on mycorrhizas can be measured by the soil P concentration that enables plants without mycorrhizas to reach 10 % of their asymptotic growth. The higher the concentration of P required for growth without mycorrhizas, the more 'dependent' the plant species. Occasionally, negative concentrations may be calculated for dependence, suggesting that less P than is available in non-fertilized, base soil allows 10 % of asymptotic growth.

In contrast to dependence, responsiveness is a property of both plant genotype and the effects of mycorrhizal fungus species on the growth of the plant. Unlike dependence, it is not directly susceptible to natural selection. Responsiveness is measured as the magnitude of plant growth improvement resulting from colonization by mycorrhizal fungi. Responsiveness to mycorrhizas may change with different P fertilities, with high soil fertilities resulting in a small growth difference between inoculated and non-

inoculated plants. Therefore, maximum responsiveness can be assessed by using a gradient of P concentrations to find the largest difference in growth. Additionally, the slopes of such P-response curves reflect P-uptake and use efficiency when a plant is colonized by AM fungi or not, potentially indicating the maximum rate of P conversion to biomass of which a species is capable. Because P use efficiency affects plant performance and interactions (Koide 1991), it can be considered a plant functional trait influenced by mycorrhizas.

Mycorrhiza dependence as defined by Janos (2007), determines where plant species lie along the continuum from facultative to obligate mycotrophy, with ecologically obligate mycotrophs incapable of growth at the highest fertility that they naturally encounter. Even though mycorrhizas are beneficial to most plant species in low-fertility environments, the AM association can range from beneficial, through neutral, to disadvantageous, depending on plant species and environmental conditions (Peng *et al.* 1993; Johnson *et al.*1997; Janos 2007; Smith & Smith 2014). Mycorrhiza disadvantage can result when the carbon cost to the host of sustaining mycorrhizal fungi outweighs the mineral nutrient uptake benefits of mycorrhizas. Such disadvantage often is observed if plants have become heavily mycorrhizal prior to mineral nutrient enrichment (Peng *et al.* 1993; Janos 2007). Otherwise, in high nutrient environments, facultatively mycotrophic plants can limit the cost of mycorrhizas by suppressing root colonization (e.g., Amijee, Tinker & Stribley 1989; Schroeder & Janos 2004; Treseder 2004; Grman 2012).

Grasses are likely to be facultatively mycotrophic thanks to their highly-branched, extensive fine root systems (Janos 1980; Baylis 1972; Maherali 2014), but C4 grasses, for

example *Andropogon gerardii* Vitman, have been described as 'obligately dependent' on mycorrhizas because of their observed inability to grow without mycorrhizas at various single, elevated P availabilities (Hetrick, Kitt & Wilson 1986; Hetrick, Kitt & Wilson 1988; Hetrick, Wilson & Hartnett 1989; Hartnett *et al.* 1994; Wilson & Hartnett 1997; Wilson & Hartnett 1998). Hetrick, Wilson and Hartnett (1989) compared the growth of a C3 grass, *Elymus cinereus*, and *A. gerardii*, and found *E. cinereus* growth was depressed by only 46.6 % in the absence of AM fungi at a P availability at which *A. gerardii* growth was depressed by 99.5 %. Subsequently, Hartnett, Hetrick and Wilson (1993) found that a lack of mycorrhizas did not significantly affect growth of *Elymus canadensis* L. and that *E. canadensis* was able to outcompete *A. gerardii* when mycorrhizas were absent (but not when they were present). Thus, those authors concluded that C3 grasses generally are less dependent on mycorrhizas than are C4 grasses (Hetrick, Kitt & Wilson 1988; Hartnett, Hetrick & Wilson 1993). Although early research suggested that of *A. gerardii* and *E. canadensis*, only the latter could grow without mycorrhizas (e.g., Hetrick, Kitt & Wilson 1986; Hetrick, Kitt & Wilson 1988; Hetrick, Wilson & Hartnett 1989), recent studies that repeatedly supplied *A. gerardii* with soluble P have found it too is able to grow without mycorrhizas (Grman 2012; Thorne, Rhodes & Cardina 2013), hence both species are facultatively mycotrophic (see Janos 2007). Nevertheless, recent work investigating C3 and C4 grasses has concluded that C3 grasses exhibit less of a growth response to mycorrhizas than C4 grasses or exhibit no response at all (Reinhart et al. 2012, Yang et al. 2016). It also is possible that C3, cool-season grasses are better able to suppress root colonization than highly mycorrhiza-dependent, C4, warm-season grasses

(Grman 2012), but highly mycorrhiza-responsive C4 species may show the greatest increases in foliar P content per unit of mycorrhizal root (Treseder 2013).

We sought to assess how allometric and stoichiometric plant functional traits of *A. gerardii* and *E. canadensis* are affected by AM fungi across a gradient of P fertilities. Although many studies have characterized subsets of responsiveness for both species (which those studies called 'dependence'), few, if any, studies have characterized dependence as defined by Janos (2007). We sought to assess both dependence and responsiveness, and to determine the level of P at which each species is maximally responsive to mycorrhizas. We hypothesized that: 1) *A. gerardii* would be strongly dependent and *E. canadensis* would be weakly dependent on mycorrhizas for growth, 2) *E. canadensis* would also be weakly responsive to mycorrhizas and have P and N uptake least improved by mycorrhizas (although mycorrhizas would improve the P and N nutrition of both species, and 3) *E. canadensis* would reduce mycorrhizal colonization at high P but *A. gerardii* would not.

Materials and Methods

A. gerardii and *E. canadensis* are common tallgrass prairies species with contrasting ecologies. *A. gerardii* is a dominant tallgrass prairie species while *E. canadensis* is a subdominant. Often found near *A. gerardii*, *E. canadensis* is usually is found in a mixture of other grasses, constituting about 1–5 % of the mixture (Weaver & Fitzpatrick 1934). Because *Andropogon* is genus of tropical origin, its photosynthesis has a high light saturation point and it also has high water-use efficiency (Turner, Kneisler & Knapp 1995). In contrast, *E. canadensis* typically is found in lowland areas where it grows best

in saturated soils (Weaver & Fitzpatrick 1934). Some ecotypes of *A. gerardii*, however, are tolerant of high soil moisture (Weaver & Fitzpatrick 1934; Olsen *et al.* 2013).

We grew *A. gerardii* and *E. canadensis* in separate, sequential experiments in an ambient shade house with 30 % shade at the Fairchild Tropical Botanic Garden Research Center (Coral Gables, Florida, USA). In both experiments, inoculation with AM fungi and weekly soluble phosphorus fertilization treatments were combined fully factorially. Both inoculated and non-inoculated *A. gerardii* and *E. canadensis* weekly received 10 mL of seven phosphorus concentrations — 1, 2, 4, 16, 32, 64, 128 μg g^{-1} P — made with NaH_2PO_4 in distilled water, but *E. canadensis* additionally received an 8 μg g^{-1} P concentration. There were eight individually-grown, replicate *A. gerardii* plants and ten *E. canadensis* plants per treatment for totals of 112 *A. gerardii* and 160 *E. canadensis*.

A. gerardii and *E. canadensis* seeds were obtained from Ever Wilde Farms nursery (Sand Creek, Wisconsin, USA) and were germinated on moist paper towels. Approximately one-week-old seedlings were individually transplanted to Ray Leach Cone-tainers (2.5 cm diameter × 12.1 cm length; 49 mL volume; Tangent, Oregon, USA). Before transplant, we filled the cone-tainers with a homogenized mixture of 90 % sand and 10 % University of Miami Gifford Arboretum soil which was minimally fertile so that we could investigate a broad range of P availabilities. The sand comprised a 3:1 blend of 30–65 grade fine sand and 6–20 grade coarse sand from Florida Silica Sand Company (Miami, Florida, USA). The soil mixture was autoclaved at 121 °C and 1.4 kg cm^{-2} for one hour, three times, each 24 hr apart, and was used for all treatments of both experiments. The pasteurized soil mixture had a pH of 7.7, 3.2 μg g^{-1} ammonium N, 2.6 μg g^{-1} nitrate N, 12.5 μg g^{-1} Olsen P, 48.8 μg g^{-1} K, 355.8 μg g^{-1} Ca, 48.3 μg g^{-1} Mg, and

1.1 μg g^{-1} Mn according to analyses by the Kansas State Agronomy Soil Testing Laboratory (Manhattan, Kansas, USA).

We inoculated cone-tainers with multiple species of mycorrhizal fungi contained in pieces of fine roots of *Stenotaphrum secundatum* (Walt.) Kuntze lawn grass collected from the Gifford Arboretum. The roots contained *Sclerocystis rubiformis* Gerd. & Trappe, *Glomus clarum* Nicholson & Schenck (now *Rhizophagus clarus* Walker & Schußler) and several species of *Glomus sensu lato* (Weremijewicz & Janos 2013). Freshly-collected roots were cut into 1–2 cm pieces by hand, and 110 g of roots were mixed uniformly throughout the entire soil volume before the cone-tainers were filled. Non-inoculated cone-tainers received the same weight of root pieces that had been autoclaved for one hour, three times, 24 hr apart to partially control for the organic matter addition to inoculated plants. Non-inoculated plants also received a microbial filtrate made by soaking 110 g of freshly-collected *S. secundatum* roots in 1 L of water for 24 hr and then filtering the solution through Whatman #4 filter paper to exclude AM fungi. The microbial filtrate was added to the soil of the non-inoculated treatment before filling cone-tainers. Soil of the inoculated cone-tainers was given the same volume of distilled water. For *E. canadensis*, we additionally inoculated each cone-tainer with 1 mL (ca. 333 spores/mL) of a commercial inoculant (Mycorrhizal Applications, Grants Pass, Oregon, USA) to try to avoid any inoculum limitation. The slurry of spores and root fragments was pipetted into the transplant hole. It contained four '*Glomus'* species (*G. intraradices* Schenck & Smith [now *Rhizophagus intraradices* (Krüger *et al.* 2012)], *G. etunicatum* Becker & Gerd. [now *Claroideoglomus etunicatum* Walker & Schußler], *G. mosseae* Gerd. & Trappe [*now Funneliformis mosseae* Walker & Schußler], and *G. aggregatum*

Schenck & Smith). At the same time, we gave non-inoculated plants an equal amount of spore slurry autoclaved as described above. Because of the potentially different suites of fungi that could have colonized *A. gerardii* and *E. canadensis*, and the different years in which we conducted their experiments, we focus on within-species allometry and stoichiometry rather than directly comparing the plant species' performance.

A. gerardii and *E. canadensis* were grown for 77 and 71 days after transplant (DAT), respectively, until growth began to slow. *A. gerardii* was grown from August 7, 2013 to October 22, 2013 (average daily temperature = 28.1 ± 1.2 °C SD) and *E. canadensis* from August 1, 2014 to October 10, 2014 (average temperature = 27.0 ± 1.2 °C SD). Beginning 7 DAT, we fertilized all cone-tainers twice per week: once with 10 mL of the designated P treatment and two days later with 10 mL of Hewitt's solution (lacking phosphate) with concentrations of: 2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM Ferric Citrate, 0.03 mM H_3BO_3, 0.011 mM $MnSO_4$, 0.002 mM $ZnSO_4$, 0.0003 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_20$, 0.1 mM $CuSO_4 \cdot 5H_20$.

Every week, we measured the length of the longest leaf of each plant to determine when growth began to slow. At harvest, we clipped shoots directly above the basal meristem and dried them for 7 days to constant weight at 60 °C for determination of shoot dry weight. We extracted root systems in gently running water over a 1 mm sieve and blotted them dry prior to determining their fresh weight. Subsequently, after randomly removing a subsample of fine roots from each root system and re-weighing the remaining roots, the remaining roots were dried to constant weight at 60 °C. We used the dry weight to fresh weight ratio to calculate the dry weights of entire root systems. Subsampled roots were preserved in 50 % ethanol until being stained and examined for

AM fungus colonization. Shoot and root dry weight data were summed to calculate the total dry weight of each plant. Dried leaf tissues were composited by treatment, and foliar N and P concentrations were determined by the Kansas State Agronomy Soil Testing Laboratory (Manhattan, KS).

We assessed the percentage root length colonized by AM fungi for the preserved, subsampled roots. We cleared the roots in 10 % KOH for 3 days, acidified them in 5 % HCl for 30 min, and then placed them in 0.05 % Trypan blue in lactoglycerol for 24 hours, all at room temperature. For *A. gerardii*, we scored mycorrhizal colonization from six randomly-selected plants per treatment. We placed 35 one-centimeter root segments from each plant on microscope slides and examined 105 magnified gridline intersections per plant (McGonigle *et al.* 1990). For *E. canadensis*, we combined root segments from all plants within a treatment, mixed them well, and mounted 67 segments from each treatment on slides, examining 201 intersections per treatment. We distinguished AM fungi at gridline intersections by the presence of blue-stained coenocytic external and internal hyphae with occasional unilateral projections, vesicles, or arbuscules.

Statistical analyses

We fitted phosphorus response curves to shoot and total dry weight data separately for *A. gerardii* and *E. canadensis* with Statistix v. 10.0 (Analytical Software, Tallahassee, FL, USA) to minimize squared deviations. The fitted curves conformed to the logistic equation given by Janos (2007), $W = A/(1 + b \bullet \exp(-SP))$, where $b = (A - I)/I$, W represents shoot dry weight, P is the concentration of added phosphorus, I is the y-axis intercept, A is the asymptote, and S is the slope of a tangent at the inflection point. We assessed normality of the residuals around the nonlinear regression fitted curve using the

Shapiro-Wilk normality test for each line. When the Shapiro-Wilk test indicated non-normal residuals, we rank ordered the residuals, and eliminated them one point at a time until the data fit the normality assumption. This resulted in the elimination of four points from the aboveground DW curve (one point from the 1ppm, 16ppm, 64 ppm and 128 ppm P treatments) and two points from the total DW curve (one point from 64ppm and 16 ppm P treatments) for the inoculated *A. gerardii* treatment.

Maximum responsiveness (denominated in grams) was calculated from the fitted curves as the largest vertical, positive difference for inoculated minus non-inoculated plants (Janos 2007). Dependence upon mycorrhizas (denominated in units of phosphorus concentration) was calculated as the phosphorus concentration at which plants without mycorrhizas attained 10 % of their asymptotic size under our experimental conditions.

We examined each host species' growth responses and foliar N and P concentrations with two-way, factorial analysis of variance (ANOVA). One *A. gerardii* individual with a root dry weight five standard deviations above the mean of the other plants in its treatment, and one *E. canadensis* with a total dry weight eight standard deviations below the mean of the other plants in its treatment were omitted from all analyses. Only total dry weights were heteroscedastic, and so were log-transformed. We calculated root-to-shoot ratios by dividing root dry weights by shoot dry weights, and then we examined the mean ratios for inoculated and non-inoculated plants using least-squares linear regressions versus log-transformed P concentrations. All statistical testing was performed with Statistix v. 10.0 using $a = 0.05$ to determine significance.

We visualized foliar N and P concentrations and contents with vector graphs (Swift & Brockley 1994; Haase & Rose 1995; Scagel 2003). For each plant species, we

relativized N and P concentrations and total dry weight versus the 1 μg g^{-1} treatment of non-inoculated and inoculated treatments separately.

We examined effects of treatments on root colonization by AM fungi with two-way, factorial ANOVAs for each host species separately, using P amendment and inoculation as factors. After testing percent colonized root length for heteroscedasticity with Levene's test, we arcsine-square root transformed all colonization data. We examined potential associations between non-transformed percentage root length colonized by AM fungi and log-transformed P concentrations (to reduce curvilinearity) by calculating Pearson's correlation coefficients. Association analyses of mycorrhizal colonization involved only inoculated plants.

Results

Plant growth and allometry

Plants in all treatments began the experiment similarly sized. At the first measurement at 7 DAT prior to beginning fertilization, mean longest leaf lengths of *A. gerardii* (two-way ANOVA, MYC: $F_{1,98} = 3.18$, $P = 0.08$, P concentration: $F_{6,98} = 0.39$, $P = 0.8821$, MYC x P concentration: $F_{6,98} = 0.17$, $P = 0.9834$) and of *E. canadensis* (two-way ANOVA, MYC: $F_{1,144} = 0.46$, $P = 0.4999$, P concentration: $F_{7,144} = 1.18$, $P = 0.3184$, MYC x P concentration: $F_{6,98} = 1.02$, $P = 0.4216$) were not statistically distinguishable.

At harvest, the main effect of P addition and the interaction between inoculation and P addition were significant for shoot, root and total dry weight of *A. gerardii*, and the main effect of inoculation was significant only for root and shoot dry weights (Table 4.1). Both shoot and root dry weights of *A. gerardii* increased with increasing P fertilization (Fig. 4.1a). Phosphorus addition had a significant main effect on *E. canadensis* shoot,

root and total dry weights (Table 4.1). Shoot and root dry weights showed a significant effect of inoculation, but total dry weights did not (Table 4.1). Root dry weights and total dry weights also showed a significant interaction between P addition and inoculation (Table 4.1). Inoculated *E. canadensis* plants were consistently heavier in shoot weights than non-inoculated plants, and shoot weights of both non-inoculated and inoculated plants increased with increasing concentrations of P fertilizer (Fig. 4.1b). Root dry weights of non-inoculated plants remained approximately the same at P additions 16 µg g^{-1} or less but exceeded inoculated plants at P additions of 32 µg g^{-1} or greater, thereby increasing total dry weights of non-inoculated plants at these P additions (Fig. 4.1b).

Root-to-shoot ratios decreased with increasing P fertilization for non-inoculated *A. gerardii* ($F_{1,38} = 10.78$, $P = 0.022$; R/S = -0.01*P concentration + 4.35; Fig 4.2a) but were not affected significantly for inoculated plants of either *A. gerardii* ($F_{1,42} = 0.09$, $P = 0.7693$; Fig. 4.2a) or *E. canadensis* ($F_{1,77} = 0.87$, $P = 0.3544$; Fig. 4.2b). Non-inoculated *E. canadensis*, however, showed a significant increase in root-to-shoot ratio as P fertilizer concentration increased ($F_{1,78} = 62.81$, $P < 0.0001$; R/S = 0.006*P concentration + 0.97; Fig. 4.2b). Although we did not compare them statistically, it is important to note that the grand mean root-to-shoot ratio of *A. gerardii* was 3–4 times the mean ratio for inoculated *E. canadensis* (Fig. 4.2).

We fitted logistic curves to both total dry weights and shoot dry weights to compare their interpretations, particularly because root-to-shoot ratios differed among inoculation and P addition treatments. When we fitted curves for shoot dry weights of non-inoculated plants, we calculated that the dependence of *A. gerardii* was at -5.0 µg g^{-1} P. In other words, 10% of its asymptotic growth was predicted to occur at an estimated

5.0 μg g^{-1} P less than was available in the base substrate. Mycorrhiza disadvantage occurred above 18.1 μg g^{-1} P and *A. gerardii* had maximum responsiveness to mycorrhizas at 7.0 μg g^{-1} P, with the shoots of inoculated plants 1.5 times heavier than those of non-inoculated plants (Table 4.2; Fig. 4.3a). Curves fitted to total dry weights revealed that mycorrhiza dependence was estimated to be -19.7 μg g^{-1} P, mycorrhizas were disadvantageous above 7.1 μg g^{-1} P (Fig. 4.3b), and inoculated *A. gerardii* plants were 1.1 times heavier than non-inoculated plants at 1.0 μg g^{-1} P (Table 4.2). We calculated *E. canadensis*'s mycorrhiza dependence to be -16.6 μg g^{-1} P (Table 4.2) and a maximum responsiveness to mycorrhizas of 1.1 times the shoot weight of non-inoculated plants at 7.0 μg g^{-1} P (Table 4.2; Fig. 4.3c). Although mycorrhizas were not disadvantageous for *E. canadensis* based upon shoot dry weights (Table 4.2; Fig. 4.3c), total plant dry weights revealed that mycorrhizas were disadvantageous above 20.8 μg g^{-1} P (Fig. 4.3d). *E. canadensis* total dry weight mycorrhiza dependence was estimated as -52.6 μg g^{-1} P (Table 4.2), maximum responsiveness at 4.4 μg g^{-1} P at which inoculated plants were 1.2 times heavier than non-inoculated plants.

Phosphorus and nitrogen stoichiometry

Mycorrhizas improved P and N nutrition for *A. gerardii* but not for *E. canadensis*. For all P additions, non-inoculated *A. gerardii* individuals had lower mean foliar P ($F_{1,6}$ = 9.69, P = 0.0208) and N concentrations ($F_{1,6}$ = 7.91, P = 0.0307) than inoculated individuals (Table 4.3). Non-inoculated and inoculated *E. canadensis* foliar mean concentrations did not differ for either P ($F_{1,7}$ = 0.64, P = 0.4508) or N ($F_{1,7}$ = 2.68, P = 0.1348; Table 4.3).

Vector graphs of relative mean foliar P concentrations per treatment versus relative mean total dry weights (relativized by the 1 μg g^{-1} P treatment) showed that at and above P fertilizer concentrations of 32 μg g^{-1} P, both non-inoculated and inoculated *A. gerardii* tended to concentrate P in proportion to their weight (Fig. 4.4a). For inoculated *A. gerardii*, however, mean relative dry weight changed little at and above 16 μg g^{-1} P addition, but the mean relative dry weights of non-inoculated plants tended to increase with increasing P fertilizer concentration. Both non-inoculated and inoculated *E. canadensis* tended to accumulate luxury P (Fig. 4.4b), but similar to inoculated *A. gerardii*, the relative mean dry weights of inoculated *E. canadensis* changed little with increasing P fertilizer concentration while the relative mean total dry weights and foliar P concentrations of non-inoculated plants both increased.

Nitrogen vector graphs for both inoculated *A. gerardii* (Fig. 4.4c) and inoculated *E. canadensis* (Fig. 4.4d) suggest a slight dilution of foliar N with increased P fertilizer concentration, although there was little change in relative mean dry weight for either species at 8 μg g^{-1} added P or higher. Non-inoculated plants of both species, however, tended to maintain little-changed relative mean concentrations of foliar N even though relative mean total dry weights increased with increasing P fertilizer concentration.

N:P ratios decreased with increasing P fertilizer concentrations for inoculated ($F_{1,6}$ = 26.24, P = 0.0037; N:P = -0.23*P concentration + 1.17) and non-inoculated ($F_{1,6}$ = 54.35, P = 0.0007; N:P = -0.41*P concentration + 1.53) *A. gerardii* plants (Fig. 4.5a; Table 4.3), but at different rates ($F_{1,10}$ = 6.54, P = 0.0285). N:P ratios decreased at similar rates ($F_{1,12}$ = 0.18, P = 0.6814) with increasing P fertilizer concentrations for both inoculated ($F_{1,7}$ = 131.63, P <0.00001; N:P = -0.33*P concentration + 1.21) and non-

inoculated ($F_{1,7}$ = 108.04, P <0.00001; N:P = -0.36*P concentration + 1.25) *E. canadensis* plants (Fig. 4.5b). At low P fertilizer concentrations below 8 µg g^{-1}, non-inoculated *A. gerardii* exhibited N:P ratios greater than 20, suggesting potential P limitation of growth, but inoculated *A. gerardii* and all *E. canadensis* may have been co-limited by P and N. At and above 8 µg g^{-1} P (except for non-inoculated *A. gerardii* at 16 µg g^{-1} P) all plants of both species had N:P ratios consistent with potential N limitation.

Root colonization

Root colonization by AM fungi was negatively correlated with weekly P addition (n = 42, Pearson's r = -0.47, P = 0.0084) only for *A. gerardii*, and not significantly so for *E. canadensis* (n = 8, Pearson's r = -0.63, P = 0.0960). *A. gerardii* had the greatest colonization by AM fungi at 4 µg g^{-1} P with an average of 51 % root length colonized, and *E. canadensis* had the greatest colonization at 1 µg g^{-1} P with 19 % root length colonized despite having received the commercial inoculum in addition to locally-collected inoculum. Inoculated *A. gerardii* individuals fertilized with 64 µg g^{-1} P had 6 % colonization, their lowest, but *E. canadensis* had their lowest colonization, 10 %, at 4 µg g^{-1} P. Non-inoculated plants of both species mostly remained without mycorrhizas for the duration of the experiments. Only four *A. gerardii* among 42 non-inoculated plants had any colonization: three of those had less than 1.5 % colonization and the forth had 5 % colonization. Non-inoculated *E. canadensis* (composited by P fertilizer concentration) had 0.4 % colonization at only a single P fertilizer concentration. Because colonization was minimal, we retained these plants in analyses.

Discussion

Plant growth and allometry

A. gerardii and *E. canadensis* were facultatively dependent on mycorrhizas for growth. Although *A. gerardii* was strongly dependent on mycorrhizas as we hypothesized, it was not literally an 'obligate' mycotroph as previously suggested (Hetrick, Kitt & Wilson 1986; Hetrick, Kitt & Wilson 1988; Hetrick, Wilson & Todd 1990; Hartnett, Hetrick & Wilson 1993). In our study, non-inoculated *A. gerardii* were able to equal the growth of inoculated plants with as little as an estimated 18.1 μg g^{-1} P supplied weekly (totaling 28 mg L^{-1} soil over our entire experiment), making it a facultative mycotroph as found recently by others (Miller *et al.* 2002; Grman 2012; Thorne, Rhodes & Cardina 2013). When the total amount of P applied throughout an experiment is summed, our results are similar to those of Grman (2012) who found mycorrhiza disadvantage for *A. gerardii* at total P additions above 86 mg L^{-1} soil and Thorne, Rhodes and Cardina (2013) who found disadvantage above 49 mg L^{-1} soil total P addition. Thorne, Rhodes and Cardina (2013) used field collected soil, but Grman (2012) used a nutrient-poor sand-soil mixture like ours, so base substrate differences in P availability undoubtedly contribute to the somewhat different threshold values for mycorrhiza disadvantage. Although Hetrick, Kitt and Wilson (1986) had found non-inoculated *A. gerardii* did not respond to 30 μg g^{-1} P with improved growth, they added dry, soluble P only once at the start of their experiment, providing a total P addition of only 0.01 mg L^{-1} soil. Hence, we suggest that *A. gerardii* is physiologically facultatively mycotrophic when substrate P concentrations are maintained artificially high, but it may be 'ecologically obligately mycotrophic' (Janos 1980), incapable of survival and growth without mycorrhizas at naturally

occurring concentrations of P, which generally are 5–20 μg g^{-1} in tallgrass prairies (Bray 1; Johnson *et al.* 2010).

The least dependent species, *E. canadensis,* was not the least responsive as hypothesized, which underscores the merits of distinguishing between 'dependence' and 'responsiveness.' Both *A. gerardii* and *E. canadensis* achieved maximum responsiveness, at similar P fertilizer concentrations of 1.0–7.0 μg g^{-1}, likely within the natural range of P concentrations in prairies, because our base substrate contained 12.5 μg g^{-1} Olsen P and our soluble P additions effectively were pulsed because of leaching by subsequent watering. We found that *E. canadensis* can greatly increase carbon allocation belowground, thereby potentially compensating for a lack of mycorrhizas, which is consistent with it being less dependent on mycorrhizas than *A. gerardii*. Aboveground data alone, however, failed to indicate that mycorrhizas could be disadvantageous to *E. canadensis*, and they additionally gave a dependence estimate only one-third that calculated from total dry weight. That provides a caveat for interspecific competition studies in which the roots of different species are difficult to disentangle such that only aboveground dry weight data are considered.

Plant species with high root-to-shoot ratios have been thought to be less responsive to mycorrhizas than those with low ratios (Koide *et al.* 1988), but a recent meta-analysis by (Maherali 2014) found no relationship between root traits and responsiveness to mycorrhizas (*sensu* Janos 2007). In our study, although we found *A. gerardii* to be the most dependent on mycorrhizas, it had a high root-to-shoot ratio that was three times greater than that of *E. canadensis*. We were not able to measure specific root length, but we observed that the roots of *A. gerardii* are considerably coarser than

those of *E .canadensis*, and this probably explains the high root-to-shoot biomass ratio of *A. gerardii*. High specific root lengths, as we infer for *E. canadensis*, may characterize at least some plant species well able to take up mineral nutrients without mycorrhizas (Hetrick 1991).

Greater proportional allocation to roots by non-inoculated *E. canadensis* than by *A. gerardii* is consistent with the different abilities of the species to grow without mycorrhizas. Non-inoculated *E. canadensis* increased root production and *A. gerardii* decreased root production as P fertilizer concentration increased, but inoculated plants did not change root-to-shoot ratios. Increased biomass allocation to roots is a common response to N and P deficiencies (Güsewell 2004), although it is especially strongly associated with N limitation (Andrews *et al.* 1999). If *E. canadensis* experiences a flush of mineral nutrient release from accelerated organic matter decomposition in the early spring when it typically begins to grow (Weaver & Fitzpatrick 1934), and if mycorrhizal fungus activity is retarded by low night temperatures (Liu, Wang & Hamel 2004) or saturated soils, then *E. canadensis* might be under strong natural selection to prioritize root production.

Phosphorus and nitrogen stoichiometry

Plant tissue P and N concentrations are acknowledged plant functional traits (McGill *et al.* 2006; Westoby & Wright 2006; Friesen *et al.* 2011), and our work illustrates how arbuscular mycorrhizas can affect them. When plants were not inoculated, as soil P fertilizer concentration increased, relative tissue P concentrations and relative total dry weights tended to increase similarly for *A. gerardii*, but tissue P increased much more rapidly than dry weight for *E. canadensis*, perhaps because of its increasing root-to-shoot

ratio. When inoculated, however, both *A. gerardii* and *E. canadensis* accumulated luxury P at and above 8 μg g^{-1} weekly P fertilization, consistent with something other than P limiting growth.

Inoculation increased P-response curve slopes, which can be used to quantify P uptake and use efficiency (Koide 1991) because they reflectthe maximum rate of uptake and conversion of a unit increase in P availability into plant biomass. Surprisingly, both plant species without mycorrhizas had similar P uptake and use efficiencies based upon total dry weight. As the less mycorrhiza-dependent species, we expected *E. canadensis* to have an ability to acquire and use P efficiently without mycorrhizas. Similar slopes for both *E. canadensis* and *A. gerardii* without mycorrhizas suggest similar physiologies between the two plant species, and so the differences between them when mycorrhizal are most likely because of differences in P acquisition.

In accord with our hypothesis, mycorrhizas increased the foliar concentrations of both P and N in *A. gerardii*. Despite our use of a nutrient-poor sand mixture, both *A. gerardii* and *E. canadensis* had foliar P and N concentrations similar to or in excess of previously reported values for plants in native soils, suggesting that our plants were not abnormal. Kemp *et al.* (1995), Loaiza, Jonas and Joern (2011), and Griffin and Jung (1983) reported *A. gerardii* P concentrations of 0.09–0.2 %, and most of our values fall within or above that range except for non-inoculated plants at the three lowest P fertilizer concentrations. Our values also mostly fall within the 1.0–1.5 % range of N concentrations reported for *A. gerardii* by Delucia, Heckathorn and Day (1992), Loaiza, Jonas and Joern (2011), and Owensby, Coyne and Auen (1993). Our *E. canadensis* grown at P fertilizer concentrations above 8 μg g^{-1} had P tissue concentrations higher

than the 0.09–0.17 % reported for plants in native soils (Klabi *et al.* 2014), while N concentrations at all P fertilizer concentrations exceeded the 0.83–1.43 % reported by Klabi *et al.* (2014). It is puzzling how *E. canadensis* was the most responsive of our two host species when neither its foliar P nor N concentrations differed between non-inoculated and inoculated plants across all P fertilizer concentrations. Perhaps inoculated *E. canadensis* tissue P concentrations were elevated in roots instead of in shoots, or another element other than P or N limited growth and mycorrhizas increased its uptake.

Generally low and decreasing N:P ratios suggest that N limitation increased for both plant species as P fertilizer concentration was increased. Vector analyses, however, show relative N concentrations remained essentially constant with increasing relative dry weights of both plant species when not inoculated and generally diminished with increased relative dry weights of inoculated plants. Increased P concentrations at high soil P fertilizer additions and constant N uptake likely led to decreasing N:P ratios for both plant species. As the less dependent plant, *E. canadensis* was able to increase its root-to-shoot ratio to capture N at high P fertilizer, which may have resulted in N:P ratios similar to those of inoculated plants. For *A. gerardii*, the presence of mycorrhizas may have increased N uptake, as suggested by higher N:P ratios for inoculated plants than for non-inoculated plants. N:P ratios reflect the balance between N, P, and C within plant tissues (Güsewell 2004), and at high P fertilizer concentrations, it is likely that the carbon cost of mycorrhizas limited the growth of both host species. Even though increasing P fertilization did diminish percentage colonized root length, neither host species fully suppressed mycorrhizas, perhaps because our soluble P additions were pulsed.

Root colonization

Facultatively mycotrophic plant species are thought to suppress colonization and to decrease carbon allocation to AM fungi to reduce the cost of non-beneficial mycorrhizas (Treseder 2004; Kiers *et al.* 2011). Nevertheless, positive growth responses to mycorrhizas by both host species in our experiments suggests that the AM fungus species we used were effective mutualists at least at low soil P availability.

As we increased phosphorus fertilizer concentration, however, percent colonized root length did decrease significantly for *A. gerardii*. The high Pearson's correlation coefficient of -0.63 for *E.* canadensis suggests that its colonization also diminished with increased P fertilization and that its lack of significance most likely was a consequence of a small sample size. Colonization of *E. canadensis* in our study was similar to that reported by Wilson and Hartnett (1998), around 15 %.

The significant negative relationship (Pearson's r = -0.47) between P fertilization and root colonization for *A. gerardii* appears to contradict Grman's (2012) finding that highly mycorrhiza-dependent, C4 plants are unable to suppress mycorrhizal colonization. Abundant root production by *A. gerardii* at P fertilizer concentrations of 16 μg g^{-1} and higher, however, effectively may have 'diluted' colonization by AM fungi that may not have spread as quickly as roots grew. The greatest mean root colonization of both *A. gerardii* and *E. canadensis* coincided with their lowest mean root dry weights, but because we did not measure fine root length, we do not know if the total length of mycorrhizal roots remained constant or diminished as P fertilizer concentration was increased. Regardless of possible reductions of mycorrhizal root length, both host species when inoculated were mycorrhizal at all P fertilizer concentrations.

Both host species received similar wild-collected inoculum, but the four additional *Glomus* species provided to *E. canadensis* might have enhanced its P acquisition, thereby making its responsiveness to mycorrhizas similar to that of *A. gerardii.* Different species of AM fungi can differentially benefit hosts (Kiers *et al.* 2011). Nevertheless, total root colonization was low for *E. canadensis*, consistent with it being less dependent upon mycorrhizas than *A. gerardii.*

Conclusion

Plant functional traits that are influenced by mycorrhizas —P uptake and use efficiency, root-to-shoot ratios, and foliar P and N concentrations — likely strongly influence the ecological niches of plant species. In particular, requirement of mycorrhizas, 'mycorrhiza dependence,' can restrict establishment of a species in sites lacking AM fungal inocula, such as post-agricultural fields (Kurle and Pfleger 1994, Richter and Stutz 2002) and mine reclamation sites (Gould and Liberta 1981, Thorne et al. 2013). We have shown that both *A. gerardii* and *E. canadensis* are able to grow without mycorrhizas at high P fertilities, which is consistent with both species being facultatively mycotrophic (Janos 2007). Although *A. gerardii* is more dependent upon mycorrhizas than is *E. canadensis*, both plant species are likely to be maximally responsive to mycorrhizas under the P availability conditions of native tallgrass prairies. Even though in our experiments mycorrhizas most improved the P uptake and use efficiency of *E. canadensis*, that could have been a consequence of the additional species of AM fungi with which we inoculated it. This underscores that 'responsiveness' is influenced not only by plant species P-use physiology but also by the complement of AM fungus species

forming mycorrhizas together with the soil environmental conditions in which they are functioning (Janos 2007).

Although dependence and responsiveness must be determined experimentally, we have shown that they reveal otherwise hidden aspects of plant functional traits with respect to mineral nutrition. For example, if mycorrhiza function is impeded by cold temperature or soil saturation when *E. canadensis* emerges in the early spring, this relatively little-dependent species can grow without mycorrhizas, especially by favoring root over shoot growth. In contrast, the mycorrhiza dependent *A. gerardii* emerges in late spring (Weaver & Fitzpatrick 1934) and may join established common mycorrhizal networks that increase its mycorrhizal colonization (Weremijewicz & Janos 2013). *A. gerardii* allocated proportionally more energy and materials to root production than did *E. canadensis*, and that strategy likely helps *A. gerardii* to thrive in hot, dry, late-summer environments by providing for extensive mycorrhizas. Effects of mycorrhizas on functional traits of *E. canadensis* and *A. gerardii* might contribute to their respective sub-dominant and dominant status in tallgrass prairies.

Table 4.1. Two-way, factorial ANOVA (MYC = presence/absence of mycorrhizas and P = concentration of weekly phosphorus addition) results for shoot and root dry weights and total dry weights at harvest 76 days after transplant (DAT) for *Andropogon gerardii* and 70 DAT for *Elymus canadensis*

		Andropogon gerardii			*Elymus canadensis*		
Growth parameter	Factor	*df*	*F*	*P*	*df*	*F*	*P*
Shoot Dry Weight	MYC	1,70	2.64	0.1084	1,143	**21.51***	**< 0.0001**
	P	6,70	**6.92**	**< 0.0001**	7,143	**11.67**	**< 0.0001**
	MYC x P	6,70	**2.84**	**0.0156**	7,143	0.98	0.4505
Root Dry Weight	MYC	1,70	**9.77**	**0.0026**	1,143	**24.58**	**< 0.0001**
	P	6,70	**5.15**	**0.002**	7,143	**13.28**	**< 0.0001**
	MYC x P	6,70	**2.68**	**0.0211**	7,143	**7.70**	**< 0.0001**
Total Dry Weight	MYC	1,70	**8.19**	**0.0056**	1,143	3.17	0.0771
	P	6,70	**5.85**	**0.0001**	7,143	**15.17**	**< 0.0001**
	MYC x P	6,70	**2.81**	**0.0166**	7,143	**4.01**	**0.0005**

* Significant differences ($\alpha = 0.05$) are shown in bold

Table 4.2. Parameters, dependence and responsiveness from logistic phosphorus-response curves fitted to shoot dry weights (DW) and total dry weights versus concentrations of weekly phosphorus additions for non-inoculated (Non-inoc.) and inoculated (Inoc.) *Andropogon gerardii* and *Elymus canadensis* plants

	Andropogon gerardii				*Elymus canadensis*			
	Shoot DW		Total DW		Shoot DW		Total DW	
Parameter	Non-inoc.	Inoc.	Non-inoc.	Inoc.	Non-inoc.	Inoc.	Non-inoc.	Inoc.
Intercept (g)	0.02	0.02	0.09	0.11	0.10	0.06	0.22	0.15
Slope (g DW/ P µg g^{-1})	0.07	0.37	0.09	0.12	0.20	1.3	0.05	0.91
Asymptote (g)	0.10	0.05	0.43	0.21	0.14	0.15	0.37	0.28
Dependence (P µg g^{-1})	-5.0		-19.7		-16.6		-52.6	
Maximum responsiveness (g)	0.015		0.015		0.015		0.043	
P concentration for maximum responsiveness (µg g^{-1})	7.0		1.0		7.0		4.4	
Upper P concentration (µg g^{-1}) at which non-inoculated and inoculated P-response curves intersect	18.1		7.1		NA*		20.8	

* NA = not available

Table 4.3. Phosphorus and nitrogen concentrations (%) and N-to-P ratios (N:P) of non-inoculated (Non- inoc.) and inoculated (Inoc.) *Andropogon gerardii* and *Elymus canadensis* individuals across a gradient of weekly P concentration additions

	Andropogon gerardii						*Elymus canadensis*					
	Phosphorus (%)		Nitrogen (%)		N:P		Phosphorus (%)		Nitrogen (%)		N:P	
P ($\mu g\ g^{-1}$)	Non-inoc.	Inoc.	Non-inoc.	Inoc.	Non-inoc.	Inoc.	Non-inoc.	Inoc.	Non-inoc.	Inoc.	Non-inoc.	Inoc.
1	0.05	0.12	1.12	1.57	23.75	12.56	0.10	0.10	1.88	2.01	19.79	19.15
2	0.04	0.10	1.27	1.33	28.52	13.63	0.12	0.15	1.95	1.84	15.77	12.54
4	0.04	0.13	1.10	1.71	26.70	13.62	0.18	0.17	1.87	1.79	10.40	10.56
8	NA*	NA	NA	NA	NA	NA	0.27	0.26	1.94	1.86	7.10	7.22
16	0.10	0.15	1.28	1.25	13.50	8.40	0.33	0.31	1.84	1.82	5.56	5.86
32	0.17	0.30	1.38	1.53	7.95	5.02	0.39	0.41	1.80	1.81	4.65	4.44
64	0.22	0.27	1.22	1.48	5.61	5.45	0.42	0.42	1.83	1.72	4.37	4.14
128	0.27	0.23	1.15	1.32	4.25	5.64	0.49	0.42	1.85	1.63	3.78	3.86

* NA = not available

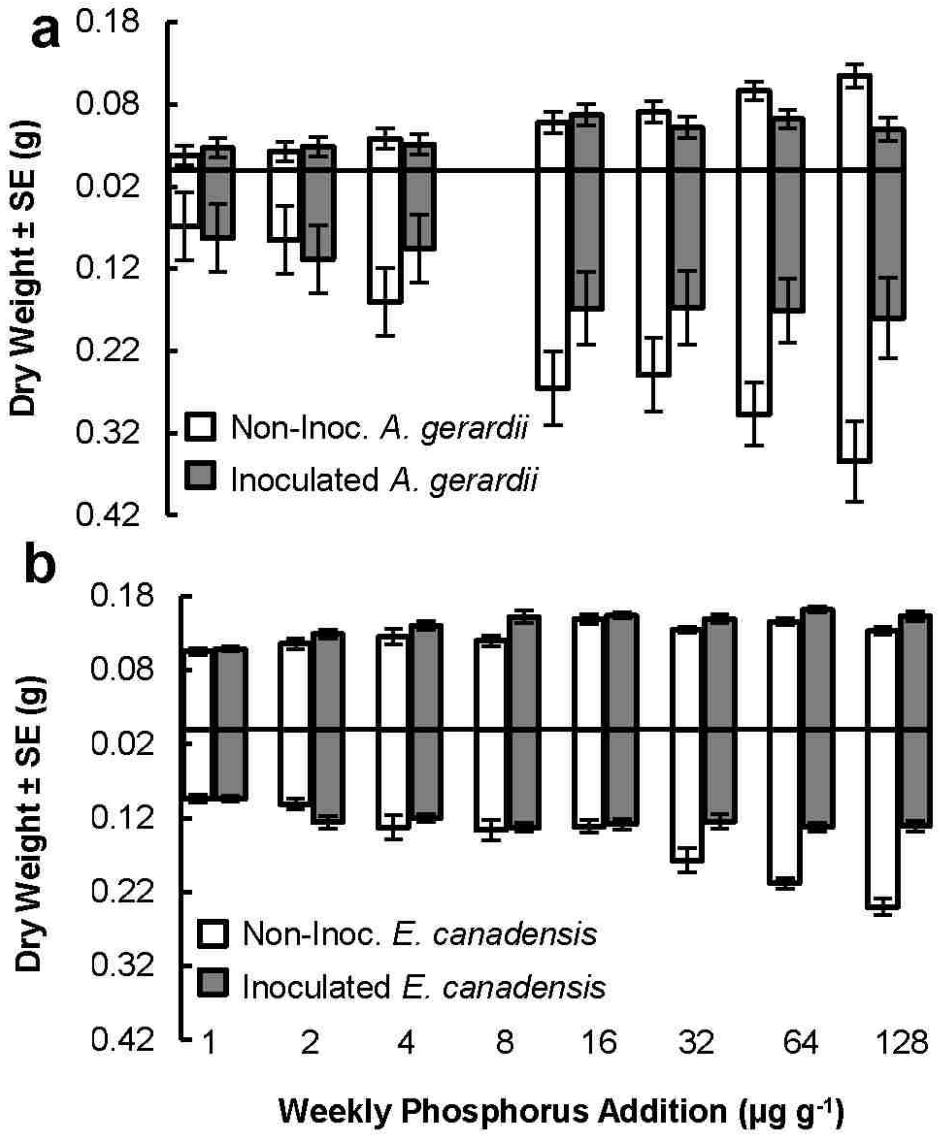

Figure 4.1. Mean dry weights ± SE (g) aboveground (bars above the x-axis) and belowground (positive-value bars below the x-axis) of non-inoculated plants or inoculated plants versus the concentrations of 10 mL weekly phosphorus additions (µg g^{-1}) for *Andropogon gerardii* (**a**) and *Elymus canadensis* (**b**). ANOVA results are shown in Table 4.1.

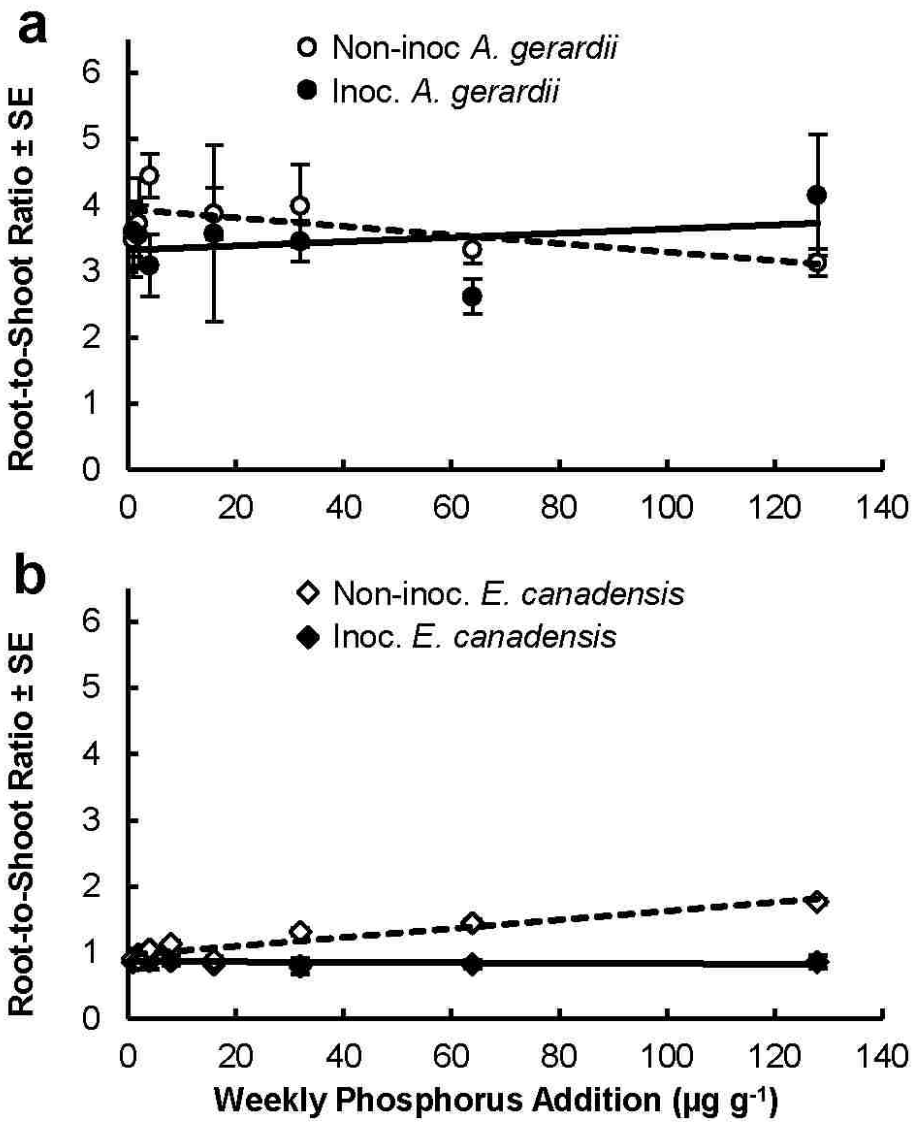

Figure 4.2. Mean root-to-shoot ratios (root dry weight/shoot dry weight ± SE) versus concentrations of 10 mL weekly phosphorus additions (µg g^{-1}) for inoculated (solid lines) and non-inoculated (dashed lines) *Andropogon gerardii* (**a**) and *Elymus canadensis* (**b**) plants.

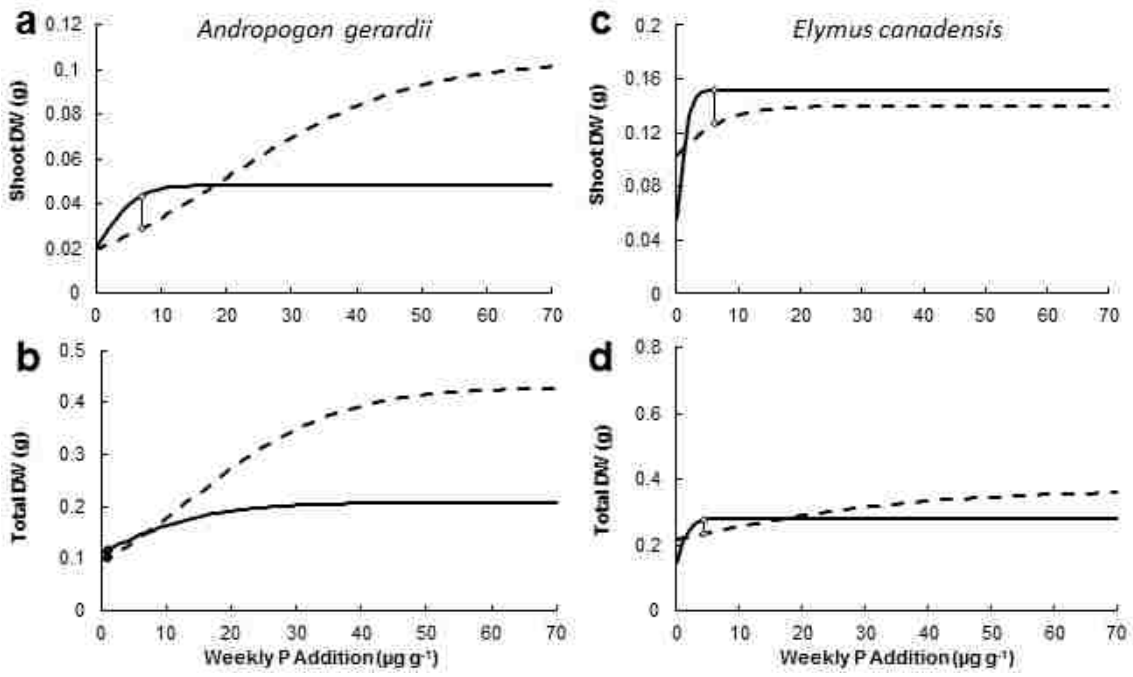

Figure 4.3. Logistic phosphorus-response curves fitted to shoot dry weights (DW, g; **a**, **c**) and total dry weights (**b**, **d**) versus concentrations of 10 mL weekly phosphorus additions (µg g^{-1}) for *Andropogon gerardii* (**a**, **b**) and *Elymus canadensis* (**c**, **d**) non-inoculated (dashed lines) and inoculated (solid lines) with arbuscular mycorrhizal fungi. Absolute maximum responsiveness to mycorrhizas is represented by solid vertical lines connecting open diamonds in each panel. Parameters for each curve are shown in Table 4.2.

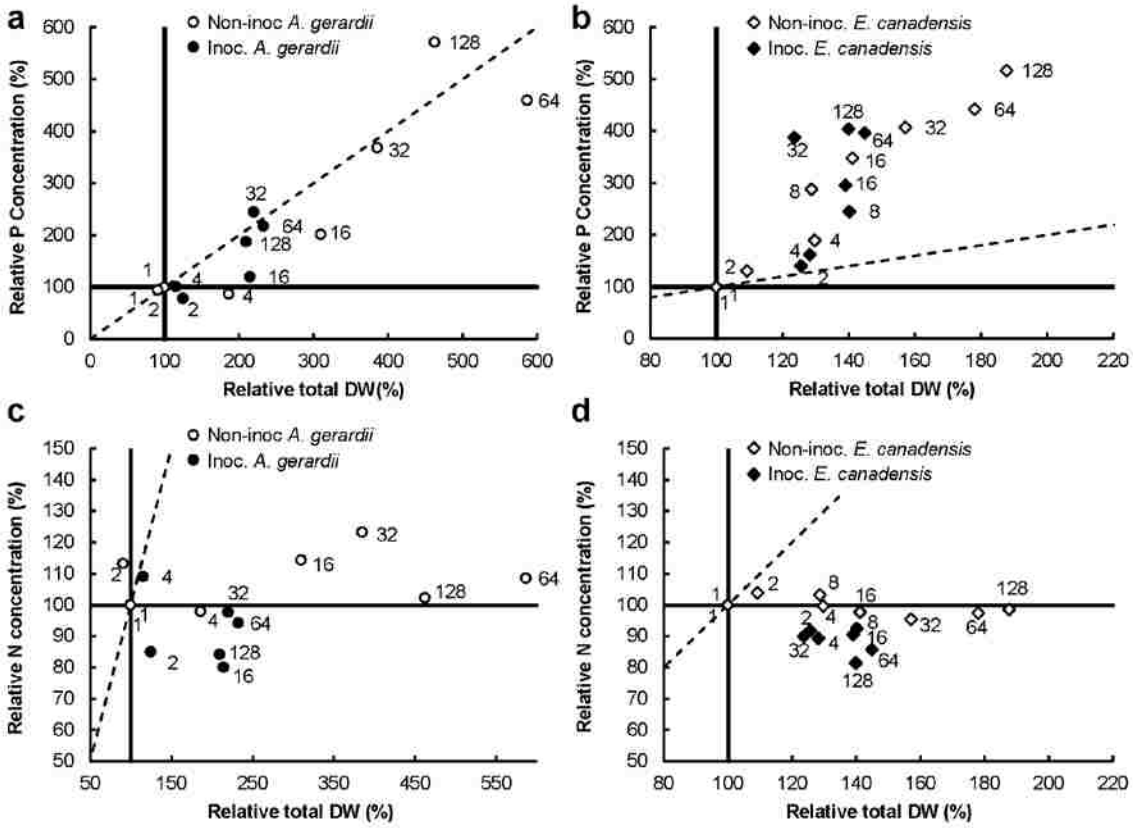

Figure 4.4. Comparison of leaf tissue relative phosphorus (**a, b**; 100 * $[P_{conc}]/[P_{1\ \mu g\ g^{-1}}]$ where P_{conc} represents each P addition concentration) and nitrogen (**c, d**; calculated similarly to relative P) concentrations versus relative total dry weight (DW; calculated similarly to relative P concentrations) for *Andropogon gerardii* (**a, c**) and *Elymus canadensis* (**b, d**) non-inoculated or inoculated with arbuscular mycorrhizal fungi at different concentrations of weekly 10 mL P additions (μg g^{-1} noted next to symbols). The vertical and horizontal heavy solid lines are at 100 % DW and P or N, respectively. The dashed diagonal line represents equal proportional changes in leaf tissue relative phosphorus concentration and relative total dry weight; points above the diagonal line, between it and the heavy vertical line, suggest luxury accumulation and storage, while

points between the diagonal line and the heavy horizontal line suggest dilution by plant growth (see Scagel 2003).

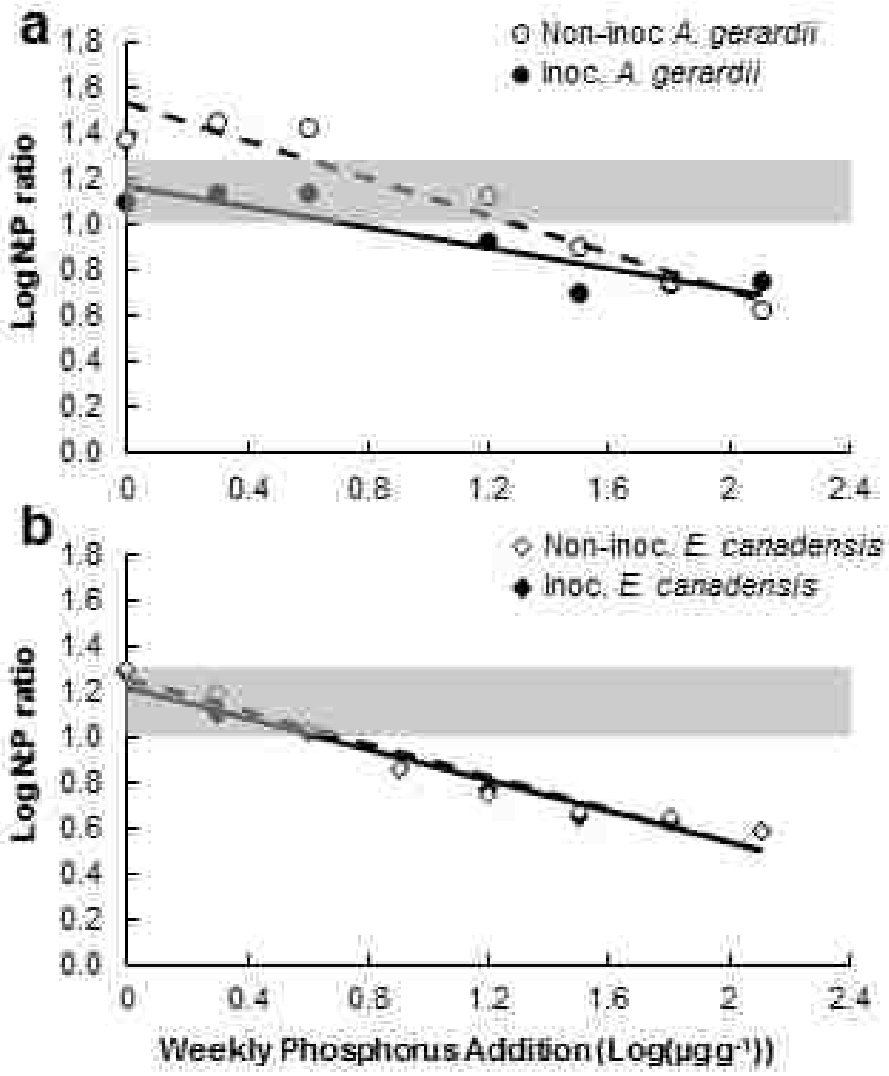

Figure 4.5. Log-transformed N:P ratios (Log_{10}([N]/[P]) versus log-transformed concentrations of weekly P additions (Log_{10}[µg g^{-1}]) for inoculated (solid lines) and non-inoculated (dashed lines) *Andropogon gerardii* (**a**) and *Elymus canadensis* (**b**). The shaded area indicates N:P between 10 and 20 (Güsewell 2004) which separates potential N limitation (above the shading) from potential P limitation (below the shading). N and P are potentially co-limiting within the shaded area.

Chapter Five

Common mycorrhizal networks foster overyielding by *Andropogon gerardii* and enhance survival of *Elymus canadensis*

Summary

- Arbuscular mycorrhizal fungi associate with roots of the majority of land plants and supply up to 80% and 25% of their P and N requirements, respectively. These fungi form extensive common mycorrhizal networks (CMNs) while foraging for mineral nutrients in the soil, and may interconnect neighboring root systems of many different plant species. The importance of CMNs is their influence on the distribution of limiting mineral nutrients among plants, affecting the symmetry of competition among interconnected individuals.
- In a factorial experiment, we examined how CMNs affect belowground interactions between populations of *Andropogon gerardii*, a highly mycorrhiza-dependent, dominant prairie grass and *Elymus canadensis*, a weakly dependent, subordinate prairie species. We examined if CMNS amplify intraspecific competition for both species, resulting in skewed size hierarchies, and if CMNs contribute to an increased competitive ability of *A. gerardii* over *E. canadensis* in mixture.
- We found CMNs improved survival of both *A. gerardii* and *E. canadensis* overall, but intensified intraspecific competition for *A. gerardii*. When grown in mixture with *E. canadensis, A. gerardii* overyielded total aboveground biomass, suggesting CMNs contributed to stronger intraspecific than interspecific

interactions. We found that severing CMNs increased mortality in *E. canadensis* populations, and this was likely caused by a reduced ability in water uptake.

- CMNs may have different roles in plant performance depending upon species' physiological requirements. Even in the absence of significant growth improvement, CMNs can enhance plant survival. In spite of amplifying asymmetric competition belowground, CMNs were associated with overyielding by *A. gerardii* in mixtures.
- Our findings suggest that it is not just the presence of AM fungi which has implications for grasslands, but it is essentially the interconnecting hyphae of CMNs that mediate plant interactions and consequently affect population structure and community composition.

Background

Over the last century of plant research, it has remained elusive when plant interactions will be competitive, neutral, or facilitative. This enigma of plant interactions may persist because most investigations have failed to consider the role of ubiquitous common mycorrhizal networks (CMNs) formed by symbiotic arbuscular mycorrhizal fungi.

Arbuscular mycorrhizal (AM) fungi associate with the majority of all plant species (Smith & Read, 2008) and do not form on individual plants in isolation, the way they often have been investigated. Instead, arbuscular mycorrhizas form when hyphae connected to one root system branch throughout the soil while foraging for mineral nutrients, encounter, and colonize the roots of another plant, forming CMNs. The importance of CMNs in plant interactions is their ability to influence the distribution of limiting mineral nutrients among plants, affecting the symmetry of competition among

interconnected individuals. It is likely that most research on plant interactions in natural soils may have incorporated CMNs unwittingly, but until recently, few investigators have attempted to distinguish the effects of CMNs.

Most research investigating the role of AM fungi in competitive interactions has compared treatments with AM fungi to those without fungi (e.g. Hartnett *et al.*, 1993; West, 1996; Smith *et al.*, 1999) instead of comparing plants interconnected by CMNs or severed from CMNs, thereby leaving ambiguous how CMNs contribute to belowground interactions. Because many species differ in their responsiveness to mycorrhizas – the change in growth when colonized by mycorrhizas – (sensu Janos, 2007), the presence of mycorrhizal fungi can alter the outcomes of competition between species. Dominant species, such as some C4 grasses, are likely to be highly responsive to AM fungi and to grow very poorly when lacking mycorrhizas, thereby entirely crippling their ability to compete (Hetrick *et al.*, 1989; Hartnett *et al.*, 1993; Hetrick *et al.*, 1994). Thus, the presence of mycorrhizas has been shown to increase the abundance of dominant species (Fitter, 1977; Hartnett *et al.*, 1993; Hartnett & Wilson, 1999; Smith *et al.*, 1999) and to diminish plant species diversity (Urcelay & Diaz, 2003) on a local scale. In contrast, when subdominant species, such as forbs, are highly responsive to mycorrhizas, the presence of AM fungi may increase diversity, as was found by Grime *et al.* (1987). Furthermore, the diversity of AM fungi within a community may also influence the diversity of plant species (Gange *et al.*, 1993; van der Heijden *et al.*, 1998). Studies investigating the effect of the presence or absence of AM fungi, however, focus more on individual host species responses and reveal little about how AM fungi and their CMNs may mediate plant interactions.

In nature, CMNs may have consequences for population structuring. CMNs have been found to amplify intraspecific competition for *A. gerardii* populations (Weremijewicz & Janos, 2013), ultimately affecting the shapes of size distributions. The shapes of size distributions can reveal the type of competition within plant populations. Populations are known to change through time from symmetric, normal size distributions shortly after germination to right- skewed ones as plants grow and age. Asymmetric competition, also known as dominance and suppression, is a major factor that can skew a size distribution because large individuals obtain disproportionate shares of a limiting resource, thereby suppressing the growth of small individuals. Although belowground competition has been widely considered "symmetric" by being proportional to root system size (Weiner, 1990; Weiner *et al.*, 1997; Cahill & Casper, 2000), the roles of roots and CMNs had not been separated prior to Weremijewicz and Janos (2013). In their experiment, asymmetric competition in high-density microcosms resulted from large individuals preempting mineral nutrient acquisition by small plants through CMNs. Individuals severed from CMNs had more uniform size distributions – a feature of symmetric competition – than those in CMNs.

Recent research suggests that CMNs may amplify competition among plants when mineral nutrients are limiting by preferentially supplying them to those individuals that provide the most carbon to AM fungi. Root organ cultures have found that *in vitro*, CMNs limit phosphorus supply to small, carbon-poor roots but reward large, carbon rich roots with up to ten times more phosphorus than carbon-limited roots (Lekberg *et al.*, 2010). When connected to carbon-limited hosts, mycorrhizal fungi can accumulate phosphorus in their storage structures (vesicles) and hyphae instead of providing it to the

host (Hammer *et al.*, 2011). Unfortunately, root organ culture studies preclude transpiration, shoot phosphorus sinks, and diurnal changes in carbon supply. Consistent with *in vitro* root organ culture work but using whole plants, Weremijewicz *et al.* (*in review*) used ^{15}N tracing to find CMNs intensify competition among *A. gerardii* seedlings by preferentially distributing Mn and N to large, abundantly carbon-fixing host plants. Individuals that were small or shaded received little to no nitrogen that only could be acquired from neighboring soil by CMNs. "Reciprocal rewards" of mineral nutrients for carbon resulted in increased size inequalities for populations with intact CMNs versus those with severed CMNs.

Although reciprocal rewards may explain interactions among conspecific neighbors, other factors, such as the AM fungal species composition of CMNs, may affect nutrient dynamics in CMNs. AM fungus species differ in their rates of mineral nutrient for carbon exchange with host plants (Kiers *et al.*, 2011). Walder *et al.* (2012) found that although sorghum provided CMNs of *R. intraradices* or *F. mossae* with large amounts of carbon, it did not receive P or N in large quantities in return. Instead, flax, which provided little carbon to the fungi, received 94 % of ^{32}P and 80 % ^{15}N from the CMN when competing with sorghum which was not consistent with reciprocal rewards. Consistent with reciprocal rewards, however, Merrild *et al.* (2013) found that independent of species, it was large, abundant carbon-supplying plants that benefited most from CMNs. Tomato seedlings in a CMN of *Rhizophagus irregularis* with larger, older cucumber plants were suppressed in growth and P uptake. Clipping cucumbers or severing the CMN alleviated suppression of seedling growth and resulted in a 6.5-fold increase in P uptake. In nature, root systems are likely to be colonized by many species

of fungi, so a suite of AM fungus species may be needed to fully elucidate the role of CMNs in plant interactions.

We set out to investigate if differences in dependence on mycorrhizas – a plant species' reliance on AM fungi for mineral nutrient uptake – affect the outcomes of competition via CMNs. If CMNs benefit highly dependent species, dependence may be selected for because dependence is a function of plant genotype. Using two species with different dependencies on AM fungi – *A. gerardii*, a strongly dependent C4 grass species and *E. canadensis*, a less dependent C3 species (Hartnett *et al.*, 1993; Weremijewicz & Seto, *in revision*) – we investigated the relative strengths of interspecific and intraspecific interactions, and examined inequalities of size distributions. We hypothesized that CMNs would amplify intraspecific competition of both species, that when CMNs were intact, intraspecific interactions would have stronger effects on plant size than interspecific interactions, and that *A. gerardii* would be the stronger competitor within mixtures but severing CMNs would diminish suppression of *E. canadensis*.

Materials and Methods

We examined plant interactions across CMNs among *Andropogon gerardii* Vitman and *Elymus canadensis* L. seedlings within monocultures and mixtures by imposing two CMN treatments – intact CMNs and severed CMNs. Each treatment had three replicates; thereby 2 CMNs X 3 COMP. ("competition;" either of two monocultures or 1:1 mixture) X 3 replicates = 18 microcosms of 100 plants each in a square array. Species alternated in the 1:1 mixture such that the four nearest neighbors of any individual were the other species. Every seedling in a microcosm was individually grown in a modified Ray Leach Cone-tainer (2.5 cm diameter x 12.1 cm length; 49 mL volume).

We modified cone-tainers and constructed microcosms similarly to Weremijewicz and Janos (2013). Briefly, cone-tainers were drilled to have two openings on opposite sides that were wrapped with a silk screen mesh to confine roots (thereby preventing root overlap) but allow arbuscular mycorrhizal hyphal crossover among cone-tainers. Wooden box microcosms (52 cm × 52 cm × 10 cm deep) were constructed with a drilled, plywood bottom which precisely positioned cone-tainers 2.5 cm away from each of their nearest neighbors. Cone-tainers were arranged in a twelve-by-twelve square array, and the central one hundred (ten rows by ten columns) were surrounded by forty-four non-modified cone-tainers used to mitigate aboveground edge effects among plants (Weremijewicz and Janos, 2013). A nutrient-poor sand mixture of a 3:1 blend of 30–65 grade fine sand and 6–20 grade coarse sand (Table 5.1; Surface Prep Supply Co, Miami, FL) was poured into the interstices between cone-tainers. We manually rotated each cone-tainer in the severed CMNs treatment through a complete revolution once a week, watering immediately after rotating to eliminate gaps between cone-tainers and interstitial sand.

We filled cone-tainers with a homogenized mixture of 90 % sand and 10 % University of Miami Gifford Arboretum soil as used by Weremijewicz and Seto (*in revision*). The sand for the cone-tainer mixture comprised a 3:1 blend of 30–65 grade fine sand and 6–20 grade coarse sand (Florida Silica Sand Company, Miami, Florida, USA). We inoculated each cone-tainer with 1 mL (ca. 333 spores/mL) of a commercial inoculant (Mycorrhizal Applications, Grants Pass, Oregon, USA) that contained four '*Glomus*' species (*G. intraradices* Schenck & Smith [now *Rhizophagus intraradices* (Krüger *et al.*, 2012)]*, G. etunicatum* Becker & Gerd. [now *Claroideoglomus etunicatum*

Walker & Schußler], *G. mosseae* Gerd. & Trappe [*now Funneliformis mosseae* Walker & Schußler], and *G. aggregatum* Schenck & Smith). We pipetted the slurry of spores and root fragments into the center of each cone-tainer when approximately half full with the soil mixture and then filled the remainder of the cone-tainer with the sand-soil mixture. We sowed pre-treatment seeds one day after pipetting the slurry of spores and seedlings germinated within one week of sowing.

We established CMNs among cone-tainers during a pre-treatment by growing one *A. gerardii* and one *E. canadensis* (Ever Wilde Farms,Sand Creek, Wisconsin, USA) individual together in each cone-tainer for eight weeks. We then sowed fresh seed based upon each microcosm's assigned treatment (monoculture of *A. gerardii*, monoculture of *E. canadensis*, or mixture). Once every cone-tainer had at least one germinant, we began counting "days after germination" (DAG) and clipped pre-treatment plant shoots beneath the basal meristem to eliminate them, leaving only one germinant in each cone-tainer. We randomized microcosms at 5 and 52 DAG.

We fertilized cone-tainers similarly to Weremijewicz and Seto (*in revision*), using a phosphorus concentration that maximized both species' growth response to arbuscular mycorrhizal fungi. Beginning at 15 DAG, we fertilized cone-tainers twice per a week – once with 5 mL of Hewitt's solution (lacking phosphate) with concentrations of: 2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM Ferric Citrate, 0.03 mM H_3BO_3, 0.011 mM $MnSO_4$, 0.002 mM $ZnSO_4$, 0.0003 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_20$, 0.1 mM $CuSO_4 \cdot 5H_20$ and three days later with Hewitt's solution with 65 mM NaH_2PO_4. We did not water for two days following either fertilization.

We took three sets of measurements of longest leaf lengths of seedlings 10 DAG, 51 DAG, and at harvest, at 98 DAG. Longest leaf length was measured from the leaf sheath to the leaf tip. At harvest, we clipped seedlings below their basal meristems and dried them to constant weight for determination of shoot weight. We measured stomatal conductance of *A. gerardii* at 89 DAG, 4 days after watering, and of *E. canadensis* at 90 DAG, 5 days after a watering. For A. gerardii, we measured stomatal conductance of nine individuals, from each CMNs and competition treatment; each individually selected from one of nine evenly divided sections of a microcosm. For *E. canadensis*, the five largest and smallest individuals (based on longest leaf length) in every microcosm were measured using a leaf porometer (Model SC-1, Decagon Devices, Pullman, WA, USA) to assess possible water stress.

Statistical analyses

For comparable assessment of monocultures and mixtures, we halved monoculture data by considering individuals similarly positioned to conspecifics within mixtures for all statistical analyses. We examined the effects of CMNs and competition type (monoculture or mixture) over time on number of individuals per halved microcosm and mean longest leaf length with two-way, repeated-measures ANOVAs followed by Least Significant Difference (LSD) *post-hoc* tests to compare treatments ($\alpha \leq 0.05$). We assessed the assumption of sphericity using Mauchly's Statistic and associated Chi-square value. When sphericity was violated, we used Minimum Epsilon tests with corrected probabilities.

We assessed total yield per treatment by summing aboveground dry weights within microcosms for each species. We examined aboveground dry weight size-

hierarchy differences among CMN treatments by combining aboveground dry weight data from monocultures and mixtures for each species and using pairwise Kolmogorov-Smirnov tests. We examined the following size-hierarchy descriptors of aboveground dry weights for each treatment: standard deviation, coefficient of variation, skew, kurtosis, Gini mean of differences, Gini coefficient and Lorenz coefficient of asymmetry. We calculated the Gini mean of differences, Gini coefficients, and Lorenz asymmetry coefficients for each species within each microcosm with the "ineq" package version 0.2-12 (Zeileis & Kleiber, 2014) in R version 3.1.0 (2014; Vienna, Austria). We tested for differences among treatments using two-way ANOVAs after testing for heteroscedasticity with Levene's test with CMNs and competition type as factors, followed by LSD *post-hoc* tests.

To examine if CMN treatments affected stomatal conductance, we combined data within competition treatments and used a one-way analysis of covariance with aboveground dry weight as a covariate for *E. canadensis* and *A. gerardii* separately. All statistical analyses were conducted with Statistix v. 10.0 (Analytical Software, Tallahassee, FL).

Results

The number of individuals per microcosm significantly decreased over time for *A. gerardii* (Fig. 5.1a; Minimum Epsilon $F_{2, 16} = 37.70$, $P = 0.0003$) and *E. canadensis* (Fig. 5.1b; Minimum Epsilon $F_{2, 16} = 24.19$, $P = 0.0012$). Although treatments did not differ at the start of the experiment, the number of individuals ultimately was affected by an interaction between CMN treatment and competition type (*A. gerardii*: Minimum Epsilon $F_{2, 16} = 8.99$, $P = 0.0171$; *E. canadensis*: Minimum Epsilon $F_{2, 16} = 7.61$, $P =$

0.0247). CMN treatment also had a significant main effect on the number of *E. canadensis* individuals per microcosm ($F_{1, 16} = 20.42$, $P = 0.0020$).

Longest leaf lengths increased in size over the course of the experiment (*A. gerardii:* Fig. 5.2a; Minimum Epsilon $F_{2, 16} = 568.61$, $P < 0.0001$; *E. canadensis*: Fig. 5.2b; $F_{2, 16} = 251.66$, $P < 0.0001$). CMN treatment significantly affected leaf lengths (*A. gerardii*: $F_{1, 16} = 23.93$, $P = 0.0012$; *E. canadensis*: $F_{1, 16} = 7.85$, $P = 0.0231$), with a significant interaction between CMN treatment and time (*A. gerardii*: Minimum Epsilon $F_{2, 16} = 12.35$, $P = 0.0079$; *E. canadensis*: $F_{2, 16} = 6.36$, Minimum Epsilon $P = 0.0093$). Although individuals with intact CMNs did not have significantly longer leaf lengths than those with severed CMNs at 10 DAG, they were significantly longer at 51 and 98 DAG.

At harvest, total yield differed significantly among treatments for *A. gerardii*, with a significant interaction between CMN treatment and competition type (Tables 5.2 & 5.3) in which mixture increased the proportional reduction of total yield caused by severing CMNs. Surprisingly, when compared to half-monoculture yields, *A. gerardii* total yield within mixture with intact CMNs was significantly greater than that of any other treatment, which did not differ significantly from one another (Fig. 5.3). For *E. canadensis* microcosm total yields were diminished only by CMN severing (Table 5.2). Aboveground mean individual dry weights of *A. gerardii* were affected similarly to microcosm totals by both main treatments and by their interaction (Tables 5.2 & 5.3). *E. canadensis* aboveground mean individual dry weights were not affected by treatment (Table 5.3).

Size distributions of aboveground dry weights (Fig 5.4) for *A. gerardii* with intact CMNs differed from those with severed CMNs (Kolmogorov-Smirnov two-tailed test

statistic = 0.19, $P < 0.0001$) but not for *E. canadensis* (Kolmogorov-Smirnov two-tailed test statistic = 0.10, $P = 0.7284$). Size-hierarchy descriptors such as standard deviation, coefficient of variation, Gini mean of differences and the Gini coefficient of *A. gerardii* aboveground dry weights were significantly affected by CMNs (Table 5.2). *A. gerardii* individuals with intact CMNs had significantly larger standard deviations, coefficients of variation, Gini mean of differences, and Gini coefficients than those with severed CMNs (Table 5.3). Standard deviation and Gini mean difference of *A. gerardii* aboveground dry weights also were significantly affected by competition type, with plants in mixtures having larger standard deviations and Gini mean of differences than those in monocultures. There were no significant interaction effects for size-hierarchy descriptors of *A. gerardii*. *E. canadensis* size-hierarchy descriptors showed no significant main effects of either treatment. Only an interaction between CMNs treatment and competition type significantly affected the Lorenz asymmetry coefficient for *E. canadensis* (Table 5.2) by having opposing effects in monoculture versus mixture (Table 5.3).

Stomatal conductance was not significantly affected by CMN severing for *A. gerardii* (Fig. 5.5a; $F_{1, 35} = 3.14$, $P = 0.0893$) but was for *E. canadensis* (Fig. 5.5b; $F_{1, 58} = 4.76$, $P = 0.0338$). *E. canadensis* individuals with intact CMNs had significantly higher mean stomatal conductance than those with severed CMNs.

Discussion

CMNs may have different implications for plant performance, contingent upon species' physiological requirements and dependence upon AM fungi for mineral nutrient uptake. In our experiment, CMNs improved growth and survival of both species but may have

done so through different mechanisms. For *A. gerardii*, CMNs likely increased mineral nutrient uptake by accessing neighboring cone-tainer soils, but for *E. canadensis*, CMNs principally may have facilitated uptake of water, thereby primarily improving plant survival.

A. gerardii *asymmetric belowground competition and overyielding*

Replication of large experiments in ecology is rare, but two of our treatments, intact and severed CMNs in *A. gerardii* monoculture, essentially repeated a portion of the experiment reported by Weremijewicz and Janos (2013) and found similar effects of CMNs. CMNs in both experiments benefitted *A. gerardii* growth but also intensified competition overall, as suggested by amplified size inequalities of plants with intact CMNs versus those with severed CMNs. Furthermore, values of each treatment's Gini coefficient were similar in both studies. Weremijewicz and Janos (2013) attributed the size inequalities to CMNs having supplied the limiting mineral nutrient, manganese, preferentially to large, carbon-proffering plants, thereby amplifying asymmetric competition belowground. It is possible that *A. gerardii* individuals in the present experiment also were competing asymmetrically via CMNs, but we have yet to conduct mineral nutrient analyses in order to investigate this possibility. The vertical growth of *A. gerardii* and use of modified cone-tainers in our experiment, however, likely minimized aboveground interactions and precluded direct root interactions.

A. gerardii overyielded in mixture with *E. canadensis* when CMNs were intact, suggesting that CMNs contributed to stronger intraspecific than interspecific competition. This phenomenon most often has been reported for crop polycultures (Vandermeer, 1981; Schroeder-Moreno & Janos, 2008). It is likely that differences in the species'

requirements for certain resources relaxed competitive interactions within mixtures (Firbank *et al.*, 1990). Moora and Zobel (1996) found that even when plants are of different sizes and ages, intraspecific interactions are more intense than interspecific interactions when AM fungi are present. In their study, intraspecific competition with a large neighbor was amplified when AM fungi (and presumed CMNs) were present, resulting in growth suppression of small plants. The presence of AM fungi in interspecific interactions, however, improved the performance of seedlings in the presence of a large neighbor. In our study, *A. gerardii* with intact CMNs in mixture was 66 % larger than predicted from its performance in monoculture. This competitive release when grown with *E. canadensis* might facilitate co-existence of the species in nature (Vandermeer, 1981). In a study in which *A. gerardii* and *E. canadensis* were in the presence of AM fungi or not, Hartnett *et al.* (1993) found that AM fungi enhanced *A. gerardii*'s ability to compete with *E. canadensis*. Our study suggests that one mode of competition between these species is through interconnecting hyphal networks that likely most benefited *A. gerardii* nutritionally in our experiment. When CMNs were severed, *A. gerardii* did not overyield in mixtures, approximately producing the total microcosm yield predicted from its respective monoculture yield. Severing CMNs likely minimized competitive interactions with neighbors, regardless of their species.

E. canadensis *survival and improved water relations*

Although size inequalities were greater for *E. canadensis* than for *A. gerardii,* there was no significant effect of CMNs severing on *E. canadensis* size inequalities. High Gini coefficients for *E. canadensis* populations likely were a consequence of a limited ability of the majority of individuals to grow under the conditions of our experiment. A lack of

differences in size inequalities among populations with intact versus severed CMNs suggests that individuals may not have competed strongly across CMNs. Because of an overall lack of a potentially limiting resource such as water, stress could have retarded growth regardless of CMNs treatment, and thereby minimized competition. It also is possible that because *E. canadensis* is only somewhat responsive to AM fungi (Weremijewicz & Seto, *in revision*), CMN-facilitated mineral nutrient uptake does not influence plant size as much as symmetric uptake by root systems. In a study investigating the effects of presence versus absence of AM fungi on populations of a highly responsive plant species and a weakly responsive plant species, Allsopp and Stock (1992) found that the presence of AM fungi increased the coefficient of variation, a measure of plant size variability that correlates closely with the Gini coefficient (Weiner & Solbrig, 1984), only for the highly responsive species.

Reduced stomatal conductance for *E. canadensis* individuals with severed CMNs suggests that CMNs may have reduced plant mortality by improving water-relations for this species. AM fungi are known to affect the water balance of both well-watered and water-stressed host plants (Simpson & Daft; Auge, 2001) in a variety of ways. In particular, as soil dries around root systems, water retreats from large pores to small capillary spaces through which AM fungal hyphae may extend (Tisdall, 1991; Auge, 2001). AM fungus species used in our study, such as *R. intraradices, C. etunicatum*, and *F. mosseae* (formerly *G. intraradices*, *G. etunicatum*, and *G. mosseae*, respectively), all have been shown to increase host stomatal conductance (Auge, 2001). Our study suggests that it is not just the presence of AM fungi that can enhance water uptake, but especially the presence of extensive, intact extraradical mycelium. Decreased water

uptake with severed CMNs likely limited photosynthesis, resulting in little growth and high mortality. Furthermore, carbon stress may have been exacerbated if AM fungi colonizing *E. canadensis* roots continued to draw carbon from their host plants (Olsson *et al.*, 2010).

Conclusions

CMNs have been suggested to be important agents of ecosystem processes by being pathways for ecological interactions (Simard & Durall, 2004; Bever *et al.*, 2010). We found that CMNs among two tall-grass prairie species affected their survival and growth of seedlings, a life-stage that is crucial for community composition. Relaxed interspecific interactions via CMNs might help to explain partially how ecotypes of *A. gerardii* adapted to wet sites and *E. canadensis* co-exist in tallgrass prairie if in proximity to one another. Overyielding by *A. gerardii* in the presence of *E. canadensis* is consistent with the dominance of *A. gerardii* in tall-grass prairies. At the same time that they provide an advantage to *A. gerardii* in interspecific competition, CMNs also may intensify intraspecific competition among *A. gerardii*, thereby accelerating size-hierarchy development by favoring large individuals that are potentially able to reproduce most vigorously. Furthermore, CMNs may enhance the survival of *E. canadensis* spring germinants during the transition to a dry summer. Thus, our findings suggest that it is not just the presence of AM fungi and formation of mycorrhizas which have implications for grasslands, but it is especially the interconnecting hyphae of CMNs that mediate plant interactions and consequently affect both plant population structure and community composition.

Table 5.1. Soil characteristics of the sand mixture surrounding cone-tainers in experimental microcosms and for the soil within cone-tainers

	Concentration (ppm)		Content (mg)	
Soil characteristic	Sand mixture*	Soil (within cone-tainers)†	Sand mixture	Soil (within cone-tainers)
Nitrate	1.0	36.0	35.0	247.0
Phosphorus	2.0 (Bray 1) 1.2 (Olsen)	4.9 (Mehlich 3)	70.1 (Bray 1) 42.0 (Olsen)	33.6 (Mehlich 3)
Potassium	7.0	57	245.3	391.1
Calcium	155	6398	5430.9	43902.8
Magnesium	25.0	121	875.9	830.3
Manganese	0.1	15	3.5	102.9
pH	8.1	7.4	–	–
Cation exchange capacity (meq/100g)	0.9	33.3	–	–

***** Sand mixture samples were analyzed by the Kansas State University Soil Testing Laboratory, Manhattan, KS, U.S.A.

† Soil within cone-tainers was analyzed by Waypoint Analytical Virginia, Inc., Richmond, VA, U.S.A.

Table 5.2. Two-way ANOVA main effects (CMNs = common mycorrhizal networks; COMP. = "competition": monoculture vs. mixture) and interactions for total and mean individual dry weights and for size-hierarchy descriptors calculated with aboveground dry weights, 98 days after germination for *Andropogon gerardii* and *Elymus canadensis**

		Andropogon gerardii		*Elymus canadensis*	
Descriptor†	Effect	$F_{1,14}$	*P*	$F_{1,14}$	*P*
Microcosm Total	CMNS	**19.87**	**0.0005**	**8.70**	**0.0106**
	COMP	**35.46**	**0.0000**	0.07	0.8004
	CMNs x COMP	**10.08**	**0.0068**	0.00	0.9498
Individual Mean	CMNS	**8.60**	**0.0109**	3.79	0.0720
	COMP	**25.83**	**0.0002**	0.04	0.8354
	CMNs x COMP	**5.30**	**0.0372**	0.24	0.6319
Standard deviation	CMNS	**16.16**	**0.0013**	1.15	0.3019
	COMP	**7.04**	**0.0189**	0.25	0.6230
	CMNs x COMP	1.50	0.2413	0.81	0.3821
Coefficient of variation	CMNS	**10.91**	**0.0052**	0.40	0.5387
	COMP	0.45	0.5143	2.15	0.1646
	CMNs x COMP	0.21	0.6548	1.50	0.2410
Skew	CMNS	2.05	0.1742	1.38	0.2601
	COMP	0.17	0.6872	1.03	0.3283

	CMNs x COMP	0.70	0.4156	1.29	0.2748
Kurtosis	CMNS	0.27	0.6135	2.16	0.1634
	COMP	0.40	0.5376	1.12	0.3081
	CMNs x COMP	0.48	0.5014	0.07	0.7933
Gini mean difference	CMNS	**14.82**	**0.0018**	0.91	0.3559
	COMP	**7.08**	**0.0187**	0.04	0.8430
	CMNs x COMP	1.86	0.1942	0.49	0.4960
Gini coefficient	CMNS	**10.46**	**0.0060**	1.04	0.3250
	COMP	0.27	0.6114	0.92	0.3545
	CMNs x COMP	0.04	0.8465	0.79	0.3883
Lorenz asymmetry	CMNS	0.00	0.9669	0.00	0.9649
	COMP	0.30	0.5953	0.02	0.9014
	CMNs x COMP	0.03	0.8658	**6.89**	**0.0200**

* Significant differences ($\alpha \leq 0.05$) are shown in bold

† Monoculture descriptors are based on halved monoculture data that duplicated the patterns within mixtures

Table 5.3. Values of total and mean individual dry weights and of size-hierarchy descriptors calculated with aboveground dry weights ± SE, 98 days after germination for *Andropogon gerardii* and *Elymus canadensis* with intact and severed CMNs in monocultures and mixtures

	Andropogon gerardii				*Elymus canadensis*			
	Monoculture*		Mixture		Monoculture		Mixture	
Descriptor	Intact CMNs	Severed CMNs	Intact CMNs	Severed CMNs	Intact CMNs	Severed CMNs	Intact CMNs	Severed CMNs
Microcosm Total (g)	7.48 ± 0.30	6.78 ± 0.40	12.43 ± 1.07	8.29 ± 0.61	1.57 ± 0.42	0.61± 0.09	1.67 ± 0.18	0.67 ± 0.25
Individual Mean (g)	0.17 ± 0.011	0.16 ± 0.008	0.26 ± 0.042	0.19 ± 0.014	0.04 ± 0.008	0.03 ± 0.003	0.04 ± 0.004	0.03 ± 0.006
Standard Deviation (g^2)	0.07 ± 0.007	0.05 ± 0.004	0.10 ± 0.006	0.06 ± 0.01	0.04 ± 0.01	0.02 ± 0.005	0.04 ± 0.007	0.04 ± 0.002
Coefficient of Variation (%)	40.78 ± 2.11	30.56 ± 2.68	37.72 ± 0.81	29.98 ± 3.18	90.59 ± 6.32	81.44 ± 11.91	94.32 ± 25.08	122.87 ± 25.94
Skew	0.55 ± 0.42	-0.29 ± 0.21	0.39 ± 0.24	0.18 ± 0.33	2.29 ± 0.33	0.92 ± 0.32	2.22 ± 1.33	2.20 ± 0.69

Kurtosis	1.94 ± 1.44	0.57 ± 0.46	0.44 ± 0.74	0.64 ± 0.25	6.03 ± 1.73	0.41 ± 0.78	8.59 ± 8.42	4.70 ± 2.91
Gini mean difference (g)	0.07 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.001	0.03 ± 0.003
Gini coefficient	0.22 ± 0.01	0.17 ± 0.01	0.21 ± 0.003	0.16 ± 0.02	0.41 ± 0.03	0.42 ± 0.78	0.41 ± 0.04	0.50 ± 0.04
Lorenz Asymmetry	0.90 ± 0.07	0.88 ± 0.05	0.93 ± 0.09	0.94 ± 0.10	1.08 ± 0.05	0.93 ± 0.04	0.93 ± 0.05	1.07 ± 0.09

* Monoculture descriptors are means of halved monoculture data (n = 6) that duplicated the patterns within mixtures (n = 3)

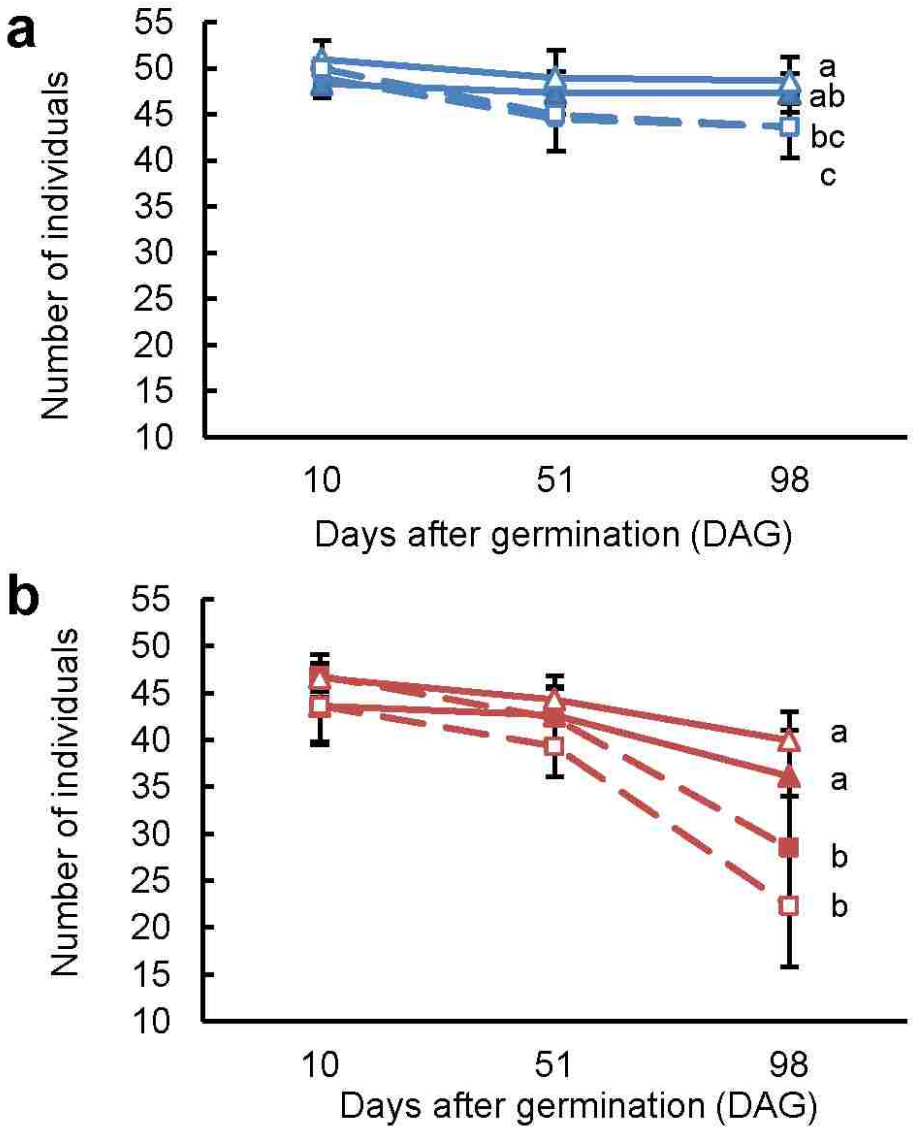

Figure 5.1. Number of individuals per microcosm (± SE) at 10, 51, and 98 days after germination for *Andropogon gerardii* (a) and *Elymus canadensis* (b) with intact CMNs (solid lines, triangles) or severed CMNs (dashed lines, squares). Data from monocultures (filled symbols) was halved to duplicate the pattern within mixed cultures (open symbols). Lines adjacent to the same letter do not differ by Least Significant Difference *post-hoc* test ($\alpha \leq 0.05$).

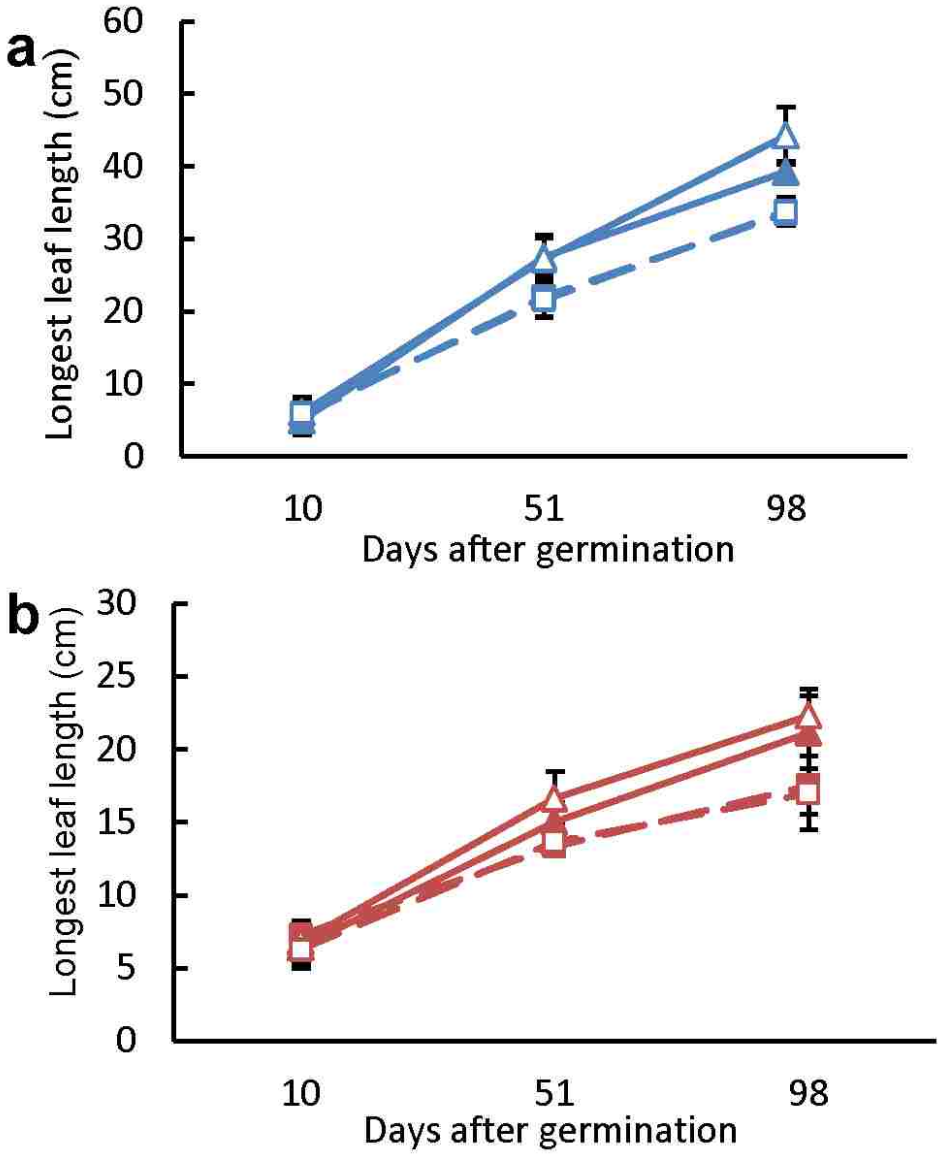

Figure 5.2. Mean longest leaf length (cm ± SE) at 10, 51, and 98 days after germination for *Andropogon gerardii* (a) and *Elymus canadensis* (b) with intact CMNs (solid lines, triangles) or severed CMNs (dashed lines, squares). Data from monocultures (filled symbols) was halved to duplicate the patterns within mixed cultures (open symbols). Lines adjacent to the same letter do not differ by the Least Significant Difference *post-hoc* test ($\alpha \leq 0.05$).

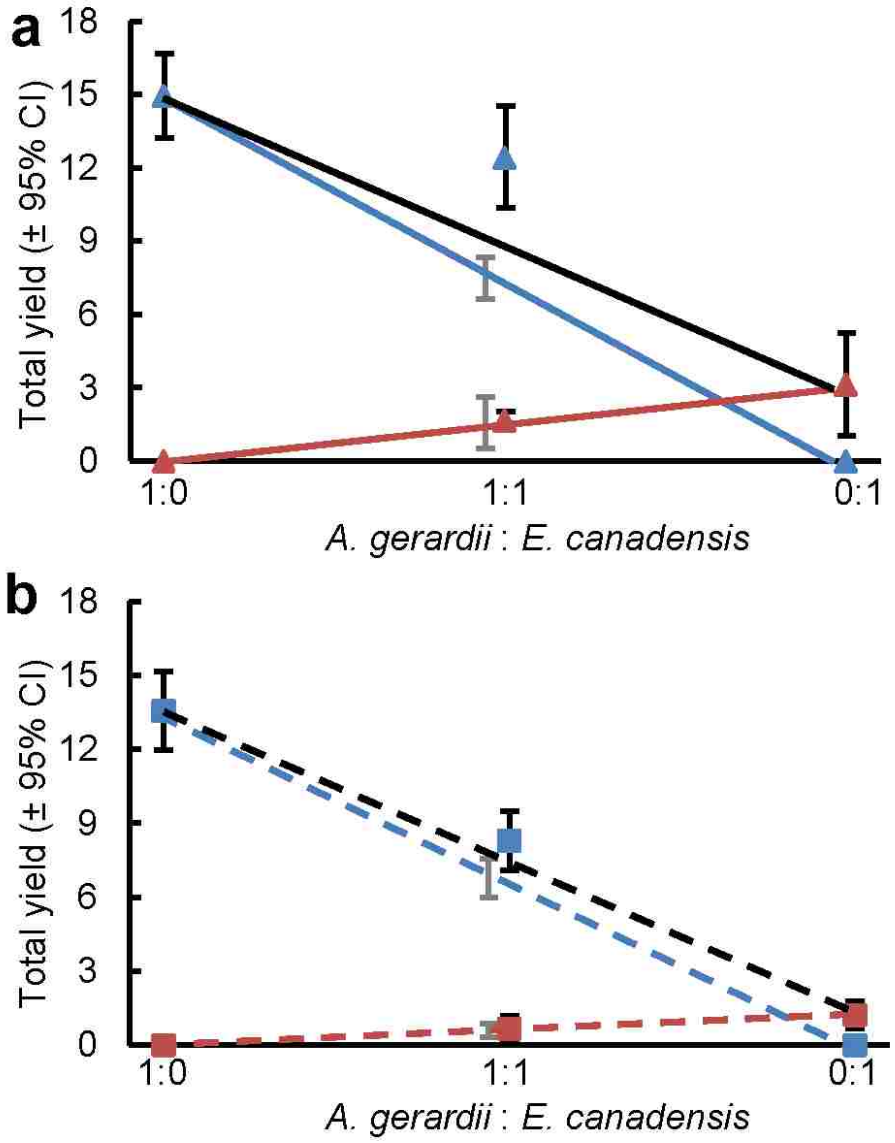

Figure 5.3. Total yields [total aboveground dry weight and 95 % CI (g)] for each competition treatment for *Andropogon gerardii* (blue symbols and lines) and *Elymus canadensis* (red symbols and lines) with intact common mycorrhizal networks (CMNs; a; triangles and solid lines) or severed CMNs (b; squares and dashed lines). Grey bars on each graph represent 95 % CI for expected half-monoculture yields.

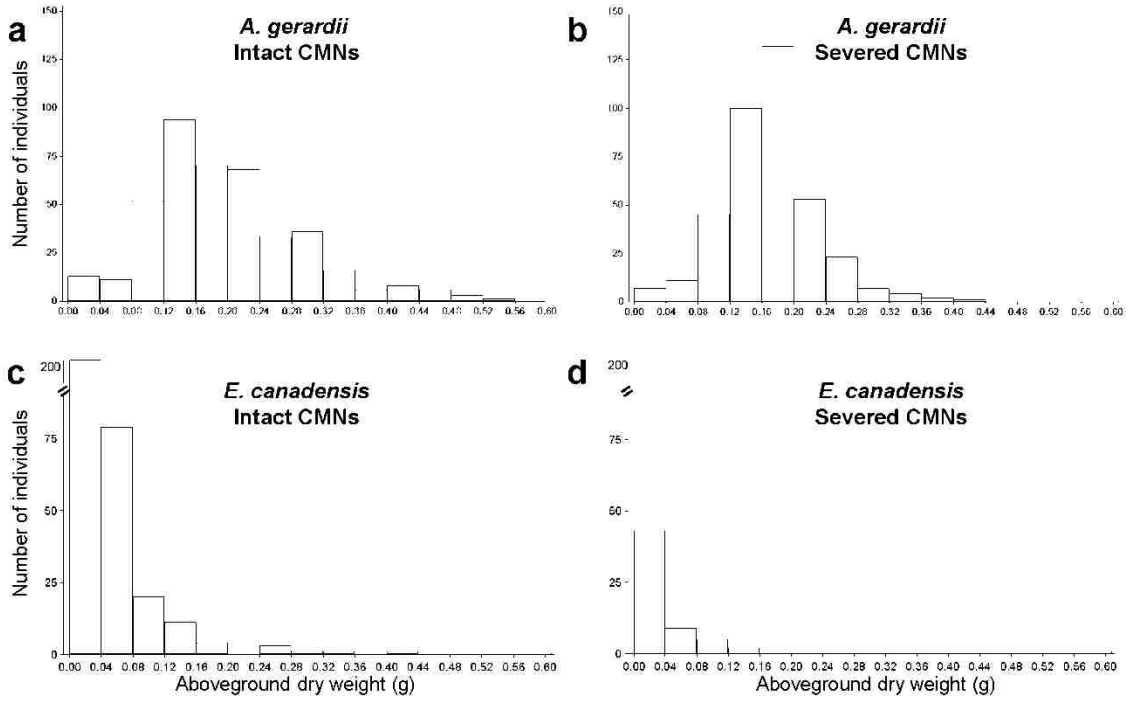

Figure 5.4. Size-frequency distributions of *Andropogon gerardii* (a, b) and *E. canadensis* (c, d) seedlings based on aboveground dry weight at harvest for all replicate microcosms combined with either intact common mycorrhizal networks (CMNs; a, c) or severed CMNs (b, d).

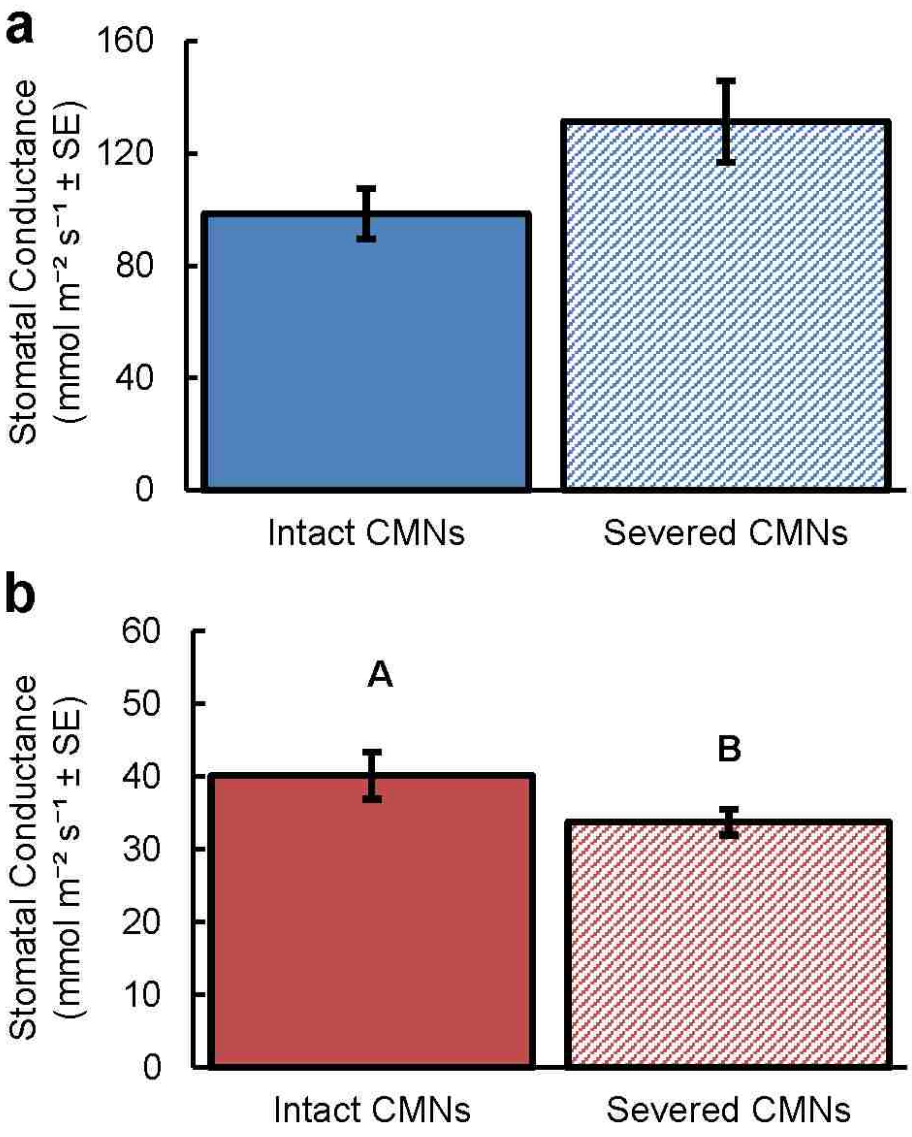

Figure 5.5. Stomatal conductance (± SE mmol m $^{-2}$ s^{-1}) of *A. gerardii* (a) and *E. canadensis* (b) seedlings with intact common mycorrhizal networks (CMNs) and severed CMNs. Stomatal conductance was not affect by CMN severing for *A. gerardii* but was for *E. canadensis*. Bars topped with the same letter do not differ by a one-way ANCOVA.

Chapter Six

Synthesis

Context

The microcosm experiments in this dissertation were intended to be model systems, and their design constrains the extrapolation of results to natural ecosystems. These experiments sacrificed the realism of belowground interactions in order to isolate and identify the effects of CMNs. The rotatable cone-tainers used in the CMN chapters (Chapters Two, Three and Five) confined root systems entirely and prevented root interactions among neighboring plants. In nature, particularly in tallgrass prairies, root overlap may influence the strength and symmetry of plant interactions. The most fertile substrates in my experiments were within cone-tainers (and any fertilization was applied directly to cone-tainers), causing mineral nutrients to be distributed patchily. Although every patch originally was occupied by an individual plant, not all individuals survived to take up mineral nutrients, consequently mineral nutrient availability in all patches might not have been equal. On the other hand, although the extent of mycorrhiza formation often differed among treatments (Figure 6.1), all contrasts among CMN treatments involved realistically comparing mycorrhizal plants to other mycorrhizal plants, a marked departure from the majority of previous mycorrhiza competition literature that used plants entirely lacking mycorrhizas as the reference treatment. Thus, the implicit question investigated in this dissertation was, "What are the consequences for plants of mycorrhizal fungus hyphae spreading to neighboring plants, to mineral nutrient rich patches, and potentially interconnecting neighboring root systems?"

In Chapter Four, experiments did involve treatments without mycorrhizas as well as pulsed fertilization with soluble phosphorus in order to reveal the interactions of mycorrhizas and phosphorus for root colonization, growth, and mineral nutrition of *Andropogon gerardii* and *Elymus canadensis*. Notwithstanding that those experiments and the others reported in this dissertation involved simplification in order to detect the roles of mycorrhizas and CMNs, the fungus species employed are likely to be encountered by *A. gerardii* and *E. canadensis* in nature. At least two of the inoculant fungus species, *Glomus mosseae* Gerd. & Trappe [*now Funneliformis mosseae* Walker & Schußler], and *G. etunicatum* Becker & Gerd. [now *Claroideoglomus etunicatum* Walker & Schußler] (Hetrick & Bloom, 1983), and probably others are found in native tallgrass prairies.

Noteworthy Findings

The experiments in this dissertation ascertained that it is not just the presence of arbuscular mycorrhizal fungi that has implications for grasslands, but it is the interconnecting hyphae their CMNs that can mediate plant interactions. My dissertation also found that CMNs improved manganese acquisition, enhanced the mycorrhizal colonization of putatively carbon-limited plants, and increased stomatal conductance of *E. canadensis*.

Manganese appears to have been a limiting mineral nutrient in both Chapters Two and Three. The soil mixture used in both chapters was a 3:1 Flatwoods:Gifford Arboretum soil that had exceptionally low concentrations of manganese (Table 6.1). Although Chapter Three was fertilized with additional manganese, that added fertilizer

did not appear to relieve limitations. Interestingly, manganese was potentially growth-limiting only for well-insolated target plants, and not for shaded target plants. Manganese demand likely declined with shading because manganese is a critical element used in Photosystem II (Yachandra *et al.*, 1996). Among shaded plants, nitrogen appeared to be limiting only for target plants with intact networks which had the lowest foliar nitrogen concentrations among the shaded treatments (Figure 6.2 a). This finding is consistent with CMNs potentially having proffered nitrogen preferentially to well-insolated, abundantly-photosynthesizing neighbors of the shaded, interconnected target plants. Chapters Two and Four also found AM fungi and CMNs assist in nitrogen and phosphorus acquisition. In Chapter Two, intact CMNs increased phosphorus uptake (Figure 6.2 b) in a soil with low phosphorus availability (Table 6.1) as they tended to do at the lowest amounts of phosphorus fertilization in Chapter Four.

The presence of intact CMNs elevated colonized root length 12 weeks after treatment in both Chapters Two and Three (Figure 6.1) in different ways. In Chapter Two, I used a field-collected inoculum that effectively may have been sparse within cone-tainers. Nevertheless, the ability of undisturbed CMNs to spread among cone-tainers increased the colonization of networked plants. In Chapter Three, the supplemental use of a commercial inoculant with four "*Glomus"* species added to each individual cone-tainer likely resulted in asymptotic colonization of *A. gerardii* across all CMN treatments in the sun (Figure 6.1). Intact CMNs, however, elevated the colonization of shaded target plants (Figure 6.1), suggesting that carbon flow from insolated neighbors may have sustained the fungi in the roots of those shaded target plants. It also is possible that limited fixed-carbon from the shaded individuals was

provided to the fungi. Olsson *et al.* (2010) found that shaded individuals continue to supply carbon to arbuscular mycorrhizal fungi, but Olsson *et al.'s* (2010) study did not involve plants interconnected by CMNs. Although arbuscular mycorrhizal fungi may have parasitize shaded, solitary host plants, connections to well-insolated individuals by CMNs might ameliorate such carbon drain. Moreover, while Chapters Two and Four showed that my field-collected inoculum colonized approximately 50 % of root length in low-phosphorus soil in solid, non-modified cone-tainers (Figure 6.1), in Chapter Three, enrichment with a commercial inoculum increased colonization to approximately 70 – 80 % in the sun, equaled by intact CMNs in the shade and in Chapter Two (Figure 6.1).

Chapter Five extended my investigation of the effects of CMNs to interspecific mixtures. I found that intact CMNs improved survival of *E. canadensis* through enhanced water supply as suggested by elevated stomatal conductance. Although it is known that arbuscular mycorrhizal fungi increase stomatal conductance of host plants (Auge, 2001), my research associates this phenomenon specifically with CMNs. Additionally, intact CMNs are associated with overyielding by *A. gerardii* in Chapter Five, but that most likely was a consequence of an effectively halved density of *A. gerardii* in mixtures which may have facilitated increased mineral nutrient acquisition from neighbor cone-tainers.

My analyses of the relationship between the sizes of "target" plants and their neighbors revealed that in Chapters Two and Three (but not Chapter Five), competition was strong among plants when CMNs were intact. Neighbor analyses through PCAs in Chapter Two, and linear regressions of target dry weight versus total neighbor dry weight per pot in Chapter Three showed that large plants were likely to be surrounded by small

neighbors only in the presence of intact CMNs. In Chapter Five, however, I could not detect such an effect. For *A. gerardii* monocultures in Chapter Five, this may have been a consequence of soluble fertilizer addition to a relatively fertile base substrate (Table 6.1) which reduced the importance of CMNs. In *E. canadensis* monocultures insignificant competition across CMNs probably was a consequence of limited plant growth because of water stress.

While Chapters Two, Three and Five attest that belowground competition is mediated by CMNs in the absence of root system overlap, Chapters Two and Three additionally suggest that belowground competition across CMNs is likely to be asymmetric. Chapter Three in particular revealed with whole plants that mycorrhizal fungi may preferentially supply mineral nutrients to large, abundantly carbon-supplying plants, consistent with "reciprocal rewards," a hypothesis most commonly tested by root organ culture experiments. Large plants may thereby preempt mineral nutrient provision through CMNs, suppressing the growth of neighbors not as able to supply fixed carbon. Chapters Two, Three, and Five both showed amplified size inequalities among populations which likely resulted from such mineral nutrient preemption.

Significance

My dissertation extends the observations of Janos (2007) which may be interpreted as reflecting opposing effects of soil fertility (especially of soil phosphorus as demonstrated by Chapter Four) and plant density, i.e., increased soil fertility is similar in effect to decreased plant density (as illustrated by Chapter Five). I found intact CMNs can enhance mycorrhizal colonization, especially of large (Chapter Two) and shaded

(Chapter Three) plants, suggesting that CMNs might partially compensate the tendency for increased fertility to diminish root colonization of facultatively mycotrophic plants. Whether that imposes an elevated demand for fixed carbon on those plants, which could diminish their benefit-to-cost ratio of mycorrhizas, needs further investigation. Alternatively, such sustained colonization might be beneficial for shaded plants in situations where canopy gaps suddenly increase photosynthesis and pre-established mycorrhizas may rapidly provide mineral nutrients.

In the absence of root system overlap, enhanced root colonization and intact CMNs favor large, well-insolated plants at the expense of their small neighbors, making belowground competition across CMNs asymmetric. In contrast, root competition generally is regarded as symmetric. Whether the asymmetric effects of CMNs or the symmetric effects of root systems predominate in belowground competition likely is determined by a plant's dependence upon and responsiveness to mycorrhizas (Chapter Four), the densities and identities of its neighbors (Chapters Two, Three, and Five), and whether intact CMNs extend among them.

Table 6.1 Base soil characteristics of interstitial sand and soil within cone-tainers for all four data chapters. Cone-tainers in Chapters Three, Four and Five were fertilized with modified Hewitt's solution (see chapters for details).

	Chapter Two		Chapter Three		Chapter Four	Chapter Five	
Soil characteristic	Interstitial sand ¥	Soil within cone-tainers	Interstitial Sand	Soil within cone-tainers *	Soil within cone-tainers	Interstitial sand	Soil within cone-tainers
Soil Type	Silica sand (Surface Prep Supply Co.)	3:1 Flatwoods : Arboretum	Acid-washed silica sand	3:1 Flatwoods : Arboretum	9:1 Silica sand (Florida Silica Co.) : Arboretum	Silica sand (Surface Prep Supply Co.)	9:1 Silica sand (Florida Silica Co.) : Arboretum
Ammonium (ppm)	2.8	4.9	–	–	3.2	–	–
Nitrate (ppm)	1	15.6	0.5	3	2.6	1	36
Phosphorus (ppm; type)	1.2 (Olsen)	6.5 (Olsen)	2.1 (Mehlich 3)	12 (Mehlich 3)	12.5 (Olsen)	2.0 (Bray 1) 1.2 (Olsen)	4.9 (Mehlich 3)
Potassium (ppm)	7	46	11	29	48.8	7	57
Calcium (ppm)	155	1505	9.2	4576	355.8	155	6398
Magnesium (ppm)	25	68	–	–	48.3	25	121
Manganese (ppm)	0.1	1.7	0.1	14	1.1	0.1	15
pH	8.1	7.3	7.0	8.0	7.7	8.1	7.4

* Soil within cone-tainers in Chapter Three and Chapter Five was analyzed by WayPoint Analytical (Richmond, VA), but soils from Chapters Two.

¥ Soils in Chapter Two, Four, and interstitial sand in Chapter Three and Five were analyzed by Kansas State Soil Testing Lab (Manahttan, KS)

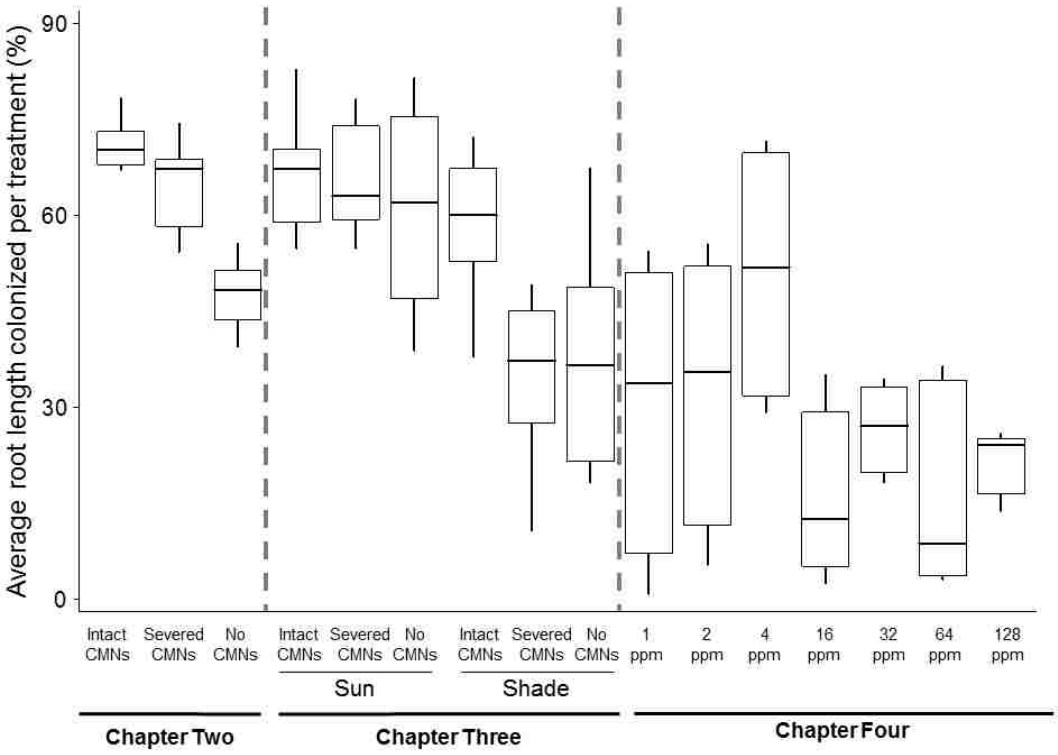

Figure 6.1. Box and whisker plot of the average root length colonized (%) by arbuscular mycorrhizal fungi across mycorrhizal treatments in Chapters Two, Three and Four. Boxes enclose the middle half of the data and bisecting lines are median values. Whiskers illustrate the range of values for each treatment.

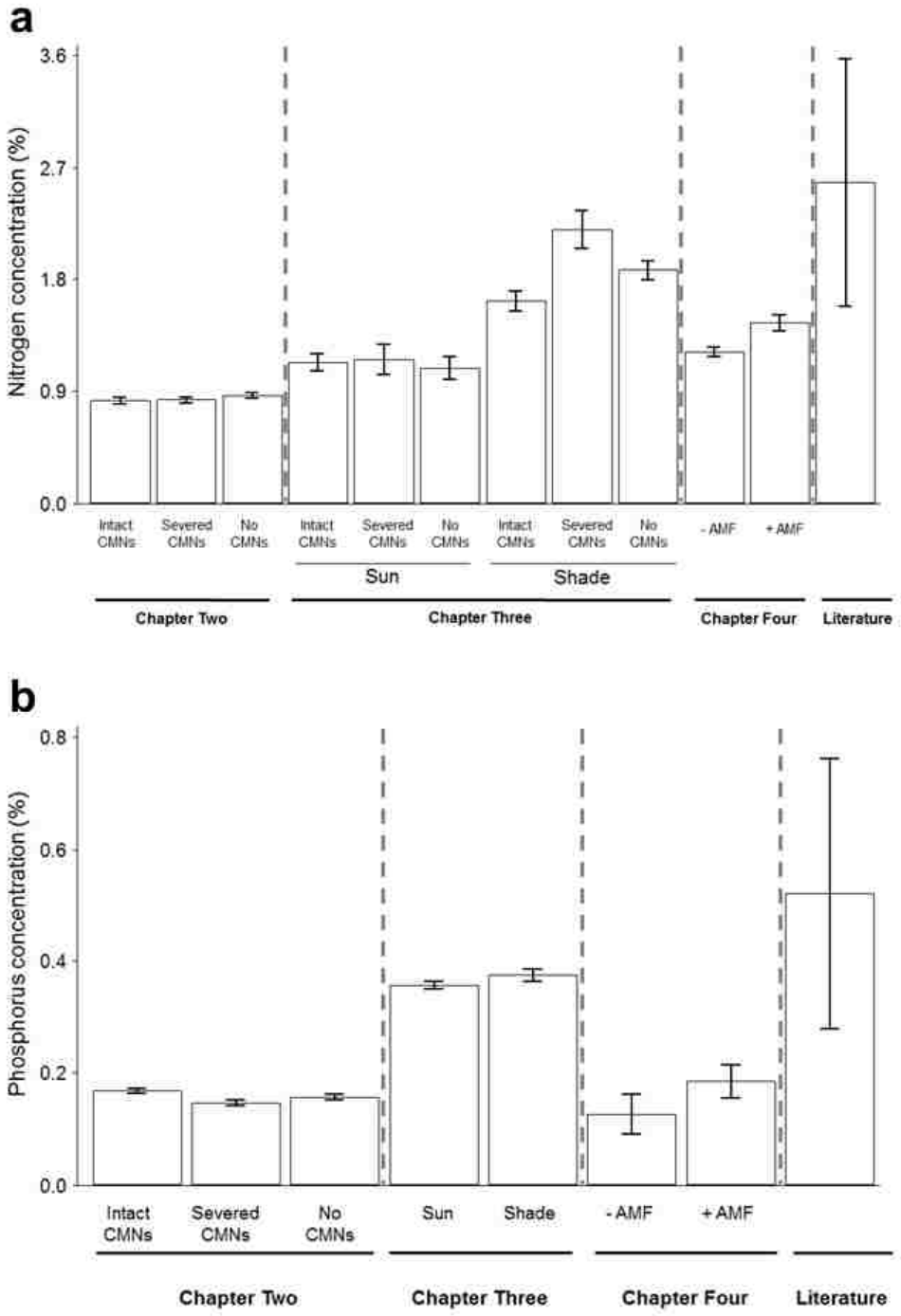

Figure 6.2. Foliar nitrogen (%, a) and phosphorus (%, b) concentrations of *A. gerardii* by treatments across chapters and from literature references.

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