

**EXPLORING GLOBAL CHANGE IMPACTS ON PLANT-PLANT AND PLANT-MICROBE
INTERACTIONS OF GRASSLAND SPECIES**

by

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Abstract

Grassland plants exist in complex environments, where in addition to coping with environmental conditions, they also interact with other plants in their vicinity as well as with microbes in the soil. How these are affected by global environmental changes need to be better characterized to predict ecosystem functions. My dissertation experimentally explores the global change impacts on plant-plant and plant-microbe interactions in grassland species.

In my first chapter, I examined how drought and a soil mutualistic microbe, arbuscular mycorrhizal fungi (AMF), affected the relationship between genetic diversity and productivity of a dominant tallgrass species, using a mesocosm experiment. I found that while genetic diversity and AMF had no effect on productivity, drought differentially affected productivity and functional traits of genotypes of a dominant grass, which implies that drought can have variable outcomes for different genotypes within a same species.

In my second chapter, I tested the Stress Gradient Hypothesis, which hypothesizes that plant-plant interactions shift from competition to facilitation with increasing environmental stress. I subjected two co-dominant grasses to drought, elevated CO₂, and varying levels of plant-plant interactions. My results demonstrated that plant-plant interactions leaned towards facilitation with decreasing stress gradient, contrary to the stress gradient hypothesis.

In the third chapter, I investigated the tripartite relationship among a legume, and two mutualistic microbes, AMF, and rhizobial bacteria, under elevated CO₂. I tested the hypothesis that the tripartite relationship depends on the cost of carbon to plants and benefit of nutrients from mutualists, and consequently, elevated CO₂ should alter this relationship. I conducted a pot experiment under different CO₂ and mutualist treatments. My findings suggest that dual inoculation of the legume with AMF and rhizobia comes with carbon costs, which decreases under elevated CO₂.

The intricate relationships between global change, plant-plant and plant-microbe interactions collectively shape the response of grassland species to global change. In summary, my dissertation advances our understanding of the context dependency of global change impacts on plant-plant and plant-microbe interactions. This research contributes not only to ecological theory but also to the development of strategies for sustainable grassland ecosystems in a changing world.

Primary Reader and Advisor: Dr. Meghan L. Avolio

Secondary Reader: Dr. Katalin Szlavecz

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Chapter 1

Effect of genotypic richness, drought and mycorrhizal associations on productivity and functional traits of a dominant C₄ grass

Abstract

While the relationship between genetic diversity and plant productivity has been established for many species, it is unclear whether environmental conditions and biotic associations alter the nature of the relationship. To address this, we investigated the interactive effects of genotypic diversity, drought and mycorrhizal association on plant productivity and plant traits. Our mesocosm study was set up at the Konza Prairie Biological Research Station, located in the south of Manhattan, Kansas. *Andropogon gerardii*, the focal species for our study, was planted in two levels of genotypic richness treatment: monoculture or three-genotype polyculture. A rainout shelter was constructed over half of the experimental area to impose a drought and Thiophanatemethyl fungicide was used to suppress arbuscular mycorrhizal fungi in selected pots within each genotypic richness and drought treatment. Genotypic richness and mycorrhizal association did not affect above-ground biomass of *A. gerardii*. Drought differentially affected the above-ground biomass, the number of flowers and bolts of *A. gerardii* genotypes, and the biomass and the functional traits also differed for monoculture versus polyculture. Our results suggest that drought and genotypic richness can have variable outcomes for different genotypes of a plant species.

Introduction

Genetic diversity within a plant species has been found to positively affect ecological functions (Bailey et al. 2009; Govindaraj et al. 2015; Hughes et al. 2008;) such as arthropod diversity (Cook-Patton et al. 2011; Crutsinger et al. 2006; Johnson et al. 2006), pollination rates (Genung et al. 2010), resistance to herbivores (Hughes and Stachowicz 2004; McArt and Thaler 2013), resilience to climate extremes (Reusch et al. 2005) and productivity (Crutsinger et al. 2006; Di Falco and Chavas 2006; Prieto et al. 2015). The effects of genetic diversity on productivity, however, are not always positive, and some studies have found no direct relationship between genetic diversity and productivity (Avolio and Smith 2013a; Avolio et al. 2015; Chang and Smith 2014; Fridley and Grime 2010). While the importance of genetic diversity has been established by numerous studies, the role of genetic diversity on influencing various ecosystem functions such as productivity remains controversial and may depend on environmental conditions and biotic interactions.

The genetic diversity effect on several ecosystem functions may vary as environmental conditions change. For instance, the effect of planted genetic diversity of *Oenothera biennis* on plant productivity was enhanced by deer herbivory (Parker et al. 2010) but that of *Taraxacum officinale* was decreased by mowing (Drummond and Vellend 2012). Environmental variability might influence the expression of genetic variance (Charmantier and Garant 2005), thus influencing the effect of genetic diversity on ecosystem function. Different genotypes of natural and experimental populations of several plant species have shown variation in their phenotypic responses to

environmental variability such as light, water and nutrients (Matesanz et al. 2010; Pigliucci et al. 1995; Westerman and Lawrence 1970). The genetic diversity effect on ecosystem functions under different environmental conditions needs to be further explored.

Drought is predicted to occur with an increasing evapotranspiration rate due to rising global temperature (Trenberth et al. 2014) and will affect all vegetation types (Farooq et al. 2009). Drought stress has been well documented in various plants and has been found to affect plant productivity (reviewed by Jaleel and Llorente 2009). Studies have found plant species diversity can buffer drought effects on plant survival (Nagase and Dunnett 2010), above-ground productivity (Craven et al. 2016; Tilman et al. 2012; Wagg et al. 2017) and below-ground productivity (Kahmen et al. 2005). Similarly, studies have found plant genetic diversity can buffer drought effects on productivity (Peleg et al. 2005) and community stability (Prieto et al. 2015). Theoretically, plant genetic diversity is essential for the adaptation of species to future environmental changes (Barrett and Schluter 2008; Raza et al. 2019) as higher genetic diversity can provide a larger trait variability and, thus, a greater chance of surviving unfavorable conditions (Westerband et al. 2021; Yachi and Loreau 1999). However, the experimental studies, exploring the role of plant genetic diversity under future global changes like drought, are underexplored.

Although relatively unexplored, genetic diversity effects could also be affected by symbiotic interactions (Aavik et al. 2021; Van Geel et al. 2021). Arbuscular mycorrhizal fungi (AMF) colonize most terrestrial plant species and provide host plants

with increased water and nutrients acquisition along with drought tolerance (Augé 2001; Johnson et al. 2010) in exchange for host plant's photosynthates (Drigo et al. 2010). Also, environmental conditions can affect AMF abundance and biomass (Avolio et al. 2014; Williams and Rice 2007; Zeglin et al. 2013) and can have consequences for ecosystem function. It is well documented that AMF can enhance productivity as well as influence above-ground plant structure and diversity (Hartnett and Wilson 1999; Maherali and Klironomos 2007; van der Heijden et al. 2008). van der Heijden et al. (2006) have reported that under higher plant species diversity, the positive effect of AMF on productivity decreased as soil nutrients were more effectively utilized. However, the effect of AMF association on the productivity of a genetically diverse pool within a plant species has not been studied before.

Plant functional traits, defined as any morphological, physiological or phenological feature of a plant that affects its fitness (PérezHarguindeguy et al. 2016; Violle et al. 2007) are known to respond to environmental changes and affect ecosystem function (La Pierre and Smith 2015; Violle et al. 2007). These traits can vary among genotypes and impact population performance and community functioning (Ellers et al. 2011), and thus, can help understand the mechanism between plant genotypic diversity and productivity. Genotypic identity has also been reported to affect population productivity (Vellend et al. 2010) and thus, some genotypes of a plant species may perform better than others under future global change scenarios. Drought effects on plant functional traits on a variety of plant ecosystems including the forests and grasslands are well studied (Cenzano et al. 2013; Jaleel and Llorente 2009; O'Brien et al.

2017). However, interactive effect of drought, AMF associations and genotypic richness on plant functional traits is not well understood. Grasslands are important ecosystems to study the effect of drought and AMF associations as they are highly susceptible to drought (Lei et al. 2020), and most grassland species have a symbiotic relationship with AMF (Johnson et al. 2010). For our study, we chose *Andropogon gerardii* Vitman, one of the dominant C4 grasses of the tallgrass prairie ecosystem, as the focal species. Contributing up to 80% of above-ground productivity (Smith and Knapp 2003), the fate of *A. gerardii* under changing biotic and abiotic conditions will have a significant effect on the community structure and prairie ecosystem (Chaves and Smith 2021; Gustafson et al. 2004). Here, we measured plant functional traits and above-ground plant productivity of *A. gerardii* at two levels of genotypic diversity (monoculture and a three-genotype polyculture) under different drought (droughted or ambient rainfall) and AMF association treatments (fungicide treated and an untreated control) to assess how genetic diversity, drought and mycorrhizal fungi interact to affect the plant functional traits and productivity of *A. gerardii*. We hypothesized that: (i) aboveground productivity of *A. gerardii* would be higher in polyculture compared with monoculture, and higher genetic diversity would offset the negative effect of drought and fungicide treatment on aboveground productivity, and (ii) above-ground biomass and functional traits of *A. gerardii* genotypes would be affected by the genetic diversity, drought and mycorrhizal association treatments.

Methods

Site characteristics

This study was conducted from 2011 to 2015 at the Konza Prairie Biological Research Station, a LongTerm Ecological Research (LTER) site, located to the south of Manhattan, KS (39.1069° N, 96.6091° W). Historically, the name 'Konza' comes from the native Americans, Kansa or, Kaw Indians who inhabited the area before the colonization by European settlers. Konza LTER is a tallgrass prairie ecosystem, and its production is primarily driven by perennial C4 grasses, including *A. gerardii* (Smith and Knapp 2003). In 2011, we established our study site in a watershed unit, AL, a lowland agricultural site that was annually burned and ungrazed prior to the study. The mean annual precipitation of the site is ~892 mm, of which 75% occurs during April–September and mean annual air temperature is 13 °C (Felton et al. 2020).

Focal species

Andropogon gerardii is a perennial, clonal grass that primarily reproduces through rhizomatous buds (Benson and Hartnett 2006) and is genetically diverse ranging from four to nine genotypes with an average of 5.2 (\pm 0.73 standard error [SE]) in a 1 m² plot (Avolio et al. 2011). Also, genotypes of this grass are phenotypically diverse and have been well documented to demonstrate a wide range of traits plasticity to water and nutrient manipulation (Avolio et al. 2018; Avolio and Smith 2013b; Chang and Smith 2014).

Experimental design

The split-plot experimental design was completely randomized. We selected a total of five genotypes (Genotypes 2, 3, 4, 5 and 12) of *A. gerardii* for this mesocosm study representing the most common genotypes found in the headquarter regions of the natural tallgrass prairie ecosystem of the LTER site (Avolio and Smith 2013a). We used meristem tissue culturing to propagate *A. gerardii* genotypes for this experiment. Original genotypes for tissue culturing collected in 2009 from the Konza Prairie Biological Research Station. After harvesting, the plant rhizomes were stored for a month at 4 °C and then established in the Marsh Botanical Gardens greenhouse at Yale University, New Haven, CT. Germplasm tissue was harvested from three individual plants of each genotype and sent to SMK Plants LCC (Billings, MT) for meristem tissue culturing to remove maternal effects. Tissue culture plants were planted in the greenhouse in 2011 for hardening and root development for 3 weeks before transplanting to the field site at Konza Prairie Biological Research Station. In mid-June 2011, the young plants were transferred to the field and planted within in 30 cm diameter collars that were buried 30 cm in the intact soil. The collars (hereafter pots) limited horizontal root spread but not vertical. Each pot was assigned a genotypic richness treatment, either monoculture (individual plants of the same genotypes) or three-genotype polyculture. For three-genotype polyculture, genotypes were selected from a pool of five genotypes such that there was an equal distribution of the five genotypes in ten different combinations of polyculture. In total, 140 pots were used which had nine individual plants each and two levels of genotypic richness—the nine

individuals were planted in a rectangular array with 9 cm between plants. There were 60 pots with plants in monoculture and 80 for polyculture. The experiment area was divided into two main plots for manipulating the amount of water received by the *A. gerardii* plants. A rainout shelter using clear, 6 mil, UV-transparent polyethylene greenhouse film was constructed on one of the two main plots to exclude rainfall by 100% such that two levels of drought treatment were (i) ambient (that received ambient rainfall) and (ii) droughted (Fig. 1.1). Fay et al. (2000) have reported a decrease in light reduction by about 21% in similar rainout shelter. Both plots, ambient and droughted, had equal numbers of monoculture and polyculture pots in a completely randomized design. Each main plot was then randomly assigned levels of mycorrhizal treatment within monocultures and polycultures. The two levels of mycorrhizal treatment were (i) untreated (only received water) and (ii) fungicide treated. Thiophanate-methyl fungicide (70% solution by weight) was used for fungicide-treated plots (Wilson and Williamson 2008). 500 mL of fungicide or water was applied every 2 weeks over the course of the growing season for the duration of the 5-year experiment. Plants under droughted treatment only received the 500 mL of fungicide or water whereas plants under ambient treatment received ambient rainfall in addition to the 500 mL of fungicide or water.

Environmental conditions measurements

In 2011, ambient temperature and humidity above the soil surface were measured daily to understand the effect of the rainout shelter on local climate using iButtons (Model DS 1923, Maxim Integrated, San Jose, CA, USA). There was no difference in air temperature ([mean \pm standard deviation] out in the open 26 \pm 8 °C

compared with under the rainout shelter 22 ± 6 °C) or relative humidity ($64\% \pm 25\%$) ambient, rainout shelter $62\% \pm 20\%$) based on a t-test (Fig. 1.S1). In 2012, we measured volumetric water content weekly from selected five pots under both drought and ambient treatments using probes from EC-20 ECH2 O soil moisture probes (Decagon Devices, Inc., Pullman, WA, USA) at 10 cm to see if there is a difference between the treatments. The shelter reduced soil moisture in average by 60% compared with ambient (Fig. 1.S2) (ambient $15\% \pm 7\%$, rainout shelter $6\% \pm 3\%$). Please note that 2012 was a drought year, and soil moisture was also low in the ambient plots.

During mid-August 2012, we collected soil from selected pots (0–10 cm) using a hand probe (2.5 cm diameter) from drought and mycorrhizal treatment and tested for phosphorus (P) and nitrogen (N) content of soil. Plant available P concentration obtained from Mehlich 3 test (Ziadi and Sen Tran 2008) was different for ambient and droughted treatments (Fig. 1.S4) with a mean of 68 ± 16 ppm for ambient treatment and 56 ± 9 ppm for droughted treatment. Phosphorus concentration did not differ for mycorrhizal treatment. Our field site being a former agricultural land had history of added soil nutrients. Ammonium N and nitrate N were extracted for 24 h in a 2 mol/L KCl solution, filtered and then analyzed colorimetrically with Alpkem autoanalyzer (Alpkem Cororation, College Station, TX). Both the extractable ammonium and nitrate N did not significantly differ between ambient and droughted treatments and between fungicide and control treatments. Our experiment site had a mean of 7.7 and 6.5 ppm of ammonium N and nitrate N, respectively.

Soil microbial community measurements

From the soil collected from selected pots during mid-August 2012, we did phospholipid-derived fatty acids (PLFA) analysis to determine the effectiveness of fungicide treatment and to see if microbial biomass differed between treatments. We assessed the biomass of gram-positive and gram-negative bacteria, AMF and saprophytic fungi. This work was done in the lab of Gail T. Wilson, Oklahoma State University, Stillwater, Oklahoma. Qualitative and quantitative PLFA analyses were done using Bligh and Dyer method (Frostegård et al. 1991) using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) and Sherlock software (MIDI, Newark, NJ, USA). The fatty acids used as indicators were: 16:1 ω 5c for AMF; 18:2 ω 6,9 for other fungal PLFAs (Schnoor et al. 2011); and 15:0, a17:0, i15:0, i16:0, i17:0, 16:1 ω 7, 17:0, cy17:0 and cy19:0 for bacteria (Moore-Kucera and Dick 2008).

A. *gerardii* biomass and functional traits measurements

For each year of the experiment, 2011–2015, aboveground biomass was clipped 2.5 cm from the ground at the end of the growing season (September–October), leaving the plant rhizome and all belowground structures intact for next year's growth. 2011 was an establishment year, and due to the small stature of the plants, these data were not included in analyses. In 2012, several functional traits that are indicators of plant growth strategies of individual plants were measured. Maximum height of each individual plant (height of the tallest tiller), number of flowers and number of bolts were measured at the end of the growing season. Plant height is associated with growth form and competitive vigor (Pérez Harguindeguy et al. 2016), number of flowers and bolts are

reproductive traits that are directly linked to plant fitness (Aguilar et al. 2008; Weltzin et al. 2003). Additionally, the weight of each individual plant was recorded at the end of the growing season and linked to planted genotype. For years 3–5 (2013–2015), only the biomass of the whole pots were recorded.

Relative yield calculation

To understand the effect of growing and competing with individuals of the same and different genetic backgrounds, we calculated relative yield as:

$$\frac{(\text{Yield in polyculture} - \text{yield in monoculture})}{\text{Yield in monoculture}}$$

We calculated relative yield only for 2012 data where we had individual plant weight. A positive value would mean higher yield of a genotype in polyculture compared with its yield in monoculture. Similarly, a negative value would mean a higher yield of a genotype in monoculture. We also looked for the mechanism explaining the difference in yield of *A. gerardii* between monoculture and polyculture. We used the equation by Loreau and Hector (2001) to calculate the complementarity and selection effects:

$$\Delta Y = N \overline{\Delta RY_1} \overline{M} + N \text{cov}(\Delta RY_i, M_i)$$

where ΔY is the difference in yield between polyculture and monoculture,

$\Delta RY_i = \frac{O_i}{M_i} - RYE$ is the relative yield difference (observed – expected) of genotype i

where O_i is the yield of ΔRY_1 genotype i in polyculture, M_i is the yield of genotype i in monoculture and RYE is the expected relative yield of each genotype in polyculture ($1/N$, where N is the number of genotypes in the polyculture). The term

$N \overline{\Delta RY_1} \overline{M}$ represents the complementarity effect and $N \text{cov}(\Delta RY_i, M_i)$ is used to

determine the selection effect. A positive complimentary effect would mean a higher yield of a genotype in polyculture compared with monoculture due to resource partitioning, and a positive selection effect would mean a higher yield of a genotype in polyculture compared with monoculture due to one or, more high yielding genotypes. We assessed differences in relative yield, and selection and complementarity effects of *A. gerardii* genotypes under different experimental conditions.

Statistical analysis

A linear mixed model for a split-plot design was used to analyze the effect of genotypic richness, drought treatment and mycorrhizal treatment on the biomass of *A. gerardii* over the years of 2012–2015 using ‘nlme’ package in R version 3.6.4 (Pinheiro et al. 2013). We used year, genotypic richness, drought treatment and mycorrhizal treatment as our main effects, and pot number and polyculture combinations as our random effects. Using the 2012 plant data, the only year we had functional trait measurements of each genotype, we did additional analyses of height, number of flowers and number of bolts for individual plant from each pot. We again used a linear mixed model with genotype, genotypic richness, drought treatment and mycorrhizal treatment as our main effects, and pot number and polyculture combinations as random effects. We used pot number that was assigned to each pot during establishment as a random effect to account for spatial variation, and the polyculture combination as our random effect on biomass, number of flowers and number of bolts to account for different polyculture types. Predictor variables were checked for multicollinearity using $VIF > 4.0$ before fitting them into our models and normality and

homoscedasticity assumptions of the model were checked and verified using diagnostic residual versus fitted and Q–Q plots. We conducted a nonmetric multidimensional scaling (NMDS) to see the difference in microbial community (gram-negative bacteria, gram-positive bacteria, AMF and saprophytic fungi) under drought and mycorrhizal treatment under *A. gerardii* genotypes from soils collected from selected pots in 2012, based on Bray–Curtis dissimilarity using the vegan package (Oksanen et al. 2005).

Results

Soil microbial community

Fungicide treatment had no effect on the biomass of arbuscular mycorrhizal or saprotrophic fungi, but fungal biomass of both AM and saprotrophic fungi was lower in droughted pots compared with pots that received ambient rainfall in 2012 (Fig. 1.S3a and b). In addition, the biomass of grampositive and gram-negative bacteria were not significantly affected by the fungicide treatment, but gram-positive bacteria biomass was slightly higher under ambient treatment than under droughted treatment (Fig. 1.S3c and d). Similarly, overall microbial biomass was higher under ambient conditions compared with the droughted treatment but did not differ under mycorrhizal treatment. Overall, microbial communities' biomass significantly differed between ambient and droughted treatments but there was no significant difference between control and fungicide-treated plants (Fig. 1.2) or among plant genotypes.

Effect of genotypic richness, drought and fungicide treatment on the overall aboveground biomass of *A. gerardii*

Our model explained about 49% of the variation in the overall above-ground biomass of *A. gerardii* over the years of 2012–2015. Year × drought treatments interaction significantly affected the above-ground biomass of *A. gerardii* (Table 1.1; Fig. 1.3). The aboveground biomass of *A. gerardii* was significantly higher under ambient than droughted treatment in 2012, 2013 and 2014. However, in 2015, the biomass did not differ significantly between drought treatments. By contrast, genotypic richness and fungicide treatment had no significant effect on the aboveground biomass of *A. gerardii* (Table 1.1).

Effect of genotypes, genotypic richness, soil moisture and mycorrhizal treatment on functional traits of *A. gerardii* in 2012

Above-ground biomass In 2012, the interaction between genotype and drought treatment had a significant effect on the above-ground biomass of individual *A. gerardii* plants (Table 1.2; Fig. 1.4). Only genotypes G2 and G3 had significantly higher above-ground biomass under ambient treatment than the droughted treatment (Fig. 1.4). Genotypic richness had no significant main effect on the above-ground biomass of *A. gerardii*, although the biomass was significantly affected by the interaction between genotypic richness and genotype (Table 1.2; Fig. 1.4). Genotype G2 had higher above-ground biomass within monoculture than polyculture while genotypic richness had no significant effect on the above-ground biomass of the other genotypes.

Height, flower and bolt number

Drought treatment and genotype had a significant effect on the height of *A. gerardii* plants (drought treatment $P < 0.0001$, genotype $P = 0.0041$). *Andropogon gerardii* plants were shorter when grown in the droughted treatment (63.34 ± 1.05 cm) compared with the ambient treatment (74.46 ± 1.09 cm). Genotype G2 was significantly taller than genotypes G4 and G5 (Table 1.3). Genotypes G3 and G12 did not significantly differ in height with either G2 or G4 and G5 (Table 1.3). There was a significant genotype \times drought treatment interaction on the number of flowers of *A. gerardii* in 2012 (Fig. 1.5) where only genotype G2 had significantly more flowers under droughted treatment than under ambient treatment (Fig. 1.5). There was also an interactive effect of genotype and genotypic richness on the number of bolts of *A. gerardii* (Fig. 1.5). Genotype G2 had more bolts under monoculture than polyculture while genotype G12 had more bolts in polyculture than in monoculture (Fig. 1.5). Drought treatment also significantly affected the number of bolts of *A. gerardii* such that the plants under droughted treatment had higher number of bolts than under ambient treatment.

Competitive outcomes of genotypes under drought treatment in 2012

Post hoc comparisons using the Tukey-HSD test indicated that the mean relative yield of the genotype G4 was significantly higher than the genotypes G2 and G3, meaning it grew more in polyculture versus monoculture, but not significantly different from G12 under ambient treatment (Fig. 1.6a). Similarly, genotype G4 had significantly higher relative yield than other genotypes under droughted treatment (Fig. 1.6a). Genotype G5 had positive relative yield under ambient treatment but had negative

relative yield under the droughted treatment. Overall, under ambient treatment, there was a positive complementarity effect but a large variation in the effect while there was a negative complementarity effect under droughted treatment (Fig. 1.6b). The selection effect was negative under ambient treatment but approximately zero under droughted treatment (Fig. 1.6b).

Discussion

Abiotic and biotic factors like drought and mycorrhizal association have the potential to affect plant productivity and plant functional traits, which can have implications for plant structure, composition and survival in the changing climate (Koerner et al. 2014; McCain et al. 2011). We assessed the effect of planted genetic diversity on the productivity a C4 grass, *A. gerardii*, under droughted and ambient water conditions, and under fungicide treated and control mycorrhizal treatments in a multi-year mesocosm study. In addition to productivity, we also assessed trait variation among *A. gerardii* genotypes to look at possible mechanism of the relationship between genetic diversity and productivity. Overall, genotypic richness and mycorrhizal association did not affect above-ground biomass of *A. gerardii* over the 4 years of our experiment. However, drought treatment significantly decreased the aboveground biomass of *A. gerardii* in all the years of the experiment. Interestingly, drought differentially affected the traits of *A. gerardii* genotypes, and the traits of genotypes also differed for monoculture versus polyculture.

Surprisingly, as evident from the PLFA tests, the biomass of AMF and saprophytic fungi in the soil collected from selected pots in 2012 did not differ for fungicide treated

and control pots. Usually, fungicide Thiophanate-methyl is used to suppress root colonization of AMF with host plants (Hartnett and Wilson 1999). Because this was a multiyear study, we did not collect the root samples for determining root colonization by AMF. However, the high phosphorus content in our soils might have suppressed the AMF abundance and colonization in the first place as has been found in several studies (Avolio et al. 2014; Balzergue et al. 2011; Breuillin et al. 2010; Carbonnel and Gutjahr 2014). Generally, the phosphorus content characteristic to the Konza Prairie Biological Station ranges from 4 to 26 ppm (Myster 2011; Rothrock and Squiers 2003) but our research site had exceptionally high amount of phosphorus content in the soil (up to 79 ppm). High phosphorus content in the soil is thought to make AMF colonization less important for plants (Avolio et al. 2014; Chen et al. 2019). Our field site being a former agricultural land had high phosphorus and thus, about twenty times lower AMF fungi biomass than is characteristic of the site which has been reported up to 60 nmol/g soil (Manoharan et al. 2017). Had our experiment been on a field site with lower soil P content, we could have seen negative effects of fungicide on biomass and functional traits of *A. gerardii*, however, the mycorrhizal treatment had no significant effect on the above-ground biomass and functional traits of *A. gerardii*. McCain et al. (2011) reported a decrease in plant productivity of dominant grasses after 4 years of AMF suppression in restored tallgrass prairie.

The hypothesized mechanism underlying the positive relationship between genetic diversity and productivity has been attributed to complementarity effects, where each individual genotype grows better in polyculture versus monoculture, and

selection effect, where the presence of a productive genotype accounts for the higher production (Loreau and Hector 2001). Complementarity effect results from niche partitioning between diverse genotypes so that resources like water and nutrients can be optimally utilized by the population. We found a small positive complementarity effect under ambient condition, but a negative complementarity effect in the drought treatment. This means that genotypes had higher above-ground biomass in polyculture compared with monoculture under ambient rainfall condition but had lower above-ground biomass in polyculture compared with monoculture in the drought treatment. Genotypes in the drought treatment seem to be competing with other genotypes for the scarce resource, water. When water was not scarce, the competition among the genotypes seems to be relaxed and slightly facilitative. Similarly, the selection effect was negative in the ambient treatment which suggests that some of the genotypes we selected for the experiment had overlapping niches for resources. Our findings add further support that environmental conditions affect the nature of the relationship between genetic diversity and productivity.

Although there was a small positive complementarity effect under ambient rainfall, the results of the study could not support our hypothesis regarding the positive relationship between genotypic diversity and productivity of *A. gerardii*. The aboveground biomass of *A. gerardii* from 2012 to 2015 did not significantly differ between monoculture and polyculture. This result is similar to the findings of Avolio et al. (2015), and Chang and Smith (2014). In contrast, Morris et al. (2016) used different cultivars of *A. gerardii* most of which are composites of various germplasms and found

positive effect of genotypic diversity on productivity of *A. gerardii*. Our study used naturally co-occurring genotypes at Konza Prairie Biological Station to create genotypic diversity as done by Avolio et al. (2015) and Chang and Smith (2014), and found similar results, and thus our findings may be more realistic of what occurs in intact *A. gerardii* populations. Level of genotypic richness and the identities of the genotypes in the polyculture can affect the relationship between genotypic diversity and productivity of a plant species. We had three genotypes randomly selected from a pool of five genotypes in our polyculture. Genotypes G4 and G5 had higher above-ground biomass compared with G2 and G3 in polyculture than in monoculture under ambient rainfall. The positive and negative relative yield of the genotypes seems to have canceled each other out and resulted in overall no significant relationship between genotypic richness and productivity of *A. gerardii*. Genotype G2 has been previously reported to have lower above-ground biomass in polyculture compared with monoculture (Avolio et al. 2015). Phenotypic differences under different genetic diversity can have important implications for understanding genetic diversity–productivity relationship (Schöb et al. 2015). Consequently, studies that use different genotypic richness and different identities of genotypes might yield different results (Vellend et al. 2010).

As expected, drought negatively impacted the above-ground biomass of *A. gerardii*. The difference in above-ground biomass of droughted and ambient treatments fluctuated each year which might be due to yearly variability in ambient soil moisture and plants root age. Decreasing growth during abiotic stress such as drought is a coping mechanism (Kim et al. 2010). Many plant functional traits such as leaf traits and

phenology are constitutive of plant strategies to drought adaptation (Chaves et al. 2003) and thus, are critical to study to understand plant growth under drought. We found that along with the above-ground biomass, drought treatment had a significant effect on the height, and number of flowers and bolts of *A. gerardii*. *Andropogon gerardii* plants under drought were smaller in height and had more bolts. Decrease in height and higher bolting under abiotic stress has been attributed to plants strategy to shorten their vegetative phase and shift the resources to the reproductive parts (Heschel and Riginos 2005; Wolfe and Tonsor 2014). For instance, genotype G2 had lower above-ground biomass but higher number of flowers and bolts in the drought treatment, and thus it is likely that genotype G2 is shifting its resources to its reproductive parts to escape drought. Genotype G2 is more responsive to water treatment and more plastic for number of buds than other *A. gerardii* genotypes (Avolio and Smith 2013b), which might have helped genotype G2 to shift its resources to reproductive phase better than other genotypes when droughted. Additionally, consistent with our results, genotype G2 has been found to grow faster and taller while genotype G4 has been found to have shorter height with a slower growth rate (Avolio et al. 2011; Avolio and Smith 2013b). Since G2 grew taller but had lower above-ground biomass than genotype G4, genotype G4 might have produced more tillers than G2. Understanding how different genotypes of the same plant species can respond differentially under various biotic and abiotic factors can inform about their fate under the global change.

Conclusion

In our study, although there was no positive relationship between genotypic richness and above-ground productivity, we found evidence of differential trait and productivity response of naturally occurring genotypes of *A. gerardii* under different environmental conditions. The results further exemplify how environmental conditions can have variable outcomes for different genotypes in different competitive environments. Understanding the competitive outcomes of genotypic diversity under various environmental conditions of a dominant grass can help with grassland restoration decisions to better cope with the present and future climate change.

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Table 1.1: ANOVA table showing results of linear mixed model for assessing the effect of drought treatment, genotypic richness and mycorrhizal association on biomass of *Andropogon gerardii* from 2012 to 2015

Variables	numDF	denDF	F	P
(Intercept)	1	289	434.43	<0.0001*
Year	3	36	18.57	<0.0001*
Drought	1	49	167.00	<0.0001*
Genetic richness	1	13	3.08	0.1029
Mycorrhizae	1	98	0.35	0.5531
Year: drought	3	49	10.84	<0.0001*
Year: genetic richness	3	36	0.45	0.7219
Drought: genetic richness	1	49	2.16	0.1481
Year: mycorrhizal treatment	3	98	0.32	0.8087
Drought treatment: mycorrhizal treatment	1	98	2.28	0.1340
Genotypic richness: mycorrhizal treatment	1	98	3.47	0.0655
Year: drought treatment: genotypic richness	3	49	0.21	0.8865
Year: drought treatment: mycorrhizal treatment	3	98	1.00	0.3940
Year: genotypic richness: mycorrhizal treatment	3	98	0.45	0.7204
Drought treatment: genetic richness: mycorrhizal treatment	1	98	0.00	0.9847
Year: drought treatment: genetic richness: mycorrhizal treatment	3	98	0.60	0.6161

denDF = denominator degrees of freedom, numDF = numerator degrees of freedom. *Significant at $P < 0.05$.

Table 1.2: ANOVA table showing results of linear mixed model for assessing the effect genotype, genotypic richness, soil moisture and mycorrhizal association on biomass of *Andropogon gerardii* in 2012

Variables	numDF	denDF	F	P
(Intercept)	1	1069	1978.1020	<0.0001*
Drought treatment	1	63	27.7140	<0.0001*
Genotypic richness	1	63	0.2900	0.5921
Mycorrhizal treatment	1	63	0.2852	0.5952
Genotype	4	1069	14.7147	<0.0001*
Drought treatment: genotypic richness	1	63	1.8403	0.1798
Drought treatment: mycorrhizal treatment	1	63	0.0810	0.7769
Genotypic richness: mycorrhizal treatment	1	63	1.1304	0.2918
Drought treatment: genotype	4	1069	3.4722	0.0079*
Genotypic richness: genotype	4	1069	6.1948	0.0001*
Mycorrhizal treatment: genotype	4	1069	1.2737	0.2785
Drought treatment: genotypic richness: mycorrhizal treatment	1	63	0.5898	0.4454
Drought treatment: genotypic richness: genotype	4	1069	1.1696	0.3226
Drought treatment: mycorrhizal treatment: genotype	4	1069	1.0764	0.3668
Genotypic richness: mycorrhizal treatment: genotype	4	1069	0.9372	0.4415
Drought treatment: genotypic richness: mycorrhizal treatment: genotype	4	1069	0.3047	0.8749

denDF = denominator degrees of freedom, numDF = numerator degrees of freedom. *Significant at $P < 0.05$.

Table 1.3: Mean values of measured plant functional traits of five genotypes of *Andropogon gerardii* in 2012 across all treatments

Genotypes	Mean trait values (\pm SE)		
	Plant height (cm)	Number of flowers	Number of bolts
G2	72.69 \pm 1.07	0.839 \pm 0.15	0.687 \pm 0.02
G3	68.3 \pm 1.50	0.004 \pm 0.06	0.483 \pm 0.10
G4	66.39 \pm 0.63	0.246 \pm 0.08	0.702 \pm 0.02
G5	68.06 \pm 0.97	0.099 \pm 0.01	0.390 \pm 0.07
G12	69.3 \pm 1.09	0.039 \pm 0.09	0.570 \pm 0.10



Figure 1.1: Research site showing the mesocosm study to assess the effect of genotypic richness, drought and mycorrhizal association in *Andropogon gerardii*. Our study area was divided into two main plots. The rainout shelter on the right were used to exclude 100% of ambient rainfall and simulate drought on one of the two main plots. Each main plot had two levels of genotypic richness: monoculture (nine individuals of the same genotype) and polyculture (nine individuals of three different genotypes). Thiophanate-methyl fungicide was used to suppress AMF in half of the pots of each genotypic richness level in each main plot. Pots that received fungicide were marked with pink flags, and other pots received water.

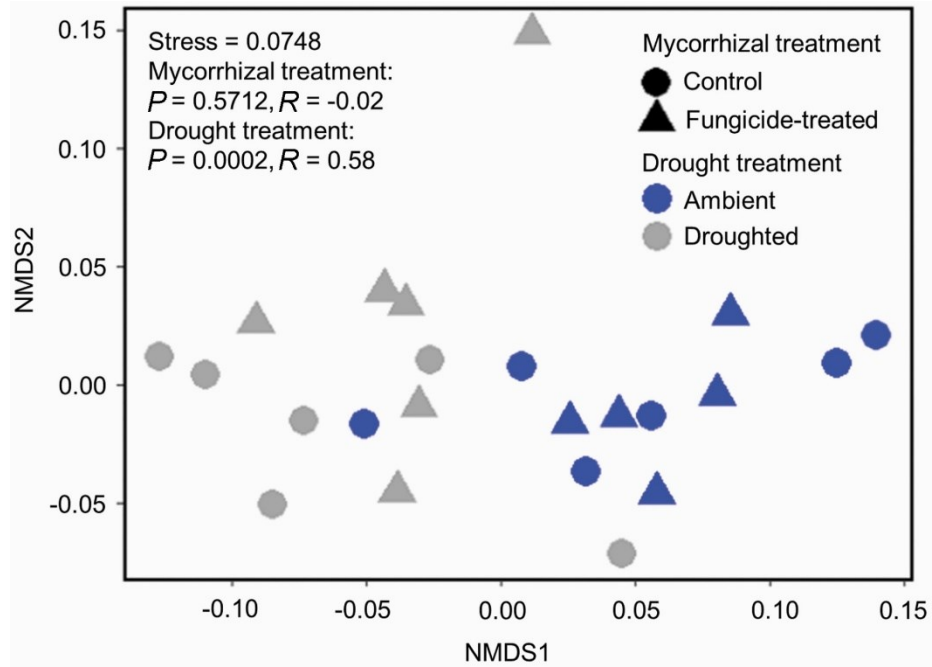


Figure 1.2: Ordination of microbial communities using NMDS generated from abundances of AMF, saprotrophic fungi, gram-positive and gram-negative bacteria. The data were obtained from PLFA tests of soil samples ($n = 22$) collected mid-August 2012 from selected pots of the experiment.

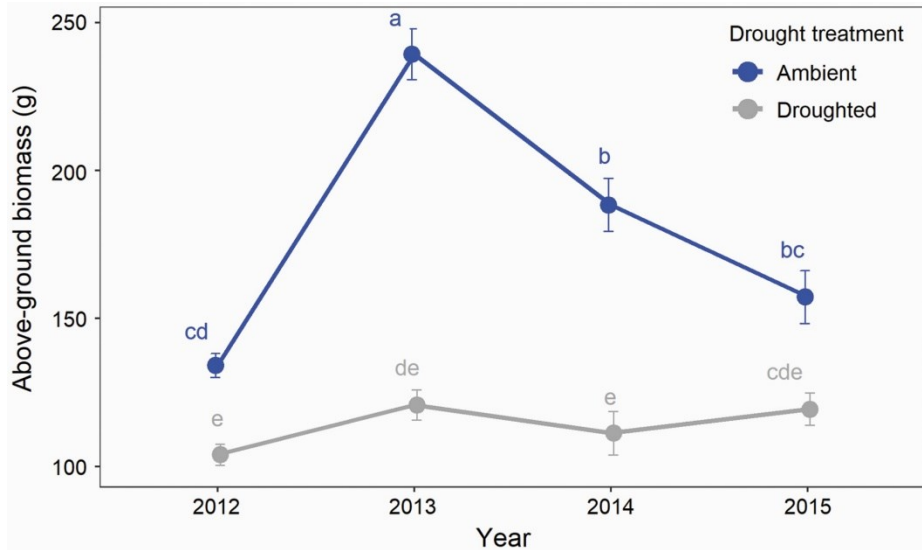


Figure 1.3: There was a significant interaction between year and drought treatment on the above-ground biomass of *Andropogon gerardii* from years 2012 to 2015. Letters show pairwise significant differences obtained by Tukey-HSD such that two points sharing no letters are significantly different to each other ($P < 0.05$). Each point represents the treatment mean and is shown with SE bars.

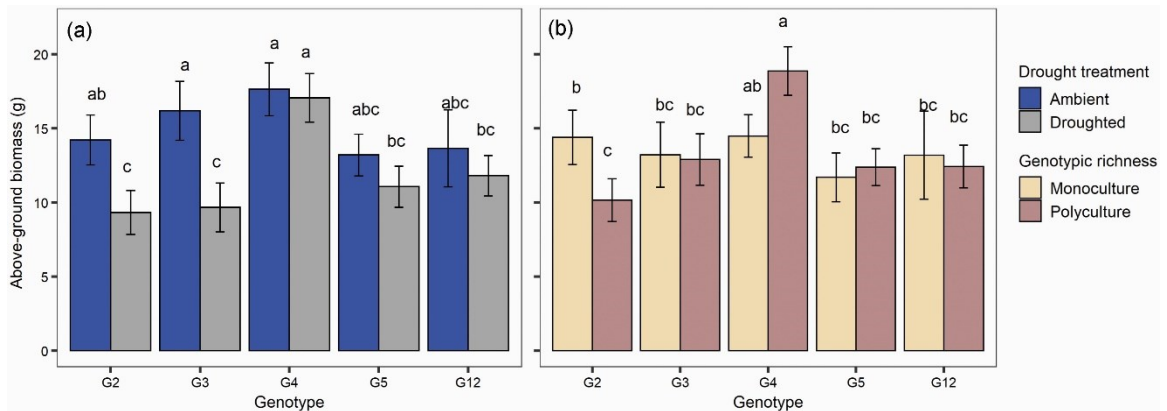


Figure 1.4: There were significant interactions between genotype and drought treatment (**a**), and between genotype and genotypic richness (**b**) on the above-ground biomass of *Andropogon gerardii* in 2012. Letters show pairwise significant differences obtained by Tukey-HSD such that two points sharing no letters in a graph are significantly different to each other ($P < 0.05$). Each bar represents the mean and is shown with SE bars.

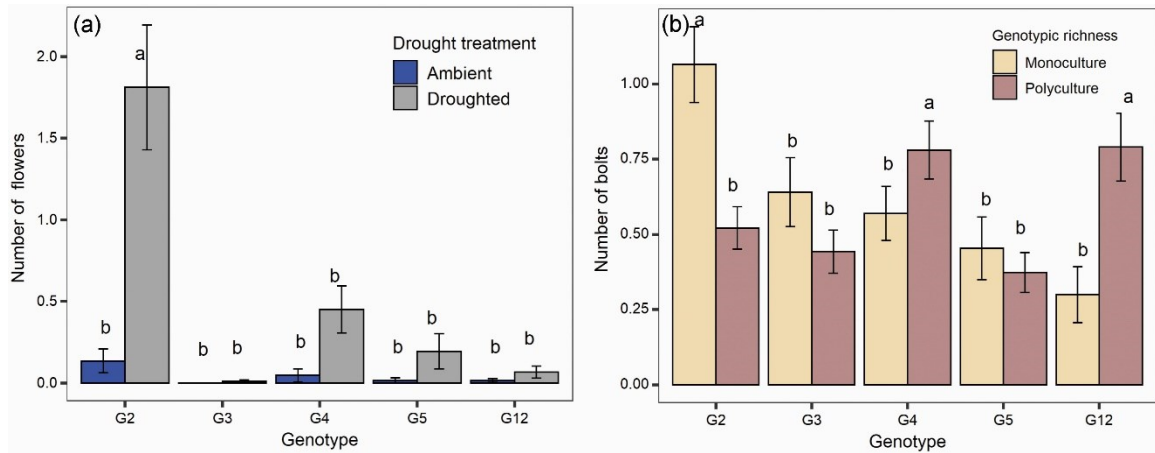


Figure 1.5: There was a significant interaction between genotype and drought treatment on the number of flowers **(a)**, and between genotype and genotypic richness on the number of bolts of *Andropogon gerardii* **(b)** in 2012. Letters show pairwise significant differences obtained by Tukey-HSD such that two points sharing no letters in a graph are significantly different to each other ($P < 0.05$). Each bar represents the mean and is shown with SE bars.

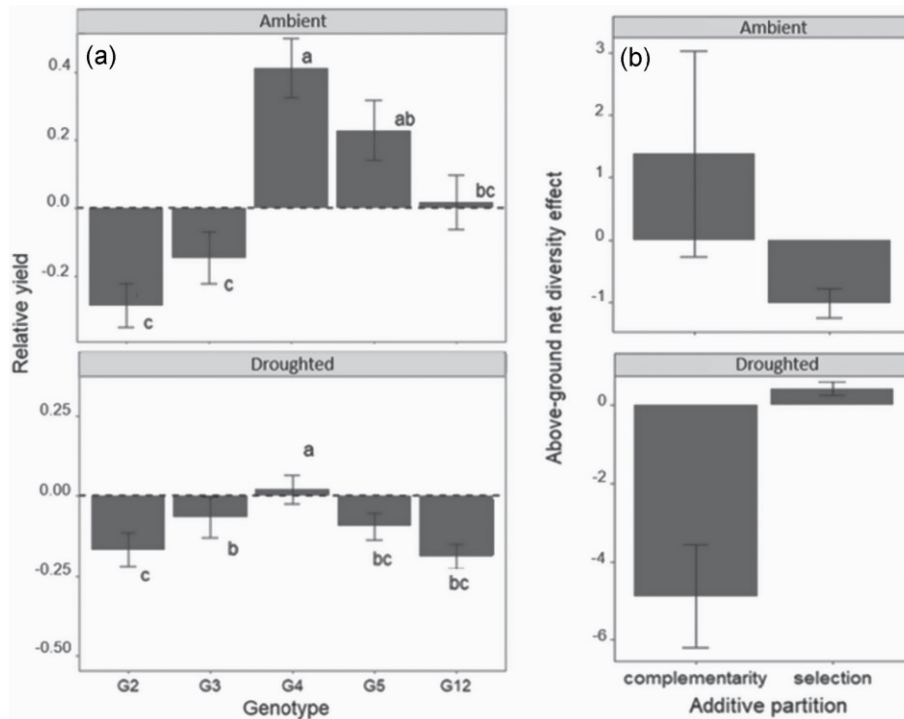


Figure 1.6: Competitive outcomes of the genotypes. **(a)** Mean relative yield biomass (± 1 SE) of genotypes under ambient and droughted treatment in 2012. Values above zero mean higher yield in polyculture than monoculture and values below zero mean lower yield in polyculture than monoculture. **(b)** Mean complementarity and selection effects (± 1 SD) on the above-ground biomass of *A. gerardii* under ambient and droughted treatment. Letters in the plots show pairwise significant differences obtained by Tukey-HSD such that two points sharing no letters are significantly different from each other ($P < 0.05$).

Supplementary figures

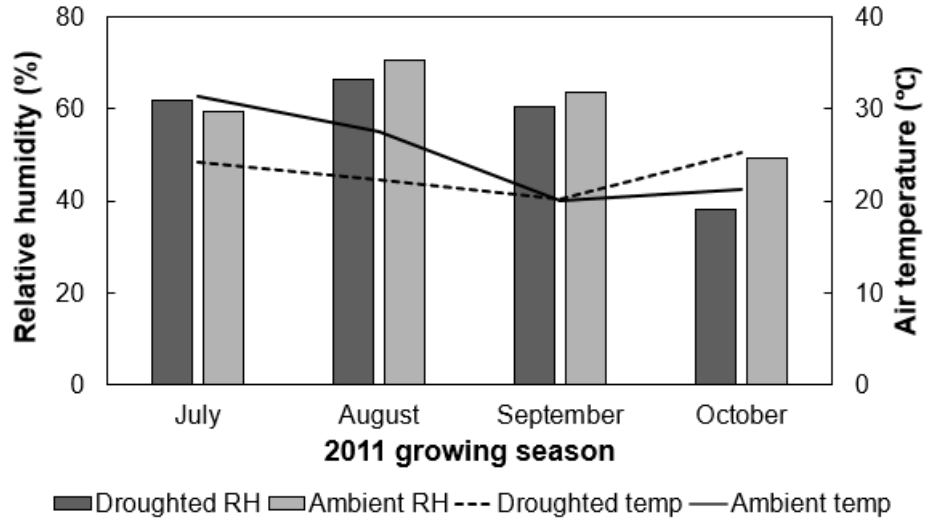


Figure 1.S1: Mean monthly temperature (temp) and mean monthly relative humidity (RH) during the 2011 growing season of *Andropogon gerardii*.

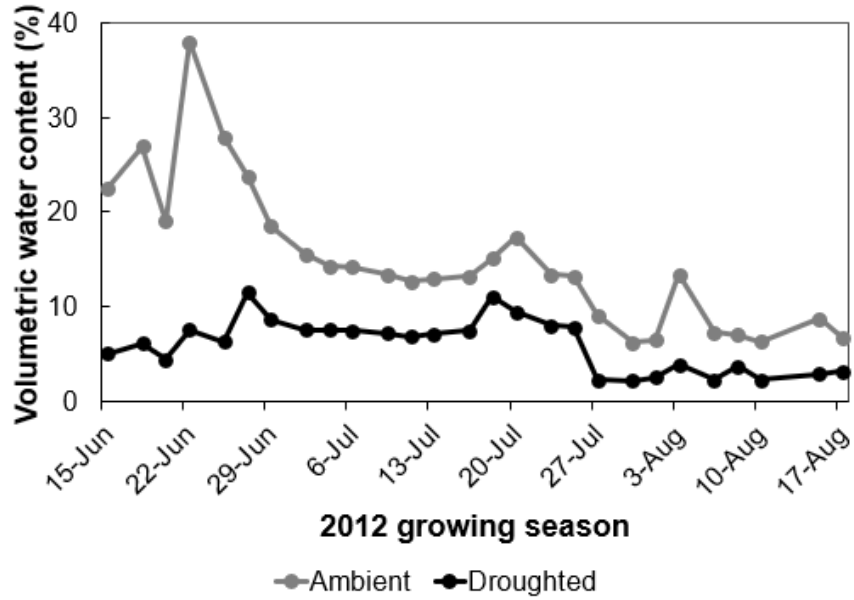


Figure 1.S2: Weekly volumetric water content across drought treatments during the 2012 growing season of *Andropogon gerardii*.

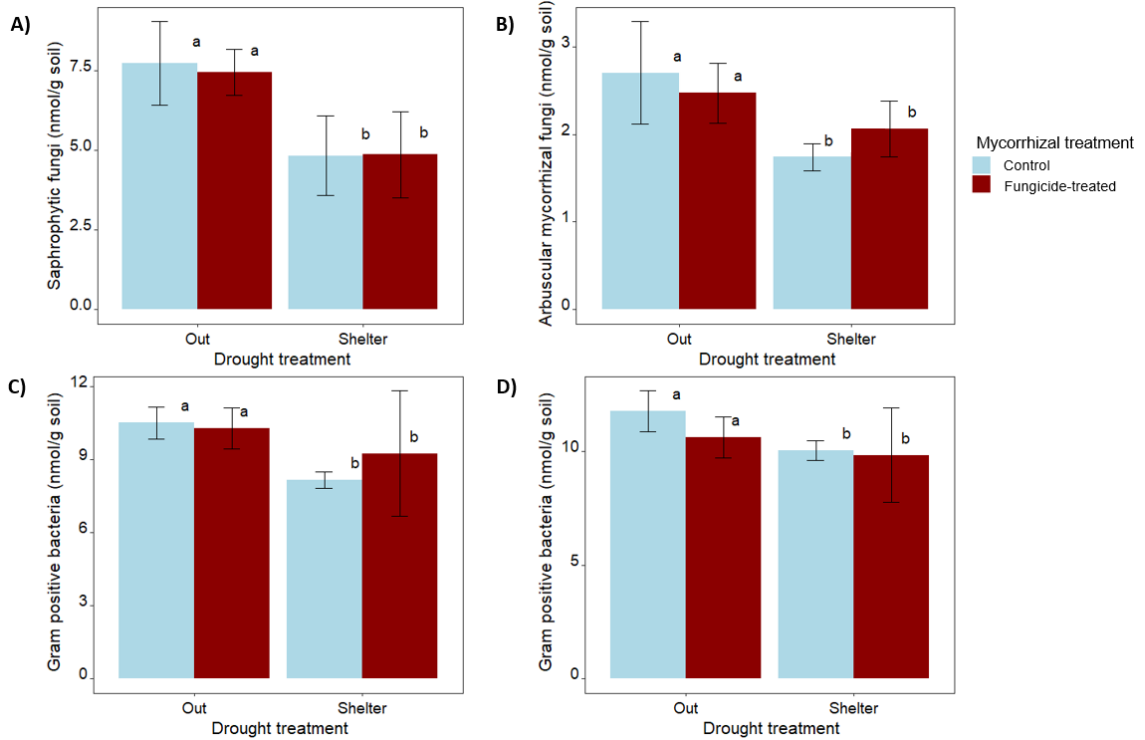


Figure 1.S3: Mean biomass (± 1 SE) of (A) saprophytic fungi (B) arbuscular mycorrhizal fungi (C) gram-positive bacteria and (D) gram-negative bacteria. The data was obtained from PLFA tests of soil samples ($n = 22$) collected mid-August 2012 from selected pots of the experiment. Letters show pairwise significant differences obtained by Tukey-HSD such that two points sharing no letters are significantly different to each other ($p < 0.05$).

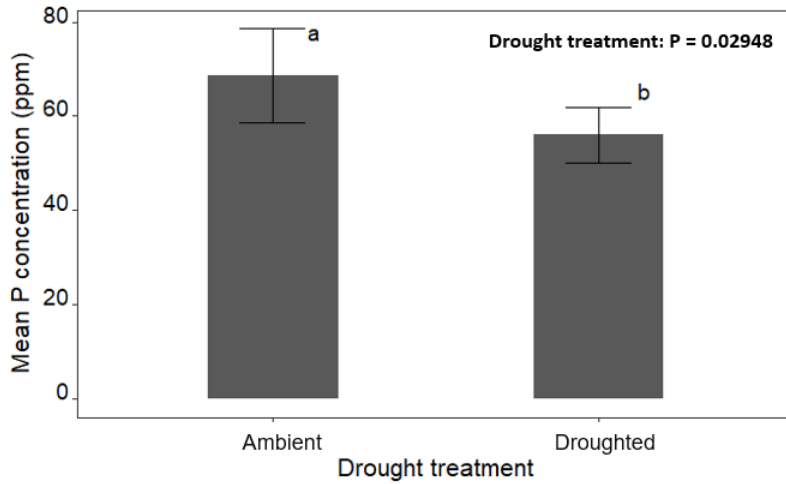


Figure 1S4: Mean phosphorus (P) concentration (± 1 SD) of soil samples ($n = 22$) collected during mid-August 2012 from selected pots of the experiment using Mehlich test. Letters show pairwise significant differences obtained by Tukey-HSD such that two points sharing no letters are significantly different to each other ($p < 0.05$).

Chapter 2

Plant-plant interactions of two codominant grasses along a stress gradient

Abstract

1. Plant-plant interactions can shape plant communities and influence ecosystem services. However, the dynamics of how these interactions shift from positive (facilitation) to negative (competition) or vice versa along a stress gradient remain less understood.
2. We tested the Stress Gradient Hypothesis, which suggests that plant-plant interactions shift from competition to facilitation with increasing environmental stress, using two co-dominant grasses of tallgrass prairie, *Andropogon gerardii* and *Sorghastrum nutans*. We conducted a pot experiment with three types of plant-plant interactions (no interactions, interspecific and intraspecific interactions), and subjected the grasses to combined effect of water and CO₂ treatments (ambient CO₂ and droughted, ambient CO₂ and well-watered, elevated CO₂ and drought, and elevated CO₂ and well-watered). Our CO₂ and water treatments created a stress gradient from most stressful (ambient CO₂ and drought) to least stressful (well-watered with elevated CO₂). We hypothesized that competition would be prominent in the least stressful condition and decrease as the stress gradient increased, eventually leading to facilitation under the more stressful condition.
3. Overall, drought and ambient CO₂ resulted in competition between plant individuals, which decreased with the increase in the stress gradient. We

observed facilitation under the least stressful condition (well-watered with elevated CO₂) for belowground biomass. Additionally, under drought and ambient CO₂, intraspecific competition was higher for both grasses than interspecific competition. Interestingly, AMF root colonization of the grasses increased with drought and decreased with elevated CO₂ in the presence of plant-plant interactions

4. *Synthesis*: Our study demonstrated a contrary finding to the Stress Gradient Hypothesis where we found increased competition under drought and decreased competition/facilitation due to elevated CO₂. These results hold significant implications for understanding the shift in plant-plant interactions of co-dominant grasses in the face of a changing climate.

Introduction

Plant-plant interactions can be defined as the effect of one individual plant on another through their influence on resource availability and habitat structure (Bakker et al., 2014; Brooker, 2006). These interactions, known as facilitation and competition for positive and negative interactions, respectively, play a key role in shaping plant communities and influencing ecosystem services by affecting soil microbial communities, plant fitness, abundance, and survival (Bakker et al., 2014; Brooker, 2006; Kunstler et al., 2011; Schöb et al., 2013). However, the impact of plant-plant interactions can be altered by environmental changes (Bilas et al., 2021; Brooker, 2006) and may shift along environmental stress gradients (Callaway & Walker, 1997), where 'stress' is used to describe environments that limit plants in converting energy to biomass (Callaway, 2007).

One of the most widely accepted conceptual models for understanding the relationship between environmental stress and plant-plant interactions is the stress-gradient hypothesis (SGH) (Callaway & Walker, 1997). According to this hypothesis, greater facilitation occurs with higher environmental stress levels while there is more competition with lower environmental stress levels (Callaway & Walker, 1997). Stress Gradient Hypothesis is supported by various studies that induce stress gradients through changes in factors like water, light, nutrients, space, and herbivory (Brooker et al., 2008; Callaway et al., 2002; Lortie & Callaway, 2006; Pugnaire & Luque, 2001). However, many studies have challenged this hypothesis, suggesting that the outcomes of plant-plant interactions may vary depending on factors such as the environmental

stress, the plant response variable selected, and the strategy of the interacting species (Goldberg et al., 1999; Kawai & Tokeshi, 2007; Maestre et al., 2005). A revised SGH proposed by Maestre et al., (2009) suggests plant-plant interactions might depend on life histories of interacting species and whether stress or the absence of it, is induced by a plant resource or not. For example, the authors propose that facilitation can become dominant at moderate stress, particularly when stress results from resource limitation (Maestre et al., 2009). Further research is needed to test the SGH hypothesis across a range of stress gradients induced by various environmental changes.

Two pressing environmental changes predicted with global change are elevated carbon dioxide (CO₂) and drought (IPCC, 2013; 2014). Understanding how these environmental changes affect plant-plant interactions will be crucial in developing strategies for preserving biodiversity in the changing climate (Brooker, 2006). Both drought and elevated CO₂ directly impact plant growth. In general, drought reduces plant growth, because plants close their stomata to minimize water loss (Chaves et al., 2003). In contrast, elevated CO₂ leads to increased plant productivity due to enhanced photosynthesis, increased carbon allocation belowground, and improved water use efficiency (Kassem et al., 2008). Thus, elevated CO₂ should offset to some extent impacts of drought, as plants will be able to acquire more CO₂ while minimizing water loss. Indeed, studies that have looked at the interactive effects of CO₂ and drought have found that elevated CO₂ ameliorates the negative impact of drought by reducing stomatal conductance, change in leaf surface, and regulating gene expression (van der

Kooi et al., 2016; Xu et al., 2013). Thus, different CO₂ levels combined with drought can create varying degrees of stress for plants.

Drought has been widely studied in terms of its effect in plant-plant interactions in various biomes. Gao et al. (2018) found that drought increased facilitation in *Cleistogenes squarrosa*, a dominant species of the typical steppe of Mongolia, whereas Butterfield et al., (2016) found that drought increased competition between shrubs in dryland ecosystems. Drought, when combined with other biotic or abiotic factors, can lead to variable outcomes in plant interactions. For instance, Alba et al. (2017) reported that invasion could mitigate drought stress, potentially altering plant-plant interactions. Similarly, elevated CO₂ has been reported to affect plant-plant interactions through their differential stimulation of growth of different plant species, changing the resource availability among them (Brooker, 2006; Valerio et al., 2011). While the impacts of drought and elevated CO₂ on plant-plant interactions have been studied more extensively, the combined impact of drought and CO₂ on plant-plant interactions is less known.

Drought and elevated CO₂ also affects arbuscular mycorrhizal fungi (AMF) associations with plants. Arbuscular mycorrhizal fungi form symbiosis with more than 80% of terrestrial plants, providing water and nutrients to plants (Augé, 2001) in exchange for the host plants' photosynthates (Drigo et al. 2010). Drought can increase or decrease AMF root colonization depending on the ability of AMF to colonize with plant roots in low moisture (Augé, 2001; Millar & Bennett, 2016; Staddon et al., 2003). Elevated CO₂ tends to increase AMF colonization due to higher host plant carbon

assimilation and increased nutrient demands (Johnson & Gehring, 2007; Cairney, 2012). Plant-plant interactions can further influence plant-AMF relationships (Van Der Heijden & Horton, 2009). Arbuscular mycorrhizal fungi associations can increase the competitive ability of one plant species over the other (Zhang et al., 2014), potentially influencing the plant-plant interactions among them. Similarly, AMF has been reported to ameliorate competition in natural ecosystems (Van Der Heijden & Horton, 2009), increase plant competition in severe drought (Koide, 1991) or increase facilitation in woody plants (Dickie et al., 2002). Additionally, although less studied, plant-plant interaction have also been reported to affect AMF root colonization depending on the interacting species (Hausmann & Hawkes, 2009; Mummey & Rillig, 2006). Further research is needed to explore how plant-plant interactions affects AMF root colonization in host plants.

Our study aimed to address how altered environmental conditions (drought and elevated CO₂) and plant neighborhood surroundings (alone, and intra- or inter-specific plant-plant interactions) interacted to affect (a) the growth of two grasses species of tall-grass prairie and (b) AMF root colonization of both species. We hypothesized that drought would be stressful for plants resulting in reduced above-and-belowground biomass, and AMF root colonization of both grasses, while elevated CO₂ would create a less stressful condition for plants by increasing water use efficiency and thus, ameliorate the negative drought effects on biomass and AMF root colonization. Furthermore, we hypothesized that drought would lead to facilitation between plant individuals while elevated CO₂ would increase plant-plant competition in line with the Stress Gradient

hypothesis (Callaway & Walker, 1997). These hypotheses suggest that competition intensifies under low-stress conditions due to increased resource demand, and facilitation prevails under high-stress conditions due to improved space and light availability.

Methods

Site description and experimental design

The study was carried out in the Fossil and Future experiment at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland in 2021. We conducted a pot experiment inside open top CO₂ chambers on two dominant species. Dominant species, defined by their high relative abundance and a proportionate impact on ecosystem functions such as aboveground productivity within the community (Avolio et al., 2019) are particularly important to study due to their disproportionate influence on ecosystem services. Both *Andropogon gerardii* Vitman (Big bluestem) and *Sorghastrum nutans* (L.) Nash (Indiangrass) are dominant species in North American tallgrass prairies, (Silletti & Knapp, 2001), and are important native rangeland species being used for erosion control, land reclamation, and biomass energy. Consequently, changes in these grasses, both present and future, carry substantial implications for the tallgrass prairie ecosystem. Additionally, both species are obligatory mycotrophic plants (Hartnett & Wilson, 1999), requiring association with arbuscular mycorrhizal fungi (AMF) for their growth and productivity.

The experiment was arranged in a split plot design, where two water treatments – well-watered and droughted were nested within CO₂ chambers. Each CO₂ chamber

was assigned one of the two CO₂ treatments – ambient and elevated CO₂ and each CO₂ treatment had three replications. Additionally, the plant treatments were: (i) no interactions: one *A. gerardii* or one *S. nutans* (ii) intraspecific interactions: two *A. gerardii* or two *S. nutans*, and (iii) interspecific interactions: one *A. gerardii* and one *S. nutans*. Each plant treatment was replicated twenty-four times resulting in a total of 120 pots.

Seeds of *A. gerardii* and *S. nutans* were obtained from Prairie Moon Nursery (Winona, MN). Following germination, seedlings of equal size were transplanted to a pot (12.7 cm × 12.7 cm × 30 cm; Stuewe and Sons, Oregon) with a hole at the bottom for drainage, containing local topsoil of Maryland. Soil was homogenized (hand-mixed) before use. Care was taken to maintain equal distances between plants (~5 cm) when two plant individuals were grown together. Transplanting was followed by watering of the plants for acclimation. The plant pots were placed over trays to capture any water runoff.

Carbon dioxide treatments were maintained at CO₂ levels of 420 ppm for the ambient CO₂ treatment and 1,000 ppm for the elevated CO₂ treatment to simulate ambient and predicted CO₂ levels, respectively (IPCC 2013, 2014) with custom-built monitoring and control equipment. However, the average daily CO₂ levels ([mean ± standard deviation]) for the ambient and elevated CO₂ treatments were 486 ± 26 and 837 ± 36 ppm, respectively (Fig. 2.1A). Because of the angled roofs of the open-top CO₂ chamber, little rainfall reached our planted pots, therefore, we constructed rainout shelters above the plants to ensure drought. Plants under well-watered treatment were

watered every other day, maintaining a gravimetric soil moisture content of approximately 45% (Fig. 2.1B). Similarly, all plants under the droughted treatment were watered when the gravimetric soil moisture content was at or below 25% (Fig. 2.1B). Inside each chamber within each water treatment, the pot locations were randomized and rotated every three weeks to reduce spatial bias. After one growing season (12 weeks), the aboveground and belowground parts of individual plants were destructively harvested, separated, and dried (60°C, 3 d) to determine biomass. About five segments (~4 cm each) were taken from the roots of each plant, and the percentage of AMF root colonization was determined by intersect gridline method (Giovannetti & Mosse, 1980) following staining the roots by trypan blue.

Statistical analyses

All statistical analyses were performed using R version 4.2.2 (R core team 2021). The response variables of individual plants without plant-plant interactions – above- and-belowground biomass, total biomass, and AMF root colonization were analyzed using a linear mixed-effects model with the “lme4” package in R (Bates et al. 2015). The model included CO₂ treatment, water treatment, and focal plant species as fixed effects, while chamber (open-top CO₂ chamber) was treated as a random effect. Root-shoot ratio for each individual plant was calculated as:

$$\text{Root-shoot ratio (RSR)} = \frac{\text{Belowground biomass (g)}}{\text{Aboveground biomass (g)}}$$

Higher RSR indicated a higher biomass allocation to roots compared to shoots, while a lower RSR indicated a higher biomass allocation to shoots compared to roots.

To determine the effect of plant-plant interactions on aboveground biomass, belowground biomass and AMF root colonization, log response ratio was calculated as:

Log response ratio (lrr) =

$$\log \left(\frac{\text{Attributes of the focal species when grown alone}}{\text{Attributes of the focal species when grown with another individual}} \right)$$

where, attributes were either aboveground biomass, belowground biomass or, AMF root colonization. Log response ratios provided a quantification of plant-plant interactions, which could either be intraspecific (e.g., *A. gerardii* grown with *A. gerardii*) or interspecific (e.g., *A. gerardii* grown with *S. nutans*). A positive log response ratio for biomass indicated facilitation, a negative ratio denoted competition, and a zero value indicated no plant-plant interactions. Regarding AMF response, a positive log response indicated a higher AMF investment when plants were grown together with an individual of same or different species compared to when grown alone, a negative value indicated a lower investment, and a zero value indicated no change in the AMF investment.

Log response ratios of aboveground plant-plant interactions, belowground plant-plant interactions and AMF response as affected by CO₂, water, and plant treatments were analyzed using linear mixed model in R (Bates et al. 2015) using the treatments and focal plant species as the fixed effects, and chamber as a random effect. Following the significant effects ($P < 0.05$) from the linear mixed models, post-hoc analyses were performed using the “emmeans” package in R (Length et al. 2018; R Core Team 2015).

Results

Effect of CO₂ and water treatments on above-and-belowground biomass, and root-shoot ratio of *A. gerardii* and *S. nutans* with no plant-plant interactions

There were significant effects of focal species, water, and CO₂ treatments on the aboveground biomass of *A. gerardii* and *S. nutans* (Table 2.1; Fig. 2.2A & B). For both focal species, there was a significant interaction between water and CO₂ treatments (Fig. 2.2A & B). Specifically, the droughted treatment significantly reduced aboveground biomass of *A. gerardii* and *S. nutans*, but it had no effect on aboveground biomass when combined with elevated CO₂. Additionally, while the belowground biomass varied depending on the species, neither water nor CO₂ treatments affected belowground biomass (Table 2.1; Fig. 2.2A & B). The root-shoot ratio of both *A. gerardii* and *S. nutans* increased when droughted under ambient CO₂ but not elevated CO₂, suggesting that CO₂ ameliorated the impacts of drought for both species (Table 2.1; Fig. 2.2C & D). We also looked for the total biomass of both grasses and we did not find significant differences than above-and-belowground biomass (data not shown).

Effect of CO₂, water, and plant treatments on above-and-belowground biomass of *A. gerardii* and *S. nutans* with plant-plant interactions

When grown with plant-plant interactions there was a significant interaction between water and CO₂ treatment on the log response ratio of aboveground biomass (Table 2.1, Fig. 2.3). Aboveground biomass was reduced relative to when plants were grown alone with plant-plant interactions for both *A. gerardii* and *S. nutans*, meaning there was aboveground competition in both CO₂ and water treatments, regardless of

whether it was intra-or-interspecific interactions. Drought alone resulted in the strongest competitive interaction (Fig. 2.3A); however, CO₂ ameliorated the negative effect of drought (Fig. 2.3A). For the log response ratio of belowground biomass, when grown with plant-plant interactions there were no significant main effects of CO₂ and plant treatments, however, there was a significant interaction among CO₂, water, and plant treatment as well as among CO₂, water, and focal species (Table 2.2; Fig. 2.3B, C). Belowground competition was stronger for intraspecific interaction compared to interspecific competition when focal species were droughted under ambient CO₂ treatment (Fig. 2.3B). We observed facilitation for both intraspecific and interspecific plant-plant interactions in well-watered condition under elevated CO₂ (Fig. 2.3B). Additionally, both *A. gerardii* and *S. nutans* had higher belowground competition when droughted under ambient CO₂. However, when well-watered under elevated CO₂, facilitative interactions were evident for both grasses. Generally, there were no substantial differences between the response of these two grasses to CO₂ and drought, except under droughted treatment with elevated CO₂ where *A. gerardii* exhibited competitive interactions, and *S. nutans* displayed facilitative interactions with another individual plants of same or different species (Fig. 2.3C).

Effect of CO₂, water, and plant treatments on AMF root colonization of *A. gerardii* and *S. nutans* with/without plant-plant interactions

Both focal species exhibited varied AMF root colonization depending on the treatments (Table 2.3). For *A. gerardii*, drought reduced root colonization regardless of the CO₂ treatment (Fig. 2.4A). However, plant-plant interactions altered this

relationship, resulting in increased AMF root colonization in *A. gerardii* under droughted condition for both CO₂ treatments (Fig. 2.4B). Additionally, under drought and ambient CO₂, AMF root colonization in *A. gerardii* was higher for intraspecific than interspecific plant-plant interactions. Conversely, for *S. nutans*, elevated CO₂ significantly increased AMF root colonization regardless of the water treatment (Fig. 2.4A) but plant-plant interactions had no or minimal impact on AMF root colonization in all cases except the droughted treatment with ambient CO₂ for intraspecific interaction (Fig. 2.4B).

Generally, there were no significant differences in AMF root colonization between intra- and interspecific interactions. However, *A. gerardii* exhibited higher AMF colonization in intraspecific interaction in droughted with ambient CO₂ treatment, as well as in well-watered with elevated CO₂ treatment. Similarly, *S. nutans* showed higher AMF colonization in intraspecific interaction under droughted with ambient CO₂ treatment.

Discussion

In this study, we explored if plant-plant interactions along a stress-gradient, induced by drought and elevated CO₂, support the Stress Gradient Hypothesis. Our findings contrasted with this hypothesis, revealing that both intra and interspecific plant-plant interactions between individuals of *A. gerardii* and *S. nutans* leaned toward competition under drought, a stressful condition while leaning toward facilitation when well-watered under elevated CO₂, comparatively a less stressful condition. Overall, our study indicates that both biotic factors (presence/absence of plant-plant interactions) and abiotic factors (drought and elevated CO₂) intertwine to shape the patterns

observed in above-and-belowground biomass and the associated root colonization of these two co-dominant grasses of tallgrass prairie.

As hypothesized, drought reduced the aboveground biomass of *A. gerardii* and *S. nutans* but elevated CO₂ reduced drought stress resulting in greater aboveground biomass, with or without plant-plant interactions. Similar observations were reported by Owensby et al. (1997) in C₄ grasses of tallgrass prairie plant communities using open-top CO₂ chambers. This ameliorating effect of elevated CO₂ has been attributed to the reduced stomatal conductance in plants, which enable them to grow while minimizing water loss through transpiration (van der Kooi et al., 2016). Had the drought sustained, a lower level of intercellular CO₂ might have ensued, leading to an incipient metabolic reduction in plants, ultimately resulting in decreased aboveground biomass (van der Kooi et al., 2016).

For belowground biomass, we observed no significant effect of drought and elevated CO₂ on the belowground biomass of these grasses when grown without plant-plant interactions, which contradicted our initial hypothesis. This begs the question if drought and elevated CO₂ created an ecologically meaningful stress gradient for the belowground biomass of the two grasses. When grown with plant-plant interactions, drought and elevated CO₂ significantly interacted with focal species. We found overall significant differences in belowground biomass of *A. gerardii* and *S. nutans* when grown without plant-plant interactions. This inherent difference in roots might have resulted in slightly varied strategies to face plant-plant interactions where *A. gerardii* faced more

competition due to drought and the drought effects were not ameliorated by elevated CO₂, whereas competition for *S. gerardii* decreased with elevated CO₂.

Similarly, we found an increased root-shoot ratio for both *A. gerardii* and *S. nutans* when subjected to drought under ambient CO₂ with or without plant-plant interactions aligning with the balanced-growth hypothesis. This hypothesis posits that when aboveground resources are limited, biomass allocation is allocated to shoots, but to roots when belowground resources are limited (Chen et al., 2018). More carbon allocation to roots than shoots or a higher root-shoot ratio might serve as a drought mitigating strategy of these grasses to increase water uptake during drought. Our finding supported previous studies where an increased root-shoot ratio due to decreased water and nutrients availability has been documented (Giardina et al., 2003; Wilcox et al., 2016).

Understanding plant-AMF symbiosis under diverse environmental conditions holds significant implications for enhancing water and nutrient use efficiency in ecologically relevant plant species. In the absence of plant-plant interactions, AMF root colonization of the two grasses showed varied response to drought and elevated CO₂; *A. gerardii* was sensitive to drought, while *S. nutans* was sensitive to elevated CO₂. However, in the presence of plant-plant interactions, both *A. gerardii* and *S. nutans* exhibited higher AMF root colonization when subjected to drought, which corresponded to higher belowground competition. This suggests that both grasses invested more in AMF root colonization when there was a higher stress gradient induced by a combined effect of an abiotic factor (drought) and a biotic factor (competition). Change in AMF

root colonization due to plant-plant interactions has been reported before to be species-specific, attributed to the fact that there is significant variation in AMF species harbored by different plant species (Hausmann & Hawkes, 2009). Further studies are needed to determine if this phenomenon is a regular occurrence.

Our findings provide evidence of aboveground competition among plant individuals, with competition intensifying as the stress gradient increases. Similar patterns emerged for belowground biomass interactions in these grasses: as drought intensified, competition increased, while facilitation was evident under well-watered conditions combined with elevated CO₂ treatment. Our results align with studies contradicting the Stress Gradient Hypothesis (Butterfield et al., 2016; Maestre et al., 2005; Tielbörger & Kadmon, 2000), but differ from those supporting it (He et al., 2013; López et al., 2016; Lortie & Callaway, 2006; Ziffer-Berger et al., 2014).

Facilitation during high abiotic stress, such as drought, is proposed to arise from plants buffering each other from stress through improved soil fertility and microclimate under canopies (Callaway & Walker, 1997; Cortina & Maestre, 2005). However, facilitation among plants sharing belowground niches is unlikely under drought due to limited water availability (Maestre et al., 2009). Similarly, increased resource availability through reduced evapotranspiration and enhanced water and nutrient supply resulted in facilitation for well-watered condition with elevated CO₂ (Maestre et al., 2009). Stronger association with AMF can also lead to facilitation (Zhang et al., 2014); however, our study found lower AMF root colonization in both *A. gerardii* and *S. nutans* when there was belowground facilitation. Plants likely adopt varied responses to stress

gradients. Drought led to lower belowground biomass but increased AMF colonization, while well-watered conditions with elevated CO₂ led to higher aboveground biomass but reduced AMF colonization. It is also important to note that the drought treatment we imposed might not be extreme enough to create high-stress conditions for these grasses. Also, the degree of stress might vary for different plant variable measured (Butterfield et al., 2016), and the combination of environmental factors accounted for in the experiment.

Additionally, intraspecific competition was higher for belowground biomass when droughted under ambient CO₂ than interspecific competition. This observation aligns with previous studies indicating a higher degree of niche partitioning between individuals of different plant species than between individuals of same species (Adler et al., 2018). This suggests that during drought, *A. gerardii* and *S. nutans* exerted more competition towards itself rather than to each other, which might have implications for their coexistence during drought. Interestingly, AMF root colonization was also higher in intraspecific competition than interspecific competition for both grass when droughted under ambient CO₂ condition.

It is important to note that these results were obtained from a one growing season study using commercial cultivars of *A. gerardii* and *S. nutans*. Genotypic diversity within species plays a crucial role in shaping species' response to biotic and abiotic factors. *A. gerardii* genotypes have been previously reported to have a differential response to drought and AMF treatment (Pehim Limbu & Avolio, 2023).

Conclusion

Our study suggests that drought and elevated CO₂ may alter plant-plant interactions between these codominant grasses and potentially change the community structure of tallgrass prairie. Mostly similar responses of these two co-dominant grasses with a few exceptions to biotic and abiotic changes may sustain their ecological co-dominance due to complementarity effect or push towards competitive exclusion. Overall, our study provides important insights into the ways in which drought and elevated CO₂ can impact the growth and interactions of *A. gerardii* and *S. nutans*. These findings have important implications for understanding the responses of plant communities to changing environmental conditions.

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Table 2.1: Linear mixed effects model results showing the effect of focal species, carbon dioxide and water treatments on aboveground biomass, belowground biomass, and root-shoot ratio of *Andropogon gerardii* and *Sorghastrum nutans* grown without plant-plant interactions. #

Source	Response variables					
	Aboveground biomass		Belowground biomass		Root-shoot ratio	
	<i>F</i> _(NdF, DdF)	<i>P</i>	<i>F</i> _(NdF, DdF)	<i>P</i>	<i>F</i> _(NdF, DdF)	<i>P</i>
Focal species	12.41 _(1, 36)	0.001**	5.25 _(1, 36)	0.027*	8.62 _(1, 36)	0.005*
CO ₂ treatment	15.87 _(1, 4)	0.016*	5.33 _(1, 4)	0.082	21.14 _(1, 4)	<0.001***
Water treatment	12.01 _(1, 36)	0.001**	2.31 _(1, 36)	0.136	36.43 _(1, 36)	<0.001***
CO ₂ treatment: Water treatment	10.11 _(1, 36)	0.003*	2.42 _(1, 36)	0.128	21.68 _(1, 36)	<0.001***
CO ₂ treatment: Focal species	0.89 _(1, 36)	0.351	1.34 _(1, 36)	0.253	8.61 _(1, 36)	0.005**
Water treatment: Focal species	1.78 _(1, 36)	0.190	1.11 _(1, 36)	0.298	15.00 _(1, 36)	<0.001***
CO ₂ treatment: Water treatment: Focal species	0.20 _(1, 36)	0.651	0.15 _(1, 36)	0.692	2.79 _(1, 36)	0.102

#Abbreviations: NdF- numerator degrees of freedom, DdF - denominator degrees of freedom, F -F-value

*** is significant at $P < 0.001$, ** is significant at $P < 0.01$, and * is significant at $P < 0.05$

Table 2.2: Linear mixed effects model results showing the effect of focal species, carbon dioxide, water, and plant treatments on log (response ratio) (lrr) of *Andropogon gerardii* and *Sorghastrum nutans*. Aboveground lrr and belowground lrr are the measures of plant-plant interactions. #

Source	Response variables			
	Aboveground lrr		Belowground lrr	
	<i>F</i> _(NdF, DdF)	<i>P</i>	<i>F</i> _(NdF, DdF)	<i>P</i>
Focal species	3.36 _(1, 124)	0.068	38.28 _(1, 124)	<0.001***
CO ₂ treatment	1.78 _(1, 4.34)	0.164	4.90 _(1, 4.21)	0.087
Water treatment	7.07 _(1, 124)	0.008**	35.01 _(1, 124)	<0.001***
Plant treatment	0.06 _(1, 124)	0.937	1.36 _(1, 124)	0.244
CO ₂ treatment: Water treatment	9.68 _(1, 124)	0.002**	0.92 _(1, 124)	0.337
CO ₂ treatment: Plant treatment	0.001 _(1, 124)	0.970	2.06 _(1, 124)	0.153
Water treatment: Plant treatment	0.24 _(1, 124)	0.623	0.24 _(1, 124)	0.623
CO ₂ treatment: Focal species	1.24 _(1, 124)	0.266	10.55 _(1, 124)	0.001**
Water treatment: Focal species	0.62 _(1, 124)	0.430	8.76 _(1, 124)	0.003*
Plant treatment: Focal species	0.71 _(1, 124)	0.400	0.13 _(1, 124)	0.712
CO ₂ treatment: Water treatment: Plant treatment	0.57 _(1, 124)	0.449	6.84 _(1, 124)	0.009**
CO ₂ treatment: Water treatment: Focal species	0.57 _(1, 124)	0.451	10.42 _(1, 124)	0.001**
CO ₂ treatment: Plant treatment: Focal species	0.68 _(1, 124)	0.410	0.29 _(1, 124)	0.590
Water treatment: Plant treatment: Focal species	0.14 _(1, 124)	0.701	1.69 _(1, 124)	0.194
CO ₂ treatment: Water treatment: Plant treatment: Focal species	2.33 _(1, 124)	0.128	0.10 _(1, 124)	0.748

#Abbreviations: NdF- numerator degrees of freedom, DdF - denominator degrees of freedom, F -F-value

*** is significant at $P < 0.001$, ** is significant at $P < 0.01$, and * is significant at $P < 0.05$.

Table 2.3: Linear mixed effects model results showing the effect of focal species, carbon dioxide, water, and plant treatments on log (response ratio) (lrr) of *Andropogon gerardii* and *Sorghastrum nutans*. Aboveground lrr and belowground lrr are the measures of plant-plant interactions. #

Source	Response variables			
	AMF		AMF root colonization	
	<u>(no plant-plant interactions)</u>		<u>(with plant-plant interactions)</u>	
	<i>F</i> _(NdF, DdF)	<i>P</i>	<i>F</i> _(NdF, DdF)	<i>P</i>
Focal species	728 _(1, 36)	<0.001***	17.38 _(1, 124)	<0.001***
CO ₂ treatment	60.3 _(1, 4)	0.001**	8.16 _(1, 4)	0.004**
Water treatment	60.6 _(1, 36)	<0.001***	469 _(1, 124)	<0.001***
Plant treatment	-	-	42.40 _(1, 124)	<0.001***
CO ₂ treatment: Water treatment	1.76 _(1, 36)	0.191	37.22 _(1, 124)	<0.001***
CO ₂ treatment: Plant treatment	-	-	4.02 _(1, 124)	0.004**
Water treatment: Plant treatment	-	-	2.4 _(1, 124)	0.122
CO ₂ treatment: Focal species	332 _(1, 36)	<0.001***	25.60 _(1, 124)	0.001**
Water treatment: Focal species	24.4 _(1, 36)	<0.001***	175 _(1, 124)	<0.001***
Plant treatment: Focal species	-	-	1.57 _(1, 124)	0.211
CO ₂ treatment: Water treatment: Plant treatment	-	-	11.63 _(1, 124)	<0.001***
CO ₂ treatment: Water treatment: Focal species	28.3 _(1, 36)	<0.001***	25.89 _(1, 124)	<0.001***
CO ₂ treatment: Plant treatment: Focal species	-	-	2.73 _(1, 124)	0.107
Water treatment: Plant treatment: Focal species	-	-	8.33 _(1, 124)	0.004**
CO ₂ treatment: Water treatment: Plant treatment: Focal species	-	-	6.28 _(1, 124)	0.013*

#Abbreviations: NdF- numerator degrees of freedom, DdF - denominator degrees of freedom, F -F-value

*** is significant at $P < 0.001$, ** is significant at $P < 0.01$, and * is significant at $P < 0.05$

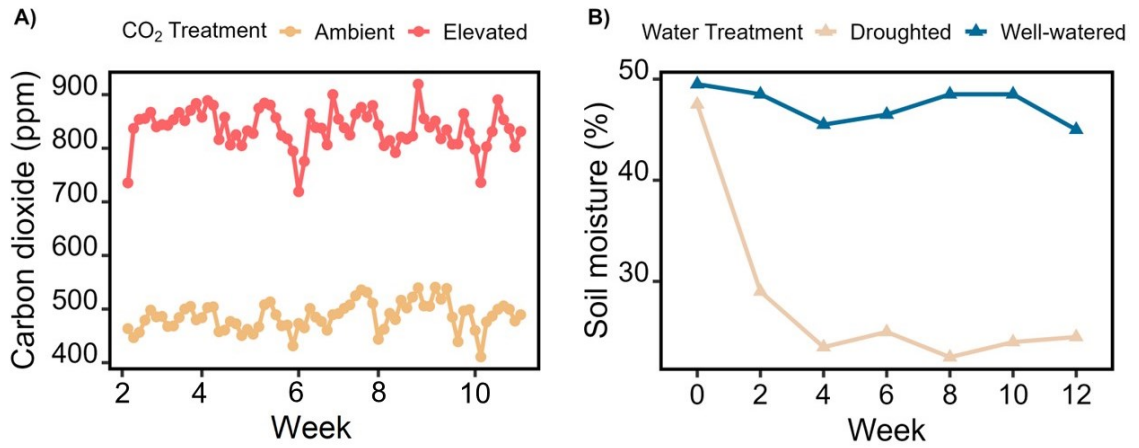


Figure 2.1: Average daily carbon dioxide concentration from about two months of the experiment (full data for the duration of the experiment is not available) (A) and gravimetric soil moisture content inside open-top chambers for the duration of the experiment (B).

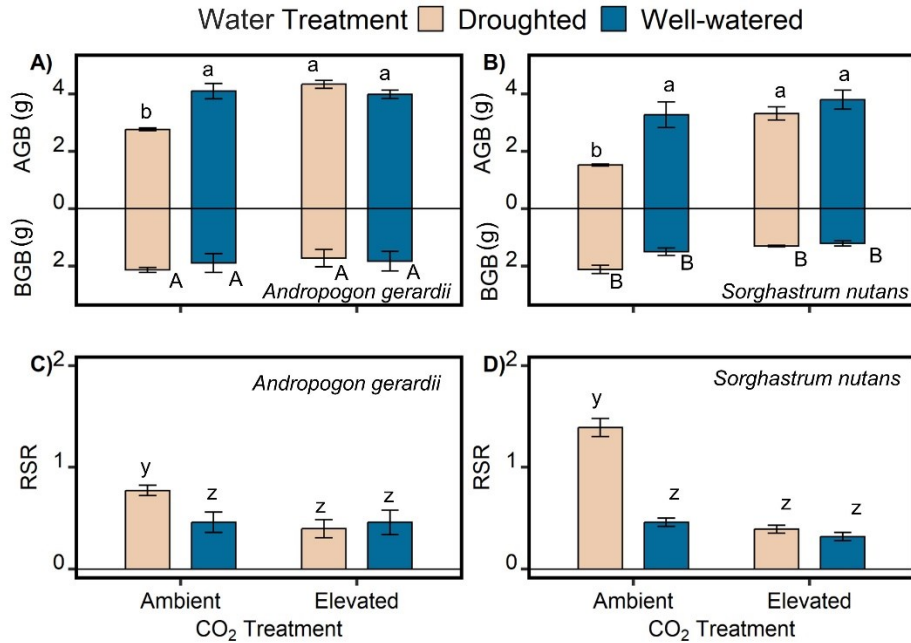


Figure 2.2: Aboveground biomass (g) (AGB) and belowground biomass (g) (BGB) of: A) *Andropogon gerardii* B) *Sorghastrum nutans* and root-shoot ratio (RSR) of: C) *Andropogon gerardii* D) *Sorghastrum nutans* as affected by drought and carbon dioxide treatments. Each bar represents the mean with error bars showing standard error. Letters show pairwise significant differences such that two points sharing no letters in a graph are significantly different to each other ($P < 0.05$).

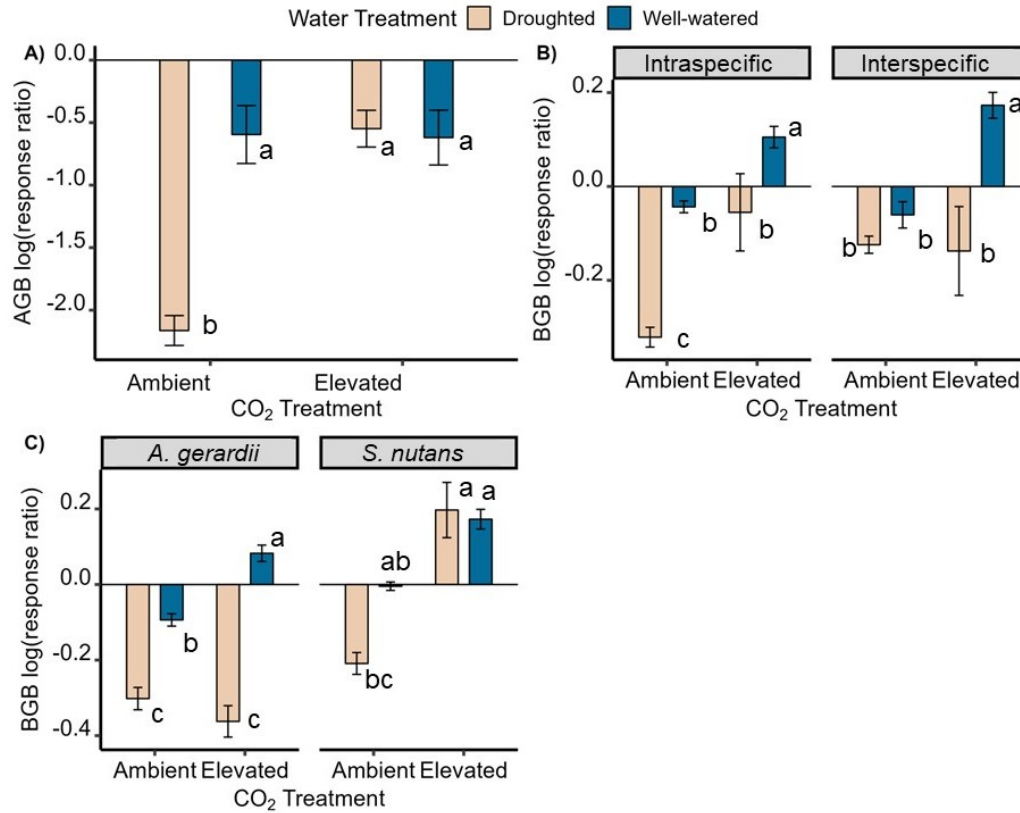


Figure 2.3: Aboveground biomass (ABG) (g) and belowground biomass (BGB) (g) response of *Andropogon gerardii* and *Sorghastrum nutans* to carbon dioxide, water, and plant treatments. The log response ratio was calculated as the logarithmic ratio of the biomass of a plant species grown alone to the biomass when grown with individual of same or different plant species. Each bar represents the mean and error bars shows standard error of mean for: (A) AGB log response ratio as affected by carbon dioxide and water treatments (B) BGB log response ratio as affected by carbon dioxide, water, and plant treatment (C) BGB log response ratio as affected by carbon dioxide and water treatments, and focal species. Letters show pairwise significant differences such that two points sharing no letters in a graph are significantly different to each other ($P < 0.05$)

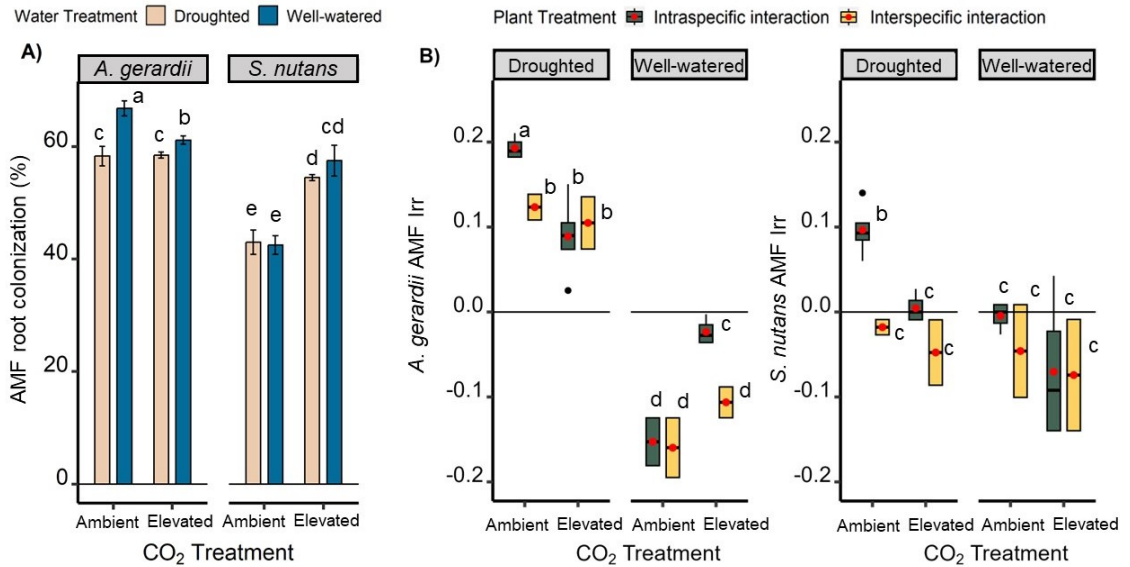


Figure 2.4. Arbuscular mycorrhizal fungi (AMF) root colonization in *Andropogon gerardii* and *Sorghastrum nutans* as affected by carbon dioxide, water, and plant treatments. (A) Each bar represents the mean of AMF root colonization % of *A. gerardii* and *S. nutans* with error bars showing standard error of mean. (B) Each bar represents the mean of AMF log (response ratio) (Irr) in *Andropogon gerardii* and *Sorghastrum nutans* with error bars showing standard error of mean. Letters show pairwise significant differences such that two points sharing no letters in a graph are significantly different to each other ($P < 0.05$).

Chapter 3

Tripartite symbiosis among legumes, rhizobia, and arbuscular mycorrhizal fungi under elevated CO₂

Abstract

Plants form symbiosis with multiple soil mutualists that affect plant productivity and function. One notable example is the symbiosis between legume plants and mutualistic microbes, specifically arbuscular mycorrhizal fungi (AMF), and rhizobial bacteria. This intricate tripartite relationship relies on the exchange of carbon from legumes and nutrients from fungal and bacterial mutualists. The strength of the legume-AMF-rhizobia relationship might depend on the balance between the carbon cost to plants and the benefits provided to plants by mutualists in the form of nutrients. With the impending rise in atmospheric carbon dioxide (CO₂) levels due to global change, there is a potential for this relationship to be altered. Elevated CO₂ levels can lead to increased carbon availability, potentially affecting this symbiosis. However, the impact of elevated CO₂ on the tripartite legume-AMF-rhizobia relationship has not been studied before. In this study, we investigated this tripartite relationship in *Lespedeza capitata*, a prairie legume under elevated carbon dioxide (CO₂). We conducted a pot experiment where we grew *L. capitata* under different CO₂ (ambient, 600 ppm, 1000 ppm) and mutualist treatments (control, AMF only, rhizobia only, AMF, and rhizobia together). After a growing season, we assessed the number of nodules – a proxy for rhizobia benefits, the percentage of AMF root colonization, and above-and-belowground biomass. We found that the dual inoculation treatment (AMF and rhizobia together) resulted in the highest number of

nodules, percentage of AMF root colonization, and biomass of *L. capitata*, with elevated CO₂ further enhancing this response. Co-limitation of nitrogen and phosphorus in the soil used in our pot experiment played a role in increasing response of *L. capitata* to dual inoculation treatment across CO₂ treatments. Our findings suggest that dual inoculation of *L. capitata* with mutualists comes with carbon costs, which decreases under elevated CO₂.

Introduction

One of the important biotic interactions in nature is that of plants with soil mutualistic microbes. Mutualistic microbes play a critical role in acquiring plant nutrients, increasing plant fitness, and providing resistance to abiotic stresses (Afkhami et al., 2020; Cordovez et al., 2019; Toju et al., 2018). Two such mutualistic microbes are arbuscular mycorrhizal fungi (AMF) and rhizobial bacteria, commonly known as rhizobia. Most legumes can associate simultaneously with both these mutualists (Johnson et al., 1997; Kaschuk et al., 2009; Larimer et al., 2010), exchanging photosynthetically-derived carbon with them in exchange for nitrogen from rhizobia obtained through biological nitrogen fixation (Cleveland et al., 1999; Vitousek et al., 2013) and primarily water and phosphorus from AMF (Brundrett, 2009; Hartnett & Wilson, 2002). However, despite the strong understanding of the independent benefits of these two types of mutualists for legumes (Afkhami et al., 2020; Burghardt, 2020; Clark & Zeto, 2000; Larimer et al., 2010, 2014), we lack a clear understanding of the tripartite interactions between AMF, rhizobia, and legumes.

The overall effect of multiple mutualists on plants and on each other can be additive (sum total), synergistic (higher than the additive effect of individual mutualists), or sub-additive (less than additive) (Afkhami et al., 2020). Despite many studies investigating tripartite relationships, the context dependency of the effect of AMF and rhizobia is not fully understood (Primieri et al., 2022). For example, a meta-analysis by Larimer et al., (2010) found additive effects of AMF and rhizobia on host plant performance in controlled studies. Similarly, Veselaj et al. (2018) reported a synergistic

effect of AMF and rhizobia on the salinity stress in pea plants. Other studies have reported synergistic effects of AMF and rhizobia inoculation on legumes (Meng et al., 2015; Pereira et al., 2019). And finally there is evidence that the prior inoculation of either AMF or rhizobia has been found to suppress colonization by the other mutualist (Catford et al., 2003, 2006; Ossler et al., 2015). What is clear is that environmental conditions are important. For example, Ficano et al. (2021) reported that higher light availability and soil nitrogen influenced the positive tripartite relationship among plant biomass, % AMF, and rhizobia investment in Neotropical legumes.

Context dependency of the legume-AMF-rhizobia relationship can be attributed to the costs and benefits associated with these interactions. A host plant's investment in its mutualists can be influenced by the carbon costs invested in the symbiosis (Mortimer et al., 2008). In cases where the nutrient benefits provided by the mutualists are smaller than the carbon costs, a host would suppress interactions with its mutualists by an auto-regulatory mechanism (Mortimer et al., 2008; Penmetza & Cook, 1997). For AMF and rhizobia, the cost of association can be as high as 32% of the carbon fixed by the host plant (Kaschuk et al., 2009). Alternately, Kaschuk et al., (2009) demonstrated that legumes can compensate for the carbon costs associated with AMF and rhizobia by increasing their photosynthetic rates to take advantage of the increased nutrient supply provided by the mutualists. Given that the fundamental underpinning of the tripartite relationship among legumes, rhizobia, and AMF is based on carbon, as both the mutualists receive carbon from the host legumes (Mortimer et al., 2012), it is expected that the relationship may be fundamentally altered under future carbon dioxide (CO₂)

concentrations. However, to our knowledge, the effect of elevated CO₂ on the legume-AMF-rhizobia has not been studied before.

Atmospheric CO₂ levels are predicted to exceed beyond 550 ppm and may reach up to 1000 ppm by the end of the century depending on various emission scenarios (IPCC 2013, 2014, 2021). Generally, elevated CO₂ has been reported to increase plant productivity by stimulating photosynthetic rate and improving water use efficiency (Kassem et al., 2008). Consequently, this increase in productivity may lead to higher demand for soil nutrients such as nitrogen and phosphorus (de Graaff et al., 2006; Newbury et al., 1995). Thus, under elevated CO₂, although the cost of mutualism should be low due to higher carbon availability, legumes' investment in AMF and rhizobia might be even more important in order to cater to the increasing demand of phosphorus and nitrogen from soil.

We investigated the effects of elevated CO₂ on the tripartite mutualism in a legume species, *Lespedeza capitata* Michx. (commonly known as Bush-clover) – a native prairie legume of south-central Kansas, U.S. *L. capitata* is usually planted in grassland restoration sites and thus, its association with mutualists under elevated CO₂ can have valuable implications for grassland management and restoration. Specifically, our research questions were (1) How does elevated CO₂ affect the tripartite association among *L. capitata*, AMF and rhizobia; the AMF root colonization % and number of nodules of *L. capitata*? (2) What are the productivity responses (above-and-belowground biomass) of *L. capitata* to inoculation of AMF and rhizobia independently versus AMF and rhizobia together and how does elevated CO₂ affect this productivity

response? We hypothesized that elevated CO₂ would increase association of *L. capitata* with both the mutualists as elevated CO₂ will increase the reliance of legumes on the microbial mutualists for critical resources of nitrogen, phosphorus, and water. Additionally, we hypothesized that there would be synergistic effects of AMF and rhizobia on above-and-belowground biomass of *L. capitata* due to increase in phosphorus and nitrogen availability and elevated CO₂ would magnify the synergistic effects of the mutualists resulting in higher biomass.

Methods

Study site and experimental design

A pot study was conducted inside CO₂ open-top chambers at the Smithsonian Environmental Research Center, MD, as a part of the Future and Fossil Atmosphere experiment. Seeds of *L. capitata* were obtained (Prairie Moon, WN) and surface sterilized followed by acid scarification (Robert & Brown, 2004). Briefly, seeds were covered with full-strength commercial bleach for 30 seconds, rinsed off with sterile water, and then covered with ethanol (95 % v/v) for another 30 seconds. Surface sterilization was done to remove any pathogens/microorganisms from the seed coats. After surface sterilization, seeds were acid scarified by covering the seeds with concentrated sulfuric acid for approximately 10 minutes and rinsed with sterile water. Any remaining acid was then neutralized with sterile sodium bicarbonate followed by rinsing with sterile water until the seeds were free of acid. Acid scarification has been reported to break seed dormancy in some plant species (Robert & Brown, 2004).

Sterilized and scarified seeds were then germinated (30 °C, 5 days) before transplanting them to the pots.

Local soil was mixed with sand (3:1), homogenized and steam sterilized (120 °C, 2 hours each day for 2 days, USDA, Beltsville, MD) to kill rhizobial and AMF spores. Two subsamples each of about 500 g of the soil and sand mixture before sterilization was oven-dried (60 °C, 2 days) and sent for analysis (Cornell Soil Health Laboratory, NY). The soil was evidently low in nitrogen and phosphorus for most plant standards and lower than what is observed for soils in tallgrass prairie where *L. capitata* is a native legume (Table 3.1). Steam sterilization has been found to be a fast and cost-effective method of soil sterilization (Dietrich et al., 2020). Approximately 4 kg of soil was packed in each pot (6.8 cm × 35.5 cm Deepot cells, Stuewe and Sons, Oregon) and three germinated seedlings were planted per pot. Seedlings were watered every day for two weeks in a greenhouse at the end of which thinning was done to maintain one seedling per pot. To study the independent and combined effects of rhizobia and AMF, four mutualist treatments were established: AMF-only (AMF), rhizobia-only (Rh), dual inoculation with both AMF and rhizobia (AMF-Rh), and control with no AMF and no rhizobia. Pots were inoculated with cultures of AMF and/or rhizobia strains known to be associated with *L. capitata*. Rhizobia strains were isolated from *L. capitata* from Cedar Creek Ecosystem Reserve, Mn and had ten strains (2037c, 2053b, 2057d, 2147b, 2157a, 2194f, 2240b, 2284a, 2307b, and 2907a). For AMF inoculation, we used commercial inoculant (BioOrganics LLC, CA) that contained AMF from *Glomus* spp., *Gigaspora* spp. and *Paraglomus* spp. No inoculation was done for control treatment, although we found one

or two nodules in some plants which might be due to contamination. These mutualist treatments were nested within three CO₂ treatments (ambient (420 ppm), 600 ppm, and 1000 ppm CO₂), each replicated three times. In total, each mutualist treatment was replicated 12 times within each CO₂ chamber, resulting in 432 pots (3 CO₂ treatments with three replicates each × 4 mutualist treatments with 12 replicates each). The pots were arranged in completely randomized design within each chamber. To minimize spatial bias, the pots were rotated every two/three weeks within each CO₂ chamber. All pots were watered and weeded regularly.

Daily monitoring of the open-top CO₂ chambers ensured that the desired CO₂ levels were maintained throughout the study. After 60 days, the plants were harvested and oven-dried (60 °C, 3 days) to determine their above- and-belowground biomass. The success of mutualist interactions was assessed by counting and measuring root nodule number (rhizobial interactions) and quantifying AMF (% root colonization). To determine the percentage of AMF root colonization, five segments of approximately 4 cm each were taken from the fresh roots of each plant, and the percentage of AMF root colonization was determined by intersect gridline method (Giovannetti & Mosse, 1980).

Statistical analyses

All statistical analyses were performed using R version 4.2.2 (R core team 2021), employing a linear mixed-effects model with the “lme4” package in R (Bates et al. 2015). In the model, CO₂ treatment and mutualist treatment were fixed effects, while chamber (open-top CO₂ chamber) was treated as a random effect. We ran these models above- and-belowground biomass, number of nodules, and AMF root colonization percentage

as response variables. To ensure the model's validity, assumptions of normality and homoscedasticity were tested and verified using diagnostic plots. Additionally, to address multiple hypothesis testing, Bonferroni's correction was applied at $P < 0.05$ per number of comparisons, which in our case was $P < 0.0125$ ($0.05/4$ analyses). After identifying significant effects ($P < 0.05$) from the linear mixed-effects models, post-hoc analyses were performed using the "emmeans" package in R (Length et al. 2018; R Core Team 2015).

Results

We found significant effects of CO₂ and mutualist treatments on all measured parameters: aboveground biomass, belowground biomass, number of nodules and AMF root colonization (Table 3.2). For aboveground and belowground biomass of *L. capitata*, there was a significant interaction between CO₂ and mutualist treatment (Table 3.2, Fig. 3.1). Specifically, AMF-Rh treatment resulted in significantly higher aboveground and belowground biomass compared to other mutualist treatments (Fig. 3.1). Across all CO₂ treatments, aboveground biomass in AMF-Rh treatment was 179% greater than the control treatment but 11% lower than the additive effect of rhizobia-only and AMF-only treatments. Similarly, the belowground biomass in AMF-Rh treatment was 315% higher than in the control treatment but approximately 25% lower than the combined effect of rhizobia-only and AMF-only treatments. Notably, the most substantial increase in the aboveground biomass in the AMF-Rh treatment was observed under 1000 ppm CO₂, yielding 246% more aboveground biomass than the control and nearly equal to the combined effect of rhizobia only and AMF only treatments. Additionally, both CO₂ and

mutualist treatment had significant effects on the number of nodules and AMF root colonization of *L. capitata* (Table 3.2, Fig. 3.2) such that number of nodules and AMF root colonization was the highest for the AMF-Rh treatment across all CO₂ levels.

Discussion

In our study, we investigated the tripartite symbiosis of rhizobia, AMF, and *L. capitata*, under ambient and two levels of elevated CO₂ treatments. Our results show significant effects of both mutualist and CO₂ treatments on above-and-belowground biomass, number of nodules, and AMF root colonization of *L. capitata*. Furthermore, we found that both rhizobia and AMF played crucial roles in facilitating a positive response of *L. capitata* to elevated CO₂. The greatest plant responses were found when *L. capitata* was dual inoculated by both rhizobia and AMF, compared to single inoculation with either of the mutualists or no inoculation at all. This implies that dual inoculation by rhizobia and AMF is the most beneficial for *L. capitata*, and these mutualistic associations are enhanced under elevated CO₂, leading to positive effects on the plant's above-and-belowground biomass.

Nodules in legume harbor rhizobia, which fix atmospheric nitrogen through the nitrogenase enzyme (Peix et al., 2015). Consequently, an increase in nitrogen fixation is often associated with a greater number of nodules (Schwember et al., 2019). There is evidence that elevated CO₂ can lead to an increase in the number of nodules (Li et al., 2017; Miyagi et al., 2007). Our findings also support this observation, as we observed a higher number of nodules in *L. capitata* under elevated CO₂ conditions, but only in dual inoculation. Across all the CO₂ treatments, the highest number of nodules was seen in

the dual inoculation treatment compared to the rhizobia-only treatment. Nodulation requires a large amount of phosphorus and hence, associating with AMF can enhance the number of nodules in legumes by providing additional phosphorus (Takács et al., 2018). Since, our soil was low in phosphorus, having both AMF and rhizobia helped in nodulation compared to the AMF only treatment.

Similar to the number of nodules, there was greater AMF colonization when *L. capitata* was dual inoculated by rhizobia and AMF and the colonization for dual inoculation increased with elevated CO₂. The increase in AMF root colonization, along with the number of nodules in dual inoculation treatment in our nutrient poor soil suggest that *L. capitata* was co-limited by nitrogen and phosphorus and addition of these mutualists helped overcome phosphorus and nitrogen limitation for nodule formation and AMF colonization. Co-limitation of nitrogen and phosphorus have been previously found to suppress legume productivity (Heiden et al., 2009; Houlton et al., 2008)

Dual inoculation resulted in higher above-and-belowground biomass of *L. capitata*, both in ambient and elevated CO₂ conditions. This can be attributed to the complementary nutrient supply provided by rhizobia and AMF, enhancing nutrient availability and utilization in legumes and subsequently promoting increased biomass production (Ossler et al., 2015). Previous studies have also reported increased plant growth with dual inoculation by rhizobia and AMF (Afkhami et al., 2021; Larimer et al., 2010). Furthermore, the positive effect of dual inoculation was more pronounced under the 1000 ppm CO₂ level, with the effect on aboveground biomass shifting from being

sub-additive to synergistic. This shift suggests that higher CO₂ levels provide more carbon for *L. capitata* to support both mutualists, reducing carbon costs to the plants and consequently reducing competition between the mutualists for carbon resources. However, we did not find synergistic effects of rhizobia and AMF on all CO₂ levels as we had hypothesized.

Stimulation of biomass production by elevated CO₂ varied among the different mutualist treatments. Elevated CO₂ is generally known to stimulate plant shoot and root biomass production by enhancing photosynthetic rate and water use efficiency (Luo et al., 2006; Reyes-Fox et al., 2014). However, in our study, we found that there was no stimulation of elevated CO₂ in the above-and-belowground biomass of *L. capitata* in the control treatment without any mutualists. The single inoculation by AMF-only and the dual inoculation by AMF and rhizobia, on the other hand, showed an increase in both above-and-belowground biomass of the legume in response to elevated CO₂. This varied response could be attributed to soil nutrient availability, as the soil used in our study had low nitrogen and phosphorus content. The lack of plant growth stimulation by elevated CO₂ in the control treatment might have been due to limited nutrient availability. When essential macronutrients such as nitrogen and phosphorus are limiting, photosynthetic biochemical process can be restricted resulting in less growth response to elevated CO₂ (Jiang et al., 2020; Warren et al., 2015). Moreover, the increase in the number of nodules, AMF root colonization, and subsequent increase in the above-and-belowground biomass with increasing CO₂ levels suggest that *L. capitata*

might have an autoregulation mechanism when it comes to investing carbon in these mutualists.

Conclusion

The understanding of legume-rhizobia-AMF symbiosis under elevated CO₂ represents a significant research gap, especially considering that legumes have ecological and economic importance as natural fertilizer, food, and livestock forage sources, and mutualists are vital for their growth and productivity. For our study, is an important first test of this, even though it is important to note that the response of these mutualists might vary for different plant species, plant genotypes, and mutualist strains, and could be influenced by other abiotic and biotic interactions (Afkhami et al., 2020). Importantly, our study suggests that the tripartite symbiosis among *L. capitata*, rhizobia and AMF under elevated CO₂ exhibits a synergistic nature.

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Table 3.1: Soil properties from local soil mixed with sand in 3:1 ratio used for the experiment

Property	Value (mean \pm standard deviation)
pH	5.3 \pm 0.05
Organic matter (%)	1.24 \pm 0.12
Extractable phosphorus (ppm)	4.2 \pm 0.29
Extractable calcium (ppm)	1423 \pm 29.6
Extractable magnesium (ppm)	130 \pm 7.51
Total nitrogen (ppm)	4.65 \pm 0.40

Table 3.2: Linear mixed effects model results showing the effect of carbon dioxide and mutualist treatments on aboveground biomass, belowground biomass, number of nodules, and Arbuscular mycorrhizal fungi root colonization.#

Source	Response variables							
	Aboveground biomass		Belowground biomass		No. of nodules		AMF %	
	$F_{(NdF, DdF)}$	P	$F_{(NdF, DdF)}$	P	$F_{(NdF, DdF)}$	P	$F_{(NdF, DdF)}$	P
CO ₂	28.5 _(2, 6)	<0.001*	10 _(2, 6)	0.012*	4.8 _(2, 6)	0.054	17.4 _(2, 6)	0.003*
Mutualist	759.7 _(3, 414)	<0.001*	393.5 _(3, 414)	<0.001*	2134 _(3, 414)	<0.001*	1896 _(3, 414)	<0.001*
CO ₂ : Mutualist	28.1 _(6, 414)	<0.001*	4.7 _(6, 414)	<0.001*	3.7 _(6, 414)	0.001*	3.2 _(6, 414)	0.004*

#Abbreviations: NdF- numerator degrees of freedom, DdF - denominator degrees of freedom, F -F-value

Bonferroni correction $P < 0.0125$ (0.05/4 comparison)

*is significant at $P < 0.0125$, after Bonferroni correction

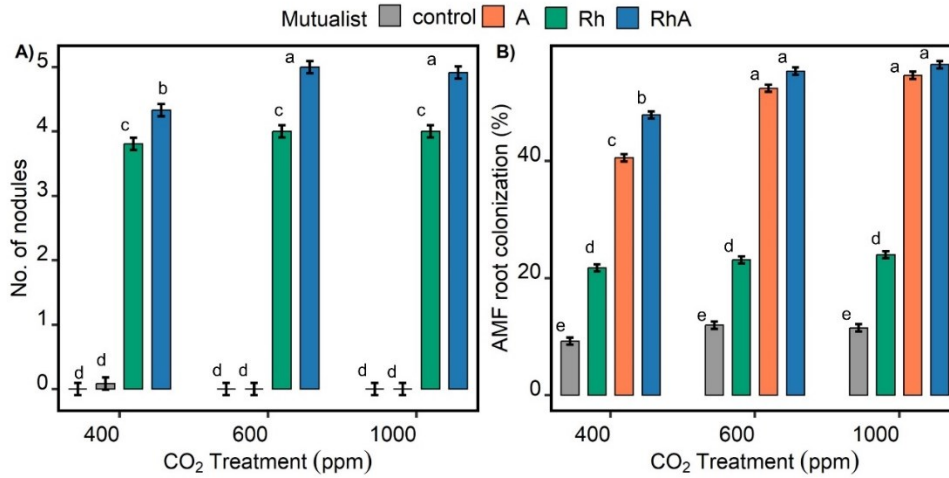


Figure 3.1: The effect of carbon dioxide and mutualist treatments on: (A) number of nodules per plant (B) AMF root colonization percentage of *Lespedeza capitata*. Each bar represents the mean with error bars showing standard error. Letters show pairwise significant differences such that two points sharing no letters in a graph are significantly different to each other ($P < 0.05$).

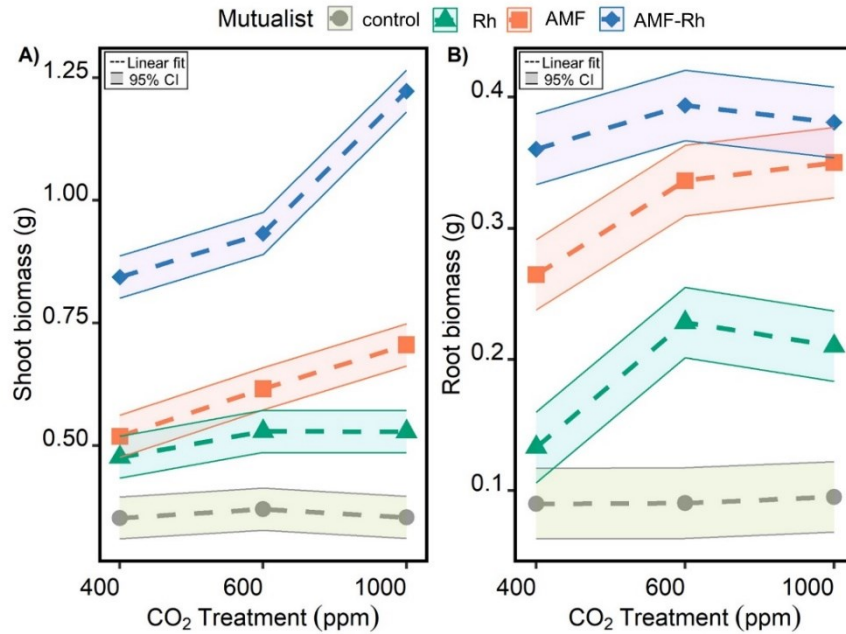


Figure 3.2: Both carbon dioxide and mutualist treatments had significant effects on: (A) Aboveground biomass (g) (Shoot biomass) (B) belowground biomass (Root biomass) of *Lespedeza capitata*. Each point represents the mean of biomass of the legume with 95% confidence intervals bands ($P < 0.05$).