

CONTEXT-DEPENDENT EFFECTS OF MUTUALISMS ON COMMUNITIES

By

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ABSTRACT

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Mutualisms can drive population dynamics and evolutionary processes, but there is still only a limited understanding of how mutualisms may be important to communities. Resource mutualisms, such as the interaction between legumes and nitrogen-fixing rhizobia, not only directly influence the partners involved in the interaction, but they also have the potential to either inhibit or facilitate other species in the community by altering the abiotic and biotic environment. Yet, understanding the role of mutualisms in a community context has typically received less attention than other species interactions. Moreover, there is substantial intraspecific genetic variation in legumes and rhizobia, including variation in legume growth, rhizobia population size, and nitrogen fixation. Therefore, not only can the presence of particular species influence the community, but the colonizing genotype could also alter these processes when they vary in traits associated to ecologically important interactions with rhizobia. My research has taken an empirical approach that combines large-scale field and greenhouse studies with smaller targeted studies to explore the role of plant-microbe mutualistic interactions on community interactions and host responses to availability and changes in these mutualisms. My findings expand our understanding of plant community dynamics by incorporating the effects of positive symbiotic interactions through nitrogen-fixing bacteria on plant communities and how they are dependent on intraspecific variation in traits related to species interactions, abiotic and biotic environmental conditions, and even the presence of other mutualists.

For Merry.

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TABLE OF CONTENTS

LIST OF TABLES	x
LIST OF FIGURES	xi
CHAPTER 1	1
INTRODUCTION	1
Introduction	1
Main Dissertation Results and Significance	3
CHAPTER 2	6
MUTUALISTIC RHIZOBIA REDUCE PLANT DIVERSITY AND ALTER COMMUNITY COMPOSITION	6
Abstract	6
Introduction	7
Methods	11
Study System	11
Experimental Design	11
Data Collection and Analysis	13
Results	14
Discussion	19
Resource Mutualism Affects Diversity	19
Mechanisms of Resource Mutualism Effects on Community Patterns	22
Conclusions	23
Acknowledgements	24
CHAPTER 3	25
WHEN MUTUALISMS MATTER: RHIZOBIA EFFECTS ON PLANT COMMUNITIES DEPEND ON HOST PLANT GENOTYPE AND SOIL NITROGEN AVAILABILITY	25
Abstract	25
Introduction	26
Methods	29
Study System	29
Greenhouse Common Garden	30
Greenhouse Mesocosm Experiment	31
Mesocosm treatments	31
Mesocosm data collection	32
Mesocosm data analysis	33
Results	34
Greenhouse Common Garden	34
Greenhouse Mesocosm	36
<i>Chamaecrista fasciculata</i> Response:	36

Diversity Responses:	36
Community Composition Responses:	38
Productivity and Nutrient Responses:	42
Discussion	43
Genetic variation in legume host plants mediates rhizobia effects on communities and ecosystems	44
Soil nitrogen availability mediates rhizobium effects on communities and ecosystems	46
Nitrogen and intraspecific variation interactively influence rhizobium effects on communities and ecosystems	47
Caveats	47
Conclusions	48
Acknowledgements	49
APPENDIX	50
 CHAPTER 4	56
RHIZOBIUM MUTUALISMS ALTER COMPETITIVE INTERACTIONS	56
Abstract	56
Introduction	57
Methods	60
Study System	60
Pairwise Competition Experiment	60
Response Surface Competition Experiment	63
Results	66
Pairwise Competition	66
Response Surface Competition	69
Discussion	71
Acknowledgements	76
 CHAPTER 5	77
EFFECTS OF MULTIPLE MUTUALISTS ON PLANTS AND THEIR ASSOCIATED ARTHROPOD COMMUNITIES	77
Abstract	77
Introduction	78
Methods	82
Study System	82
Experimental Design	83
Field Sampling	84
Statistical Analyses	85
Results	85
Rhizobia effects on ants	85
Ant effects on rhizobia	86
Rhizobia and ant effects on plant traits and fitness	87
Rhizobia and ant effects on non-ant arthropods	89
Discussion	89
Interactions between mutualists	91

Effects of mutualists on plant fitness.....	92
Multi-mutualist effects on higher trophic levels	94
Conclusions	95
Acknowledgements	95
APPENDIX	97
LITERATURE CITED.....	100

LIST OF TABLES

Table 2.1. Effects of rhizobia and nitrogen treatments and the interaction on measures of α -diversity and composition.	15
Table 4.1. F-test results from response surface analyses (sensu Zar 2010) examining how rhizobia alter the competitive responses of focal species to densities of intraspecific and interspecific competitors.	68
Table 4.2. Results from the multiple regressions for each species included in the response surface competition experiment.	70
Table 5.1. Treatment effects of rhizobia and ants on ant abundance, plant fitness (seeds and pods), and aboveground biomass analyzed with two-way ANOVA.	86
Table 5.2. Likelihood ratio tests of the effects of rhizobia and ant treatments on nodule number, arthropod abundance, and aphid abundance.	88
Table S5.1. ANCOVA tables of rhizobia and ant treatment effects with aboveground biomass or extrafloral nectary number as covariates on ant abundance and plant fitness (seeds and pods).	98
Table S5.2. Likelihood ratio tests of the effects of rhizobia and ant treatments on arthropod number.	99

LIST OF FIGURES

Figure 2.1. Mean effects of rhizobia on A) <i>C. fasciculata</i> biomass and B) Shannon Diversity (H') in experimental mesocosms.	16
Figure 2.2. Effects of rhizobia on community composition in experimental mesocosms. ...	17
Figure 2.3. Average relative community composition of A) rhizobia non-inoculated and B) inoculated mesocosms for all seven species.	18
Figure 3.1. Common garden <i>C. fasciculata</i> population differences in (a) emergence time, (b) height, and (c) nodule number.	35
Figure 3.2. Effects of <i>C. fasciculata</i> population and rhizobia treatments on (a) <i>C. fasciculata</i> relative aboveground biomass, (b) Shannon diversity index, and (c) the relationship between <i>C. fasciculata</i> relative aboveground biomass and Shannon diversity in the presence (open squares) and absence (grey squares) of rhizobia.	37
Figure 3.3. Effects of rhizobia and nitrogen treatments on (a) <i>C. fasciculata</i> absolute aboveground biomass and (b) Shannon diversity index.	39
Figure 3.4. Effects of rhizobia on community composition for six <i>C. fasciculata</i> populations in experimental mesocosms.	41
Figure 3.5. Ammonium nitrate (NH_4^+) concentrations at time of harvest for (a) rhizobia treatments and (b) <i>C. fasciculata</i> population identity treatments in experimental mesocosms.	43
Figure S3.1. Common garden <i>C. fasciculata</i> population differences in (a) aboveground biomass, (b) number of leaves (c) per nodule fitness benefit, and (d) number of branches.	51
Figure S3.2. Total aboveground biomass of <i>C. fasciculata</i> in each population treatment with and without rhizobia.	52
Figure S3.3. Effects of <i>C. fasciculata</i> population and rhizobia treatments on the Shannon diversity index of the subdominant community.	53
Figure S3.4. Effects of rhizobia and nitrogen treatments on the Shannon diversity index of the subdominant community.	54
Figure S3.5. Total aboveground biomass of all species for each <i>C. fasciculata</i> population treatment with and without rhizobia.	55
Figure 4.1. Response surface experimental design.	63

Figure 4.2. Effects of pairwise competition and rhizobia on the biomass of A) <i>M. punctata</i> , B) <i>O. biennis</i> , and C) <i>S. rigida</i>	67
Figure 4.3. 3D Response surfaces for competition between: <i>M. punctata</i> and <i>C. fasciculata</i> (A and B); <i>R. hirta</i> and <i>C. fasciculata</i> (C and D); and <i>S. rigida</i> and <i>C. fasciculata</i> (E and F).....	72
Figure 5.1. Diagram illustrating the possible interactions and effects of multiple mutualists (ants and rhizobia) on plant traits, herbivores, and plant fitness.....	82
Figure 5.2. Effects of rhizobia and ants on A) average number of ants visiting the plants; B) number of nodules formed on plant roots; C) total seed set, and C) <i>C.</i> <i>fasciculata</i> aboveground biomass.....	87
Figure 5.3. Treatment effects of rhizobia and ants on A) arthropod and B) aphid abundances.	90

CHAPTER ONE

INTRODUCTION

Introduction

Mutualistic interactions are understudied in comparison to the antagonistic interactions that ecologists have focused on for decades. Nevertheless, mutualistic interactions are nearly ubiquitous across organisms and communities (Stachowicz 2001, Bronstein 2009), and not only are the species directly involved in mutualisms affected by the positive interaction, but the effects of mutualisms also may influence other community members. This may be especially likely if the mutualism alters a species' competitive ability or resource availability within the community. Mutualisms have been shown both to increase and decrease diversity, alter community assembly trajectories, and affect ecosystem functions (e.g. Clay and Holah 1999, Bshary 2003, van der Heijden et al. 2006, Rudgers and Clay 2008, Keller 2014). Understanding when mutualisms are most important to communities and the magnitude of mutualism effects remains an important step in further incorporating these types of species interactions into community ecology.

Like other types of species interactions important to community processes such as competition and predation, mutualisms are context dependent (Chamberlain et al. 2014). Understanding this context dependency may predict when mutualists are most likely to influence diversity and community composition. Just as the benefit to the species involved in the mutualism may vary depending on environmental conditions (i.e., context dependency) (e.g., Neuhauser and Fargione 2004), the ability of these effects to then subsequently affect other species in the community may as well. Since the abiotic conditions of a site can affect the benefit a mutualist provides to a focal species, mutualists may have greater effects on species

interactions when the mutualist provides a limiting resource such as nitrogen to the environment. Biotic factors such as other types of species interactions or the density of competing species could also affect the outcome of mutualists and their importance to community properties (Gange and Smith 2005, Schroeder-Moreno and Janos 2008).

Another source of context dependency in mutualism is the genetic identity. It is widely recognized that individuals of the same species are not uniform in traits across populations within their range or in their effects on other species. Intraspecific trait variation and particular genotypes can increase productivity, influence community structure, deter invasions, and stabilize diversity (e.g., Turkington and Aarssen 1984, Rudgers and Maron 2003, Whitham et al. 2003, Schweitzer et al. 2004, Silfver et al. 2007, Lankau and Strauss 2007, Crutsinger et al. 2009, Vellend et al. 2010, Tomas et al. 2011, Breza et al. 2012). My dissertation explores the role of mutualists and specifically the mutualism between the legume *Chamaecrista fasciculata* and N-fixing rhizobia, on individuals, species interactions, and community patterns while explicitly testing the context-dependence of these effects through variation in abiotic and biotic conditions, including the genetic variability in the mutualist partners. In particular, I explore:

Chapter 2) How do rhizobia affect legume dominance and the community level

consequences of this dominance across a nitrogen gradient?

Chapter 3) Are the effects of rhizobia on communities dependent on the genetic

identity (source population) of their legume host?

Chapter 4) How do rhizobia alter the competitive interactions between a focal legume

and co-occurring non-leguminous forbs and grasses?

Chapter 5) How do rhizobia and ants independently and interactively affect C.

***fasciculata* traits, the arthropod community visiting these plants, and ultimately plant fitness?**

Chamaecrista fasciculata is an annual legume native to disturbed grasslands and high quality prairies of the Midwestern and Eastern United States (Irwin and Barneby 1982, Galloway and Fenster 2000). *Chamaecrista fasciculata* is an outcrossing, buzz-pollinated species. It forms mutualistic interactions with rhizobia, *Bradyrhizobium sp.*, which provides the plant with nitrogen in exchange for carbohydrates. In addition, *C. fasciculata* produces extrafloral nectar that it exchanges with ants for defense (Rios et al. 2008). Importantly, there is substantial intraspecific trait differentiation across the range, with variation in traits associated with these mutualisms (Galloway and Fenster 2000, Etterson 2004a, Rios et al. 2008, Keller and Lau In Review).

Main Dissertation Results and Significance

Through large-scale manipulative field and greenhouse experiments, my research has demonstrated that rhizobia increase the competitive dominance of their legume host, resulting in reduced diversity, altered community composition, increased community convergence, and altered nutrient dynamics. Moreover, there is substantial intraspecific genetic variation in how legumes such as *Chamaecrista fasciculata* interact with rhizobia, including variation in legume growth response to rhizobia, number of rhizobia-housing nodules, and rates of nitrogen fixation. Therefore, not only can the presence of a particular legume species and its associated rhizobia influence the surrounding community, but the genetic identity of the colonizing legume could also alter community processes when populations vary in traits associated with ecologically important interactions with rhizobia. My research indicates the magnitude of

rhizobia effects are context dependent on the genetic identity of *C. fasciculata* that are colonizing a newly disturbed site from the regional metapopulation, with some populations becoming more dominant than others, and on abiotic conditions (N-availability). In exploring the mechanisms through a series of experiments, rhizobia create both facilitative and inhibitory effects on other species in the community. This likely occurs from shifting nutrient and light availability or altering the niche overlap of the legume with other species by reducing the legume's dependence on soil nitrogen. Therefore, rhizobia promote coexistence between the legume and some species, promote the exclusion of others, and alter competitive hierarchies. Additionally, variation in competitive and facilitative response may be dependent on the density of *C. fasciculata*. Together, this research demonstrates the role of mutualists and intraspecific variation to community and ecosystem properties, while also uncovering the underlying mechanisms.

While my research shown the effects of rhizobia on communities, species are also often engaged in multiple mutualistic interactions at once. In addition to its mutualism with rhizobia, *C. fasciculata* forms a mutualistic interaction with ants, providing ants with nectar in exchange for defense against herbivores. Although they provide the plant with very different benefits, these two mutualisms may be connected, with one influencing the effects of the other. I have explored how rhizobia and ants independently and interactively influence the growth and fitness of the legume *C. fasciculata* as well as how these mutualists may affect the co-occurring arthropod community. From this study, I have found very large fitness benefits from associating with rhizobia, but a cost of associating with ants that is amplified in the presence of rhizobia. The ant community is positively benefited by the presence of rhizobia with ants preferentially tending plants with rhizobia, but these ants reduce allocation to rhizobia.

Additionally, rhizobia and ants interact to influence the abundance and diversity of arthropods found on the plants. Therefore, when assessing the effects of mutualists on population and community processes, it is important to consider a wide range of species interactions.

Overall, my research has demonstrated that: 1) rhizobia can act as a keystone mutualist in communities reducing diversity and altering community composition; 2) there is substantial genetic variation in *C. fasciculata* traits related to competitive ability and the resource mutualism that can drive changes in community and ecosystem processes; 3) the effects of legume intraspecific variation on communities may be dependent on the availability of rhizobia; 4) that rhizobia drive both inhibitory and facilitative effects on plant competitors, which vary depending on intraspecific and interspecific density of competitors; 5) that the effects of rhizobia may scale-up to interact with other mutualists on arthropod communities and host biomass; and 6) that rhizobia and ant mutualists may independently affect host fitness and drive asymmetric effects on each other mediated through trade-offs in the host. Together, my dissertation research expands our understanding of plant population and community dynamics by incorporating the effects of positive symbiotic interactions through nitrogen-fixing bacteria, and how they are dependent on intraspecific variation in traits related to species interactions, environmental conditions, and even the presence of other mutualists such as ants.

CHAPTER TWO

MUTUALISTIC RHIZOBIA REDUCE PLANT DIVERSITY AND ALTER COMMUNITY COMPOSITION

Abstract

Mutualistic interactions can be just as important to community dynamics as antagonistic species interactions like competition and predation. Because of their large effects on both abiotic and biotic environmental variables, resource mutualisms, in particular, have the potential to influence plant communities. Moreover, the effects of resource mutualists such as nitrogen-fixing rhizobia on diversity and community composition may be more pronounced in nutrient-limited environments. I experimentally manipulated the presence of rhizobia across a nitrogen gradient in early assembling mesocosm communities with identical starting species composition to test how the classic mutualism between nitrogen-fixing rhizobia and their legume host influence diversity and community composition. After harvest, I assessed changes in α -diversity, evenness, community composition, β -diversity, and ecosystem properties such as inorganic nitrogen availability and productivity as a result of rhizobia and nitrogen availability. The presence of rhizobia decreased plant community diversity, increased community convergence (reduced β -diversity), altered plant community composition, and increased total community productivity. These community level effects resulted from rhizobia increasing the competitive dominance of their legume host *Chamaecrista fasciculata*. Moreover, different non-leguminous species responded both negatively and positively to the presence of rhizobia, indicating that rhizobia are driving both inhibitory and potentially facilitative effects in communities. These findings expand our understanding of plant communities by incorporating the effects of positive symbiotic interactions on plant diversity and composition. In particular, rhizobia that specialize

on dominant plants may serve as keystone mutualists in terrestrial plant communities, reducing diversity by more than 40 percent.

Introduction

While mutualisms have long been recognized as important drivers of population dynamics and evolutionary processes (e.g., Wolin 1985, Bronstein et al. 2003, Stanton 2003, Thompson 2005, Frederickson and Gordon 2009, Kay and Sargent 2009), there is now growing support for the importance of mutualisms to community patterns. Mutualisms can increase (e.g. Bshary 2003, Bastolla et al. 2009, Stein et al. 2009, Wurst et al. 2011, Rodriguez-Cabal et al. 2013) or decrease diversity and evenness (e.g. Clay and Holah 1999, Hartnett and Wilson 2002, Izzo and Vasconcelos 2005, Grover et al. 2008, Rudgers et al. 2010b). For example, a cleaning symbiosis between cleaner wrasse and their client fish increases fish diversity in coral reefs (Bshary 2003), while invasive tall fescue grass and its endophytic fungus mutualist reduce plant and arthropod diversity (Clay and Holah 1999, Rudgers and Clay 2008). Identifying the factors underlying these contrasting diversity effects may yield a more predictive framework for the role of mutualisms in community ecology.

Resource mutualisms may be especially likely to influence communities because they alter the availability of limiting resources such as nitrogen and phosphorus (Afkhani et al. 2014). Mycorrhizal fungi and rhizobium bacteria are two common resource mutualists with which plants exchange carbohydrates for phosphorous and nitrogen respectively. Mycorrhizal fungi have been shown to increase phosphorous uptake into a community by 44% (van der Heijden et al. 2006b) thus alleviating phosphorous limitation to plants. Similarly, rhizobia can significantly increase soil nitrogen to the system through the fixation of atmospheric nitrogen (Vitousek and

Walker 1989). Since resources are among the most important drivers of species coexistence and competitive outcomes, these large effects on resource dynamics may lead to large effects on communities (Maron and Connors 1996).

In particular, rhizobia not only directly influence the legume, but they also have the potential to either inhibit or facilitate other species in the community by altering the abiotic and biotic environment (as outlined by van der Heijden et al. 2006a). The legume-rhizobium mutualism may provide the legume a competitive advantage over non-leguminous species through access to a nitrogen pool inaccessible to non-leguminous species (Morris and Wood 1989, van der Heijden et al. 2006a), or could decrease nitrogen limitation to other plants and promote coexistence both within a season or over longer time scales (Vandermeer 1989; Maron and Connors 1996; van der Heijden et al. 2006a, Fustec et al. 2010). Overall shifts in community composition, patterns of diversity, and changes in ecosystem function may occur due to these changes in competitive and nutrient dynamics, especially in low-nitrogen environments. For example, increased competition from the legume may reduce diversity while reduced nitrogen limitation may increase diversity by ameliorating nitrogen limitation. Yet since these changes in competition and nutrient availability are not mutually exclusive, the overall effects of rhizobia on communities may be a result of the relative strengths of these potentially opposing forces.

Given the potential for strong effects of the legume-rhizobium mutualism on plant communities, it is important to study how rhizobia may influence species interactions and community patterns, especially when legumes are highly abundant in the community. In an elegant study that manipulated generalist rhizobia associating with multiple species of legume in experimental communities, van der Heijden and colleagues (2006a) found that rhizobia increased the evenness of communities by promoting coexistence of legumes. Surprisingly, the presence of

rhizobia did not affect non-leguminous species, possibly because in this system legumes were subdominant species in communities dominated by grasses. Many other studies that focus on highly abundant species or mutualisms that interact with many species observe effects of mutualisms on entire communities (e.g., Clay and Holah 1999, Bshary 2003, Stein et al. 2009, Wurst et al. 2011, Bauer et al. 2012), suggesting that mutualisms may be especially likely to affect other community members when they involve dominant species or generalist mutualists.

Importantly, however, suitable mutualist partners may not always be available, for example, the availability of compatible rhizobia to a particular legume species can vary across habitats (Odee et al. 1995, Larson and Siemann 1998, Tlustý et al. 2004, Thrall et al. 2007, Stanton-Geddes and Anderson 2011). In agricultural systems and for invasive species, this spatial heterogeneity in the soil biotic community can limit legume establishment (Lowther et al. 1987, Parker et al. 2006). Yet, rhizobia availability can limit native species establishment or growth, too (Odee et al. 1995, Thrall et al. 2007, Stanton-Geddes and Anderson 2011). A study of 18 legume species across 12 sites in Kenya identified substantial spatial variation in the availability of suitable rhizobia across sites (Odee et al. 1995). Similarly, another study detected significant geographic variation in rhizobia presence, abundance, and effectiveness for two Australian *Acacia* species, with some sites completely devoid of compatible rhizobia (Thrall et al. 2007). This spatial variation in rhizobium availability may be especially important for species with a patchy distribution or those colonizing newly disturbed habitats. Given the spatial variation in the availability of compatible rhizobia and the large effects resource mutualisms can have on communities, the characteristic soil microbial community at a site could lead to differences in the assembly, diversity, and composition of a community in natural field environments.

The effects of rhizobia availability may be context dependent and depend on abiotic environmental variation (Bronstein 2009). For example resource mutualisms can be especially beneficial to plants when nutrients are limiting, but in higher nutrient conditions this mutualism can shift to parasitism (Neuhauser and Fargione 2004). Rhizobia provide leguminous plants with a greater benefit from the association when nitrogen is limiting in the environment (Heath and Tiffin 2007, Lau et al. 2012). These varying legume responses to rhizobia availability across abiotic conditions may result in differing competitive dynamics between legumes and non-leguminous species. In nitrogen rich environments, legumes produce fewer nodules and the competitive advantage is lost (Lauenroth and Dodd 1979, Vargas et al. 2000) while other species that compete more strongly for soil nitrogen may be at an advantage over legumes (Lawrence 1979). Since the effects of mutualists may not be consistent across environments, identifying the abiotic constraints of mutualist driven community effects could lead to a more predictive understanding of the role of these positive interactions in ecosystems. While some studies have shown rhizobia to influence various community properties (e.g. van der Heijden et al. 2006a, Bauer et al. 2012), it remains necessary to identify environmental contexts when rhizobia most greatly influence plant communities.

Here, I used a mesocosm experiment in which I manipulated the presence of rhizobia that associates with a focal dominant legume in simulated early assembling plant communities across a nitrogen gradient to ask: (1) How do rhizobia influence the dominance of a legume host? (2) Do rhizobia affect α - and β -diversity, and community composition, and productivity? and (3) Are rhizobia effects on communities more pronounced when nitrogen is limiting?

Methods

Study System

Chamaecrista fasciculata is an annual legume native to the Midwestern and Eastern United States. It is a pioneer species that establishes and can dominate grasslands and old-fields following disturbance (Holah and Alexander 1999; Galloway and Fenster 2000; Keller personal observations). This legume is found in both highly disturbed sites and high quality prairies at densities ranging from nearly 0 to 55 plants per m² (Fenster 1991), with some populations containing more than 100 plants per m² (Keller personal observation). *C. fasciculata* forms a facultative mutualistic interaction with rhizobia, *Bradyrhizobium elkanii*, which provide the plant with fixed nitrogen in exchange for carbohydrates. *C. fasciculata* has a patchy distribution and compatible rhizobia are not found consistently across potential colonization sites. Limited rhizobia availability can affect *C. fasciculata* establishment and growth at some locations (Stanton-Geddes and Anderson 2011; Keller unpublished).

Experimental Design

I created mesocosms simulating early successional prairie communities and manipulated the presence of rhizobia at three different nitrogen levels in a 2×3 full-factorial design replicated 3 times ($N=18$ mesocosms). Each mesocosm consisted of a 14.4 liter pot filled with potting mix [68.5% soil (Metro Mix 360, SunGro Horticulture, Agawam MA), 24.5% sand (Quikrete All Purpose Sand, Quikrete Companies, Atlanta GA), and 7% clay (Turface MVP, Profile Products, Buffalo Grove IL)] simulating sandy soils characteristic of *C. fasciculata* habitat in the upper Midwestern United States. I planted each mesocosm with simulated native early successional prairie communities consisting of *Chamaecrista fasciculata* (12 individuals to create a density

consistent with higher-density field observations of this early establishing legume) and 4 individuals each of *Bromus kalmii* (short-lived perennial C₃ bunch grass), *Danthonia spicata* (short-lived perennial C₃ bunch grass), *Monarda punctata* (short-lived perennial forb), *Oenothera biennis* (biennial forb), *Potentilla arguta* (perennial forb), and *Vulpia octaflora* (annual C₃ grass). I sterilized all seeds with 95% ethanol and germinated them in seedling flats filled with Metro Mix 360. Two weeks later, I transplanted seedlings into mesocosms placed in the Kellogg Biological Station (Hickory Corners, MI) greenhouse. Individuals were placed at approximately 3cm apart in the same arrangement across mesocosms. Seeds of *C. fasciculata* were greenhouse-reared progeny of field-collected seeds from 6 populations across the Midwestern United States in Illinois, Indiana, Michigan, and Ohio. Seeds from all other species were obtained from Native Connections (Three Rivers, MI).

I manipulated the presence of rhizobia, *B. elkanii* [strain 6437, isolated at the University of Minnesota (Tlusty et al. 2004)] by culturing rhizobia in TY liquid media for 5 days at 28°C and then applying 1mL of rhizobia inoculant diluted to $\sim 2.5 \times 10^6$ cells/mL based on OD600 to the base of each *C. fasciculata* seedling. *B. elkanii* strain 6437 was isolated from a Minnesota population of *C. fasciculata* not included in the seed collection for this experiment; however, this strain successfully nodulated all *C. fasciculata* populations used in this experiment. Non-inoculated mesocosms received 1mL of TY media without rhizobia to each *C. fasciculata*.

I manipulated nitrogen availability at three levels: 0g, 10g, 20g N per m² with the highest value representing high fertility sites in southwest Michigan (Foster and Gross 1998). Nitrogen was applied as ammonium nitrate granules on the soil surface, with half of the total amount applied one week after mesocosm installation and the other half applied 3 months later. No-nitrogen treatments did not receive any nitrogen fertilization. To prevent phosphorous and

potassium limitation across all three nitrogen treatments, all mesocosms received 10g per m² of P and K applied as super phosphate and potash, with half applied at the same time of each nitrogen fertilization.

Data Collection and Analysis

After six months (to mimic a single growing season and provide sufficient time for interactions between individuals), I harvested the aboveground biomass in each mesocosm. Individuals of each species were sorted, counted, and biomass was dried at 65°C for >2 days and weighed. I calculated α -diversity as both Shannon diversity (H') and Simpson's index of diversity ($1-D$) for each mesocosm, both of which incorporate richness and abundance (biomass) data into a single diversity measure. I also calculated evenness of the communities in each mesocosm with Pielou's evenness. I took three 10cm soil cores from each mesocosm, performed a KCl extraction, and estimated inorganic soil nitrogen availability with an Alpkem/ OI Analytic Flow Solution IV analyzer (Model 3550) (see Eilts et al. 2011). I also examined some *C. fasciculata* roots from each mesocosm to confirm inoculation treatments but was unable to completely measure belowground root productivity and the number of nodules produced due to the tight intermixing of plant roots between species. At the time of harvest, inoculated mesocosms exhibited successful nodulation and non-inoculated mesocosms were not contaminated by rhizobia.

To test how rhizobia and nitrogen influence diversity, aboveground productivity, individual species aboveground biomasses, relative abundances, and inorganic nitrogen availability, I performed ANOVA with rhizobia presence, nitrogen treatment, and the rhizobia $\times$ nitrogen interaction included as fixed factors. I used Pearson's correlation coefficients to

examine the pairwise relationships between *C. fasciculata* biomass and the biomass of competing species and between *C. fasciculata* biomass and diversity.

To test how rhizobia and nitrogen affect plant community composition, I performed perMANOVA ('adonis' function of *vegan* using the Bray-Curtis distance measure with 9999 permutations) on biomasses from each species, including rhizobia and nitrogen treatments and the interaction as fixed factors. Since mesocosms started with identical species composition and there were minimal extinctions resulting in similar richness values across mesocosms, I used abundance estimated from biomass with joint absences excluded to examine β -diversity as variation in community composition (sensu Anderson et al. 2011). Specifically, I analyzed among-mesocosm dissimilarity in composition by treatment by first creating a matrix of pairwise dissimilarities using the Bray-Curtis distance measure then using a multivariate test of Levene's homogeneity of variances to calculate within-treatment dispersion ('betadisper' function of *vegan*). I then tested for differences between treatments using a permutation test ('permutest' function of *vegan* with 9999 permutations) (Anderson et al. 2006). Community composition was visualized using non-metric multidimensional scaling (NMDS) using the Bray-Curtis distance measure ('metaMDS' function of *vegan*) to explore changes in both location and dispersion effects between treatments. All analyses were performed in R with the *car* and *vegan* packages (3.0.2, R core development team; Fox and Weisberg 2011; Oksanen et al. 2013).

Results

Rhizobia reduced α -diversity and evenness by 43.3% and 46.2%, respectively (Shannon diversity: $F_{1,12}=25.98$, $P<0.001$, Table 2.1, Fig. 2.1B; evenness: $F_{1,12}=30.38$, $P<0.001$, Table 2.1). Rhizobia reduced α -diversity primarily because of reductions in evenness since all

Table 2.1. Effects of rhizobia and nitrogen treatments and the interaction on measures of α -diversity and composition. Shannon diversity, Simpson's diversity, and Pielou's evenness were analyzed with ANOVA. Community composition was analyzed with perMANOVA.

Significant results are shown in bold.

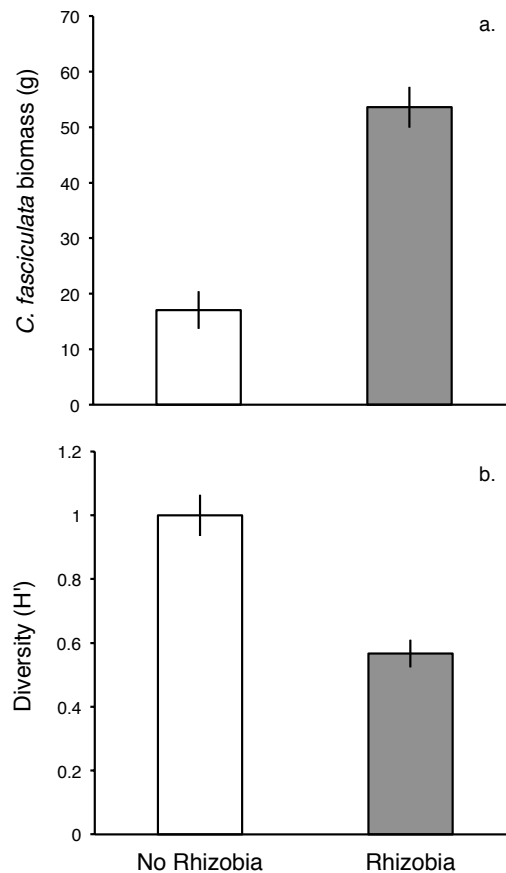
		Shannon Diversity		Simpson's Diversity		Pielou's Evenness		perMANOVA	
	<i>df</i>	<i>F</i>	<i>P</i> -value	<i>F</i>	<i>P</i> -value	<i>F</i>	<i>P</i> -value	<i>F</i>	<i>P</i> -value
Rhizobia	1,12	25.98	0.0003	30.48	0.0001	30.38	0.0001	14.75	0.0003
Nitrogen	2,12	0.72	0.51	0.81	0.47	1.00	0.40	0.28	0.95
Rhizobia $\times$ Nitrogen	2,12	0.05	0.95	0.03	0.98	0.17	0.85	0.03	0.79

mesocosms were started with the same number of species and few extinctions were observed during the experiment (there were no significant effects of rhizobia on richness, $P>0.05$).

Simpson's diversity also declined by 48.2% in mesocosms inoculated with rhizobia, further indicating an increase in dominance by few species ($F_{1,12}=30.47$, $P<0.001$, Table 2.1).

Rhizobia altered plant community composition ($F_{1,12}=14.75$, $P=0.001$, Fig. 2.2, Table 2.1) because rhizobia increased the abundance of some species and caused reductions in other species. Rhizobia also significantly reduced β -diversity; variability among mesocosms in community composition was lower for rhizobia treatments than no rhizobia treatments ($F_{1,16}=7.46$, $P=0.014$, Fig. 2.2), suggesting that rhizobia caused communities to converge. The observed effects of rhizobia on diversity and composition likely result because rhizobia increased *C. fasciculata* competitive dominance, increasing its relative abundance from 60% to 84% of the community ($F_{1,12}=15.43$, $P<0.01$, Fig. 2.3). Rhizobia inoculation also tended to decrease the

Figure 2.1. Mean effects of rhizobia on A) *C. fasciculata* biomass and B) Shannon Diversity (H') in experimental mesocosms (Error bars represent $\pm$ SE). White bar represents non-inoculated mesocosms (rhizobia absent), gray bar represents inoculated mesocosms (rhizobia present).



relative abundance of all species in the mesocosms (Fig. 2.3), with *O. biennis* ($F_{1,12}=10.75$, $P<0.01$) and *M. punctata* ($F_{1,12}=4.68$, $P=0.05$) experiencing significant reductions in relative biomass.

Overall, rhizobia significantly increased total community productivity (mean $\pm$ SE: no rhizobia: 26.4 ± 3.9 g/mesocosm; rhizobia: 62.9 ± 3.4 g/mesocosm) ($F_{1,12}=41.71$, $P<0.001$), but

this was due to increased *C. fasciculata* biomass in inoculated mesocosms since rhizobia did not affect subdominant community productivity ($P>0.05$). Rhizobia increased *C. fasciculata* biomass ($F_{1,12}=43.43$, $P<0.001$, Fig. 2.1A), and increased *C. fasciculata* biomass was associated with decreased α -diversity ($r=-0.92$, $P<0.001$). Rhizobia inoculation reduced *O. biennis* biomass ($F_{1,12}=10.5$, $P<0.01$) but tended to marginally increase *B. kalmii* biomass ($F_{1,12}=3.79$, $P=0.075$). Moreover, *O. biennis* biomass was negatively correlated with *C. fasciculata* biomass ($r=-0.53$, $P=0.02$), suggesting that rhizobia reduced *O. biennis* biomass by increasing competition from *C. fasciculata*. All other species (*D. spicata*, *M. punctata*, *P. arguta* and *V. octaflora*) were not

Figure 2.2. Effects of rhizobia on community composition in experimental mesocosms

indicating differences in both community composition and dispersion (β -diversity) between treatments, visualized with non-metric multidimensional scaling (NMDS) based on biomass of each species per mesocosm. Each point represents either a non-inoculated mesocosm (rhizobia absent, white circles) or inoculated mesocosm (rhizobia present, gray triangles).

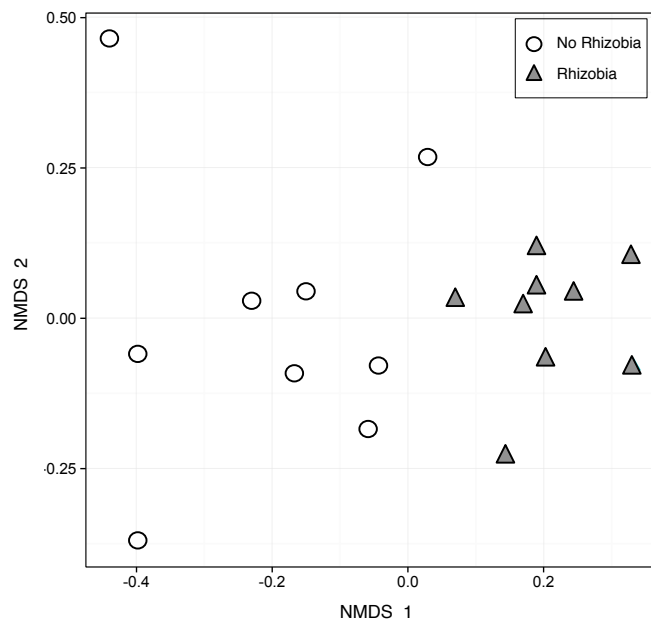
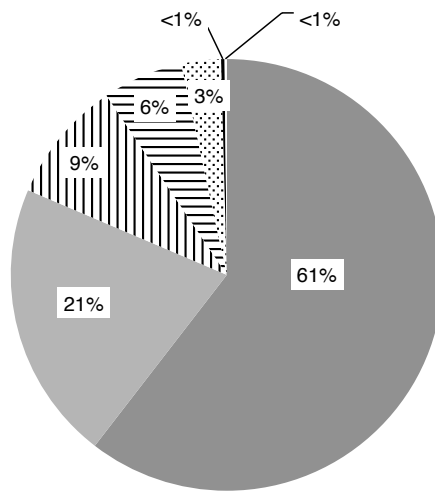
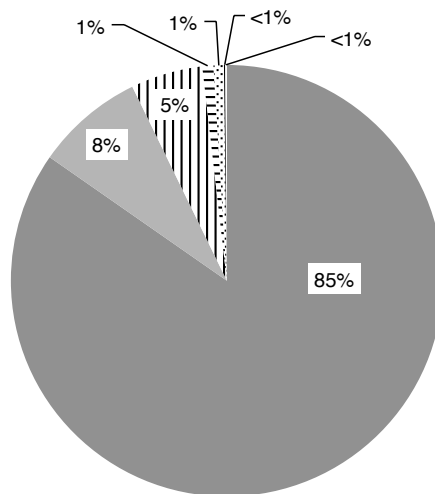


Figure 2.3. Average relative community composition of A) rhizobia non-inoculated and B) inoculated mesocosms for all seven species (in order): *C. fasciculata* (dark gray), *M. punctata* (light gray), *B. kalmii* (vertical lines), *O. biennis* (horizontal lines), *P. arguta* (dotted), *V. octaflora* (black), and *D. spicata* (white).

a. No Rhizobia



b. Rhizobia



directly influenced by the treatments applied ($P>0.1$), but the change in biomass of some species were correlated with changes in other species. As *B. kalmii* increased, *M. punctata* biomass marginally decreased ($r=-0.44$, $P=0.06$). Increased *M. punctata* biomass was correlated with decreased *P. arguta* biomass ($r=-0.48$, $P=0.04$). Biomass of the short-lived grasses *D. spicata* and *V. octiflora* were positively correlated ($r=-0.81$, $P<0.001$).

Rhizobia effects on plant communities were consistent across nitrogen treatments (non-significant rhizobia $\times$ nitrogen interaction, $P>0.1$, Table 2.1). Nitrogen main effects did not significantly affect productivity, diversity, or composition (all $P>0.1$, Table 2.1). Total available nitrogen did not vary across rhizobia or nitrogen treatments or their interactions (all $P>0.1$).

Discussion

Resource Mutualism Affects Diversity

Here I show that similar to predators and ecosystem engineers, mutualists have the potential to be keystone species. In this system, rhizobia act as a keystone mutualist by decreasing plant diversity and evenness, altering community composition, and driving convergence in community structure. Like the classic keystone species *Pisaster* starfish which alters diversity by influencing the abundance of a dominant intertidal competitor (Paine 1966, 1969) rhizobia influence diversity by changing the abundance of the dominant plant competitor *C. fasciculata*. However, while *Pisaster* decreased the abundance of the dominant competitor, relaxing competition and promoting diversity, rhizobia increased the competitive dominance of *C. fasciculata*, thus *inhibiting* diversity.

This short-term (6 month) study shows effects of mutualism on early community structure; however, transient dynamics can be important to the long-term successional trajectory

of a community through priority effects, (Fukami and Nakajima 2011). Here, the reduction in diversity observed in my study may be a transient response to the immediate success of the early successional dominant legume. Over longer time scales, increasing nitrogen levels during succession may help promote the establishment of other species and decrease the dominance of the legume (e.g., Tilman 1987, Chapin et al. 1994, del Moral and Rozzell 2005). How the effects of rhizobia on diversity and convergence in community structure observed here influence longer-term community assembly processes requires further study.

Rhizobia reduced both α -diversity and β -diversity. Shannon diversity decreased and there was also more convergence in community composition between inoculated mesocosms. Rhizobia likely drove greater community similarity by dramatically increasing *C. fasciculata* dominance from 60% to 84% of the community. Conversely, in the absence of rhizobia, there was greater divergence in community composition between mesocosms as subdominants experienced less competition from *C. fasciculata* and there was greater variation in subdominant species growth. This is consistent with research showing reduced β -diversity with increased competitive dominance (Hillebrand et al. 2008). For example, invasion by the dominant ant *Anoplolepis gracilipes* also reduces β -diversity of ant-plant mutualists and local arthropods (Savage and Whitney 2011).

While a mutualism reduces diversity in this and several other systems, numerous other studies have found the opposite pattern — that mutualists increase diversity. These contrasting effects of mutualists on diversity may be explained by the degree of specificity of the mutualistic interaction (Rudgers and Clay 2008). While my study does not permit for exploring the effects of generalist mutualists, mutualists that associate with many species in a community frequently increase overall species diversity by increasing fitness of many species and promoting

coexistence by minimizing average fitness differences across species (sensu Chesson 2000). For example, arbuscular mycorrhizal fungi increase diversity by benefiting numerous subdominant species in phosphorous-limited tallgrass prairies, especially when the competitively dominant species does not greatly benefit from AMF (Collins and Foster 2009). Similarly, generalist ant seed dispersal mutualists promote diversity (Gove et al. 2007), and declines in the abundance of similar ant generalist mutualists reduced diversity and altered community composition in the South African fynbos (Christian 2001). In contrast, specialist mutualists that associate with a single host species may make their partner species more competitive and decrease diversity, especially when their partner is a dominant species such as in the system studied here. For example, a specialized aphid-ant mutualism where ants tend honeydew-producing aphids on *Populus* trees increased ant abundance causing reduced arthropod diversity (Wimp and Whitham 2001). Also, endophytic fungi reduce diversity by making their host, the dominant plant tall fescue, even more competitive (Clay and Holah 1999) by altering small mammal herbivory (Rudgers et al. 2007).

C. fasciculata can be a dominant species in disturbed habitats, and rhizobia appear to increase that dominance. Moreover, the rhizobia mutualism is not generalist across many species in my experimental communities (*C. fasciculata* was the only legume in my experimental mesocosms); thus, this specialized interaction confers a unique competitive advantage to *C. fasciculata* over the other species. In a similar study, Bauer et al. (2012) tested how mycorrhizae and rhizobia influence simulated prairie communities, finding that mycorrhizae increased diversity while rhizobia altered community composition. Bauer's results are consistent with some results presented here: rhizobia induce shifts in community composition mediated through the legume; however, Bauer did not detect any effects of rhizobia on diversity. In Bauer's

experiment, however, legumes were not a dominant species, and rhizobia were generalists interacting with multiple leguminous species. Similarly, another study that manipulated generalist rhizobia associating with multiple species of subdominant legumes in experimental communities dominated by grasses found that rhizobia increased community evenness by promoting coexistence of legumes (van der Heijden 2006a). The degree of rhizobium specificity and partner dominance may explain the contrasting patterns between these studies and the findings presented here.

Mechanisms of Resource Mutualism Effects on Community Patterns

When associating with a dominant legume, rhizobia can positively affect other community members by relaxing nitrogen limitation on the entire community or can negatively affect competing plants by conferring competitive advantages solely to legume species. Both of these mechanisms may alter community composition and diversity. In this study, rhizobia tended to have both positive and negative effects on competitors, suggesting that both mechanisms may act simultaneously. The decline in some species, such as *O. biennis*, with increasing *C. fasciculata* biomass indicates that increased competition due to rhizobia may negatively impact other species, possibly through reduced nutrient, water, or light availability. In contrast, facilitation from rhizobia increasing nitrogen availability to competitors (directly via increased inputs or indirectly by reducing legume competition for soil nitrogen) may cause biomass increases in other species like *B. kalmii*. These differences in subdominant species responses could be due to varying degrees of niche overlap with *C. fasciculata*.

Numerous studies have shown that rhizobia are less beneficial in fertilized soils (e.g., Naisbitt and Sprent 1993, Heath and Tiffin 2007). Therefore, I expected rhizobia effects to be

more pronounced in nitrogen-limited mesocosms compared to nitrogen-fertilized mesocosms. However, rhizobia effects on diversity, composition, and biomass were consistent across nitrogen treatments. Also surprisingly, rhizobia and nitrogen fertilization did not change soil nitrogen availability despite successful nodulation, and experimental nitrogen treatments did not affect diversity or community composition. One possibility is that plants were using nitrogen quickly and allocating resources belowground, which was not measurable in this experiment due to the dense root matrix that was formed by the end of the experiment. Alternatively, small sample sizes may have limited statistical power for detecting nitrogen effects. Consistent with this latter hypothesis, while not significant, aboveground biomass tended to be highest in the high nitrogen treatment (mean $\pm$ SE: high-nitrogen: 47.8 ± 9.8 g/mesocosm; mid-nitrogen: 42.7 ± 10.1 g/mesocosm; no-nitrogen: 43.4 ± 8.0 g/mesocosm), and similar nitrogen treatments did significantly influence aboveground productivity in a separate experiment (Keller and Lau In Review).

Conclusions

In sum, rhizobia can be a keystone mutualist in communities, reducing both α - and β -diversity and altering community composition. As communities assemble, an early colonizing legume may become dominant and substantially drive subsequent species interactions depending on the biotic soil conditions of the site. Further incorporating the effects of positive symbiotic interactions on plant communities will help increase our understanding of community dynamics by looking beyond only negative interactions such as predation and competition. In particular, more research is needed to explore how plant diversity and community composition may change over time and whether facilitative effects follow these initial reductions in diversity as soil

nitrogen concentrations increase following legume senescence. Additionally, it is important to consider how generalist versus specialist mutualists may differentially influence community diversity, composition, and even stability. If this system is any indication, specialist mutualists that affect a dominant competitor may be especially likely to be keystone mutualists.

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CHAPTER THREE

WHEN MUTUALISMS MATTER: RHIZOBIA EFFECTS ON PLANT COMMUNITIES DEPEND ON HOST PLANT GENOTYPE AND SOIL NITROGEN AVAILABILITY

Abstract

Mutualistic interactions such as the relationship between legumes and rhizobia can affect community and ecosystem properties, but abiotic and biotic factors can alter the importance of these interactions. There is substantial intraspecific genetic variation in how legumes interact with rhizobia, including variation in legume growth response to rhizobia, number of rhizobia-housing nodules, and nitrogen fixation. Soil nutrient availability is also known to influence legume-rhizobium interactions. As a result, both the genetic identity of the colonizing legume and the soil nutrient environment may mediate effects of the legume-rhizobium resource mutualism on communities and ecosystems. We manipulated the presence of rhizobia, nitrogen fertilization, and population identity of the annual legume *Chamaecrista fasciculata* in mesocosms simulating native plant communities. We found that *C. fasciculata* populations differed in their effects on plant diversity, composition, productivity and soil nitrogen availability, likely because populations differ in competitive dominance. We detected greater variation among populations in their effects on communities in the absence of rhizobia than in their presence, and although rhizobia consistently reduced diversity, the magnitude of rhizobia effects on diversity varied across legume populations. Rhizobia also had the strongest effects on communities when nitrogen was most limiting. These findings show that abiotic environmental factors and intraspecific variation in a dominant host plant can influence the magnitude of mutualism effects on communities.

Introduction

Mutualisms can influence surrounding community members, including species not directly involved in the mutualism, by altering competitive dynamics or resource availability (e.g. van der Heijden et al. 2006a, Rudgers and Clay 2008, Keller 2014). For example, rhizobia engaged in a resource mutualism with the legume *Chamaecrista fasciculata* decreased species diversity, altered community composition, and increased community convergence (Keller 2014). However, other studies have found little or no effects of mutualistic rhizobia on plant communities (van der Heijden et al. 2006a, Bauer et al. 2012). In these examples, the type of mutualism was similar across studies, but the particular species studied, environmental context, and specificity of mutualistic interactions varied. In short, seemingly similar mutualistic interactions may have very different effects on communities.

Some of the variation in the effects of mutualism on communities may be due to variation in the strength of mutualistic interaction, caused by abiotic and biotic environmental conditions. While mutualisms clearly can provide substantial benefits to each partner, the interaction between species can also be suboptimal, neutral, or even negative in some contexts (Bronstein 2009). Depending on the type of mutualism, this context-dependency could be due to the density of partners (Brown et al. 2012), nutrient availability (Johnson 2010, Lau et al. 2012), or the strength of other biotic interactions such as herbivory or parasites (Cushman and Addicott 1991, Cheney and Côté 2005). For example, when nitrogen is abundant or when light is limiting, the relative benefit of the legume-rhizobium resource mutualism to one or both of the partners is reduced (Lau et al. 2012). Because of such context-dependency, it is necessary to recognize the range of possible interactions between partners when attempting to identify when and how mutualisms are most likely to scale up to influence communities and ecosystems.

In addition to the abiotic and biotic environmental context, species interactions also may be influenced by the genetic identity of the interacting species (Parker 1995, Mooney and Agrawal 2008). Because species interactions are important community drivers, intraspecific variation in traits related to species interactions may be especially likely to affect community dynamics. For example, genetic variation in pinyon pine traits mediating interactions with herbivores influenced ectomycorrhizal community composition (Sthultz et al. 2009). Similarly, *Daphnia magna* adapted to high light environments prevented the establishment of immigrant species and altered community assembly in aquatic mesocosms compared to effects of *D. magna* adapted to a turbid pond, presumably because *D. magna* from high light ponds were more competitive (De Meester et al. 2007). These examples illustrate the potential influence of within-species variation on fundamental ecological patterns and processes, but an important direction at the interface of evolution and community ecology is to go beyond documenting cases where intraspecific genetic variation influences communities to instead identifying when and how this genetic variation is most important (Hughes et al. 2008, Bolnick et al. 2011, Hersch-Green et al. 2011). While research is still limited in this area, existing case studies suggest that intraspecific genetic variation may be especially important when genotypes differentially alter abiotic and biotic environmental conditions (Schweitzer et al. 2004), when environmental conditions vary across small spatial scales (Albert et al. 2010), when a dominant species is involved (Whitham et al. 2006); or when this variation alters interactions with other species in the community such as mutualists or ecosystem engineers (Crawford et al. 2007).

Because of the context dependency of many mutualistic interactions described above, for species engaged in mutualism, the effects of intraspecific variation also likely depend on the environmental context. Extensive theory developed specifically for resource mutualisms predicts

variability in mutualism outcomes as a function of resource availability (e.g., Johnson et al. 1997, Schwartz and Hoeksema 1998, McGill 2005, Grman et al. 2012, Bever 2015). Perhaps, this theory can be extended to predict when these mutualisms and when genetic variation in mutualism-related traits will be most important for communities and ecosystems. For example, in the legume-rhizobium resource mutualism, where legumes exchange carbon fixed through photosynthesis for nitrogen fixed by their rhizobium symbionts, variation among legume genotypes in mutualism benefit may be greatest in low-nitrogen environments where the potential benefits of mutualism are likely higher. In contrast, minimal variation among genotypes may be observed in high nitrogen conditions where all genotypes likely experience minimal benefit from mutualism. As a result, when rhizobia are present, we may expect intraspecific genetic variation in legume traits to strongly influence community processes in low nitrogen but not high nitrogen soils.

Here, we investigate how the abiotic (nitrogen) environment and intraspecific variation in the legume host mediate effects of legumes and their associated rhizobia on community diversity, community composition, and soil nitrogen availability. We focus on the legume-rhizobium mutualism because 1) it has great potential to alter species interactions throughout the community via its effects on both aboveground (plant competition for light) and belowground processes (soil nutrient availability) and 2) there is tremendous intraspecific genetic variation in legume traits mediating interactions with rhizobia. For example, legumes vary in traits that can affect nodulation (Gorton et al. 2012), legume and rhizobium fitness benefits (Parker 1995, Heath 2010), and nitrogen fixation (Neuhausen et al. 1988, Burdon et al. 1999). Because rhizobia can affect diversity, alter community composition, and drive community convergence (Keller 2014), intraspecific variation in traits mediating resource mutualisms could drive intraspecific

variation in legume effects on plant communities. We first document intraspecific genetic variation in key legume traits that may influence interactions with rhizobia, interactions with competitors, or nutrient dynamics in the community. Then, because compatible rhizobia are not ubiquitous and can be limiting in the environment (Parker 2001, Stanton-Geddes and Anderson 2011), we manipulated rhizobia presence in experimental mesocosms to ask: 1) Do rhizobia alter diversity, community composition, productivity, or nitrogen availability? 2) Are the effects of rhizobia on communities dependent on the source population of their legume host? and 3) Is the magnitude of these population and rhizobia effects greater in nitrogen-limited systems?

Methods

Study System

Chamaecrista fasciculata is an early successional annual legume with a wide geographic range from the Great Plains to the Eastern United States (Irwin and Barneby 1982, Kelly 1992, Fenster 1997, Galloway and Fenster 2000). It forms facultative mutualistic interactions with nitrogen-fixing rhizobia including *Bradyrhizobium* sp. However, the availability of rhizobia is spatially variable, with some sites having no rhizobia, lower densities of rhizobia, or less beneficial rhizobia (Stanton-Geddes and Anderson 2011, Keller *pers. obs.*). *Chamaecrista fasciculata* populations are genetically divergent in many life history and morphological traits across the species range, such as leaf pubescence, leaf number, leaf thickness, specific leaf area, nectary size and volume, emergence and flowering times, biomass, fruit production, and seed size (Kelly 1992, Fenster 1997, Galloway and Fenster 2000, Etterson 2004, Rios et al. 2008), with local adaptation shown at large spatial scales (Galloway and Fenster 2000, Etterson 2004).

Greenhouse Common Garden

To explore variation among populations in traits related to the legume-rhizobium resource mutualism and competitive ability, we grew six *C. fasciculata* populations collected from across the Midwestern United States (Barry County, MI; Fair Oaks, IN; Kitty Todd Nature Preserve, OH; Loda, IL; Sand Ridge Nature Preserve, IL; and Westland, MI) in a common garden greenhouse environment in the presence and absence of rhizobia. The experiment included 36 plants from each population (six plants from each of six field-collected maternal half-sib families). The plants were grown in 656mL containers (D40 Deepots, Stuewe and Sons, Inc., Tangent OR) filled with 3:1 mixture of potting media (LP5, SunGro Horticulture, Agawam MA) and clay (Turface MVP, Profile Product, Buffalo Grove IL). Three plants from each maternal half-sib family were inoculated with 1mL of *B. elkanii* rhizobia (density of 5.7×10^8 cells), and the remaining three plants per family were not inoculated. The rhizobia used were *B. elkanii* strain 6437 which was isolated from *C. fasciculata* plants in Minnesota, is novel to each *C. fasciculata* population used here, and forms mutualistic associations with each of our study populations. We used this strain so that we could isolate *C. fasciculata* variation from variation in the benefit provided by rhizobia strains. Plants were harvested after five months, shortly before senescence. At harvest, we measured height and counted the number of branches, leaves, and nodules. We weighed aboveground biomass after drying for 48 hours at 65°C, and calculated the ratio of plant biomass to nodule number for each individual to estimate per nodule plant benefits. Trait differences between populations and rhizobia treatments were analyzed with ANOVA with population, rhizobia presence, and the population $\times$ rhizobia interaction included as fixed factors. Family nested within population and the interaction with rhizobia were included as random factors.

Greenhouse Mesocosm Experiment

We studied how *C. fasciculata* intraspecific variation, rhizobia availability, and nitrogen availability affected species diversity, community composition, and nutrient availability in greenhouse mesocosms. We simulated local native grassland communities using 14.4 liter experimental mesocosms filled with a mixture of soil (68.5% of Metro Mix 360, SunGro Horticulture, Agawam MA), sand (24.5% of Quikrete All Purpose Sand, Quikrete Companies, Atlanta GA), and clay (7% of Turface MVP, Profile Product, Buffalo Grove IL)]. Identical communities consisting of seven early successional prairie species were planted as seedlings into each mesocosm: *Bromus kalmii* (short-lived perennial C₃ grass), *Danthonia spicata* (annual C₃ grass), *Monarda punctata* (short-lived perennial forb), *Oenothera biennis* (biennial forb), *Potentilla arguta* (perennial forb), and *Vulpia octaflora* (annual C₃ grass) and the dominant legume, *C. fasciculata*. These species are commonly found to co-occur in early successional communities. All non-legume seeds were obtained from Native Connections (Three Rivers, MI). *C. fasciculata* seeds were greenhouse-reared progeny of field-collected seeds obtained from each of six source populations. Seeds were sterilized with 95% ethanol, germinated in seedling trays, and transplanted into mesocosms two weeks later.

Mesocosm treatments

We manipulated rhizobia availability (present/absent), *C. fasciculata* population identity (six populations), and nitrogen fertilization (three levels). Each fully factorial treatment (rhizobia presence/absence $\times$ six populations $\times$ three nitrogen levels) was replicated four times for a total of N=144 mesocosms. However, due seed limitation with a few populations, the final sample size was N=135 mesocosms. We manipulated the presence of rhizobia, *B. elkanii*, by applying

1mL of rhizobia inoculant ($\sim 2.5 \times 10^6$ cells based on OD670, strain 6437) cultured in TY media to the base of each *C. fasciculata* seedling in half of all pots. Non-inoculated mesocosms received 1mL of TY media without rhizobia as a control. We manipulated the population identity of *C. fasciculata* by planting twelve individuals from one of six study populations (see “Common Garden Experiment”) into each mesocosm. We used offspring of field-collected seed grown in a common garden environment to minimize maternal effects. To create a gradient of nitrogen availability that simulates conditions in early successional prairie communities in southwest Michigan, we applied ammonium nitrate at three levels: 0g, 10g and 20g N per m². We also applied 10g per m² of P and K applied as super phosphate and potash to minimize phosphorous and potassium limitation across all three nitrogen treatments. Half of the N, P, and K was added at the time of seedling establishment, and the other half was after three months.

Mesocosm data collection

After six months, we harvested all aboveground biomass, sorted biomass to species and weighed the biomass after drying at 65°C for >2 days. Based on species presence and abundances at harvest, we calculated multiple α -diversity measures for each mesocosm including species richness, Shannon diversity (H'), and Pielou’s evenness. We also examined *C. fasciculata* roots in each mesocosm to verify rhizobia treatments; rhizobia treatments were successfully maintained as inoculated and non-inoculated. At harvest, we also estimated inorganic soil nitrogen availability (NH_4^+ and NO_3^-) by collecting three 10cm soil cores from each mesocosm, homogenizing the soil samples from a given mesocosm, performing a KCl extraction, and analyzing the samples in an AlpKem/ OI Analytic Flow Solution IV analyzer (Model 3550) (see Eilts et al. 2011).

Mesocosm data analysis

To test how *C. fasciculata* population identity, rhizobia and nitrogen influence diversity, aboveground productivity, and inorganic nitrogen availability, we performed ANOVA with population identity, rhizobia presence, nitrogen treatment, and all potential interactions included as fixed factors. We used Pearson's correlation coefficients to examine the pairwise relationships between *C. fasciculata* biomass and the biomass of competing species and between *C. fasciculata* biomass and diversity. We performed ANCOVA to explore if the relationships between *C. fasciculata* biomass and other response variables vary across the treatments. We also used Pearson's correlation coefficients and ANCOVA to examine the relationships between population mean trait values estimated in the common garden experiment and the community responses in mesocosms and how these relationships vary depending on the availability of rhizobia. All analyses were conducted on both the total community and the subdominant community (all species except *C. fasciculata*).

We tested how population identity, rhizobia, and nitrogen affect plant community composition with perMANOVA ('adonis' function of *vegan* using the Bray-Curtis distance measure with 9999 permutations) using biomass from each species (Oksanen et al. 2013). Population, rhizobia, and nitrogen treatments and the interactions were included as fixed factors. Since the experimental species pool was consistent across treatments with few extinctions thereby minimizing any bias of α -diversity on β -diversity, we further analyzed among-mesocosm dissimilarity (β -diversity) in composition by calculating the average distance to the weighted mean of the community in multivariate space using a multivariate test of Levene's homogeneity of group dispersions with the Bray-Curtis distance measure ('betadisper' function of *vegan*) (Anderson et al. 2006, 2011). Statistical significance was assessed with permutation tests

(‘permutest’ function of *vegan* with 9999 permutations). Since testing for interactions in group dispersion can confound location and variance (Anderson 2001), we performed dispersion tests for differences in β -diversity on the main treatments, then further explored differences by splitting up the significant main effects by treatments. To determine which species are driving the community composition results, we also tested the individual species responses to the experimental treatments and their interactions as both total biomass of each species per mesocosm and their relative biomasses per mesocosm with ANOVA. These species responses may be direct responses to the treatments, but could also be responses to changes in other community members. To visualize treatment effects on community composition through both location and dispersion effects, we performed non-metric multidimensional scaling (NMDS) using the Bray-Curtis distance measure (‘metaMDS’ function of *vegan*). Analyses were performed in R with the *car* and *vegan* packages (3.0.2, R core development team; Fox and Weisberg 2011, Oksanen et al. 2013) and with Proc GLM and Proc Mixed (SAS Institute, 2001).

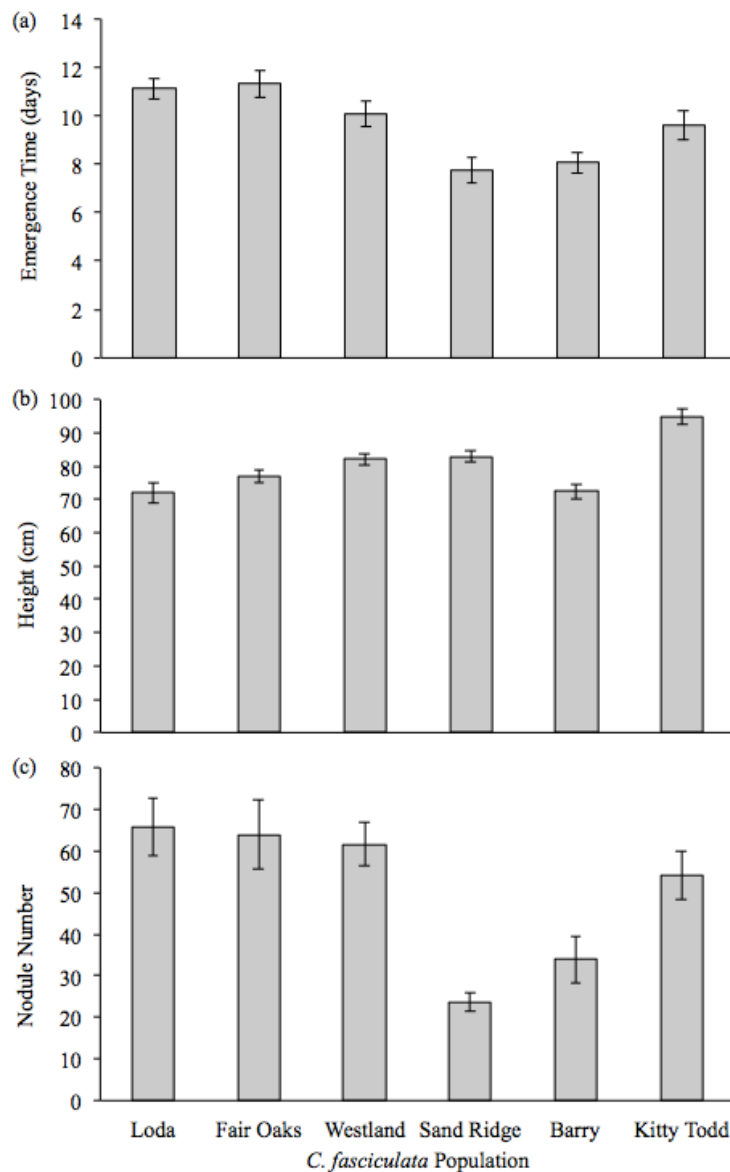
Results

Greenhouse Common Garden

C. fasciculata populations differed in phenological traits including emergence time ($F_{5,184}=4.61$, $P<0.001$, Figure 3.1A) and the number of days to develop the first leaves ($F_{5,184}=4.09$, $P<0.01$). Populations also differed in growth traits such as leaf number ($F_{5,184}=4.27$, $P<0.01$), number of branches produced ($F_{5,184}=6.58$, $P<0.001$), height ($F_{5,184}=12.50$, $P<0.001$), and aboveground biomass ($F_{5,184}=3.75$, $P<0.01$) (Figures 3.1B, S1). For plants inoculated with rhizobia, populations differed in the number of nodules they produced ($F_{5,99}=7.95$, $P<0.001$, Figure 3.1C) and the amount of plant biomass per nodule produced ($F_{5,99}=3.98$, $P<0.01$), which

is a measure of the benefit the plant receives from nodule formation (Figure S3.1). Therefore, these populations vary in growth traits that may influence competition and in traits related to mutualistic interactions with rhizobia.

Figure 3.1. Common garden *C. fasciculata* population differences in (a) emergence time, (b) height, and (c) nodule number (bars represent means $\pm$ SE).



Rhizobia influenced many traits except for germination and the number of days to the first leaves, which was expected given that rhizobia inoculant was applied to seedlings. Rhizobia increased *C. fasciculata*: leaf number ($F_{1,184}=15.44$, $P<0.001$), branch number ($F_{1,184}=7.89$, $P<0.01$), and aboveground biomass ($F_{1,184}=4.76$, $P<0.05$), and reduced plant height ($F_{1,184}=4.52$, $P<0.05$). All rhizobia effects were consistent across populations (non-significant Population $\times$ Rhizobia interactions, all $P>0.1$).

Greenhouse Mesocosm

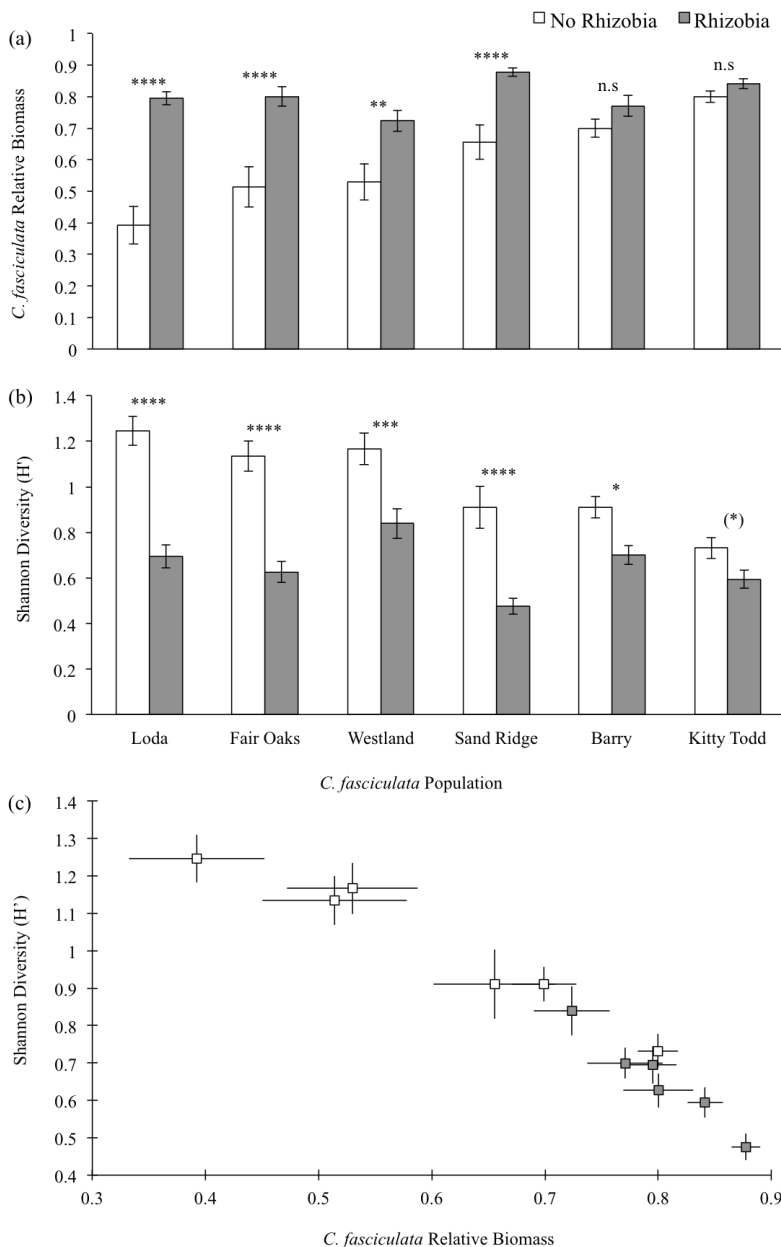
Chamaecrista fasciculata Response:

Rhizobia increased *C. fasciculata* aboveground biomass and relative biomass, but in contrast to the common garden results, the magnitude of that effect varied across populations (Rhizobia $\times$ Population interaction on absolute aboveground biomass: $F_{5,99}=3.87$, $P<0.01$, Figure S3.2 on relative biomass: $F_{5,99}=5.99$, $P<0.001$, Figure 3.2A). While rhizobia benefited *C. fasciculata* in all nitrogen treatments, the effects were most pronounced in the 0 N-addition treatment (Rhizobia $\times$ Nitrogen interaction: $F_{2,99}=3.52$, $P=0.03$, Figure 3.3A).

Diversity Responses:

The presence of rhizobia reduced diversity and evenness, but the magnitude of this reduction varied across populations (Rhizobia $\times$ Population interaction: Shannon diversity: $F_{5,99}=4.35$, $P=0.001$, Figure 3.2B; Pielou's evenness: $F_{5,99}=3.78$, $P<0.01$). Rhizobia also decreased the diversity of the subdominant community ($F_{1,99}=12.85$, $P<0.001$). The reductions in diversity appear to be largely driven by *C. fasciculata* dominance as evidenced by: 1) increased

Figure 3.2. Effects of *C. fasciculata* population and rhizobia treatments on (a) *C. fasciculata* relative aboveground biomass, (b) Shannon diversity index, and (c) the relationship between *C. fasciculata* relative aboveground biomass and Shannon diversity in the presence (open squares) and absence (grey squares) of rhizobia. Error bars represent means $\pm$ 1 SE. Pairwise comparisons of rhizobia effects per population in (a) and (b) conducted with the SLICE option in SAS [($*$): $P < 0.1$; ($*$): $P < 0.05$; ($**$): $P < 0.01$; ($***$): $P < 0.001$; ($****$): $P < 0.0001$].



dominance was correlated with reduced diversity in both the absence ($r = -0.88$, $P < 0.0001$) and presence of rhizobia ($r = -0.92$, $P < 0.0001$, Figure 3.2C); 2) the magnitude of dominance increase for each population resulting from rhizobia tended to be positively associated with the magnitude of rhizobia effect on diversity ($r = -0.745$, $P = 0.089$); and 3) diversity increased with increasing biomass of the subdominant community ($r = 0.28$, $P < 0.001$). A marginal rhizobia by *C. fasciculata* relative biomass interaction indicates that the effects of *C. fasciculata* dominance on diversity may be greater in the presence of rhizobia ($F_{1,63} = 3.49$, $P = 0.066$, Figure 3.2C), although this may also result from a nonlinear relationship between *C. fasciculata* dominance and diversity.

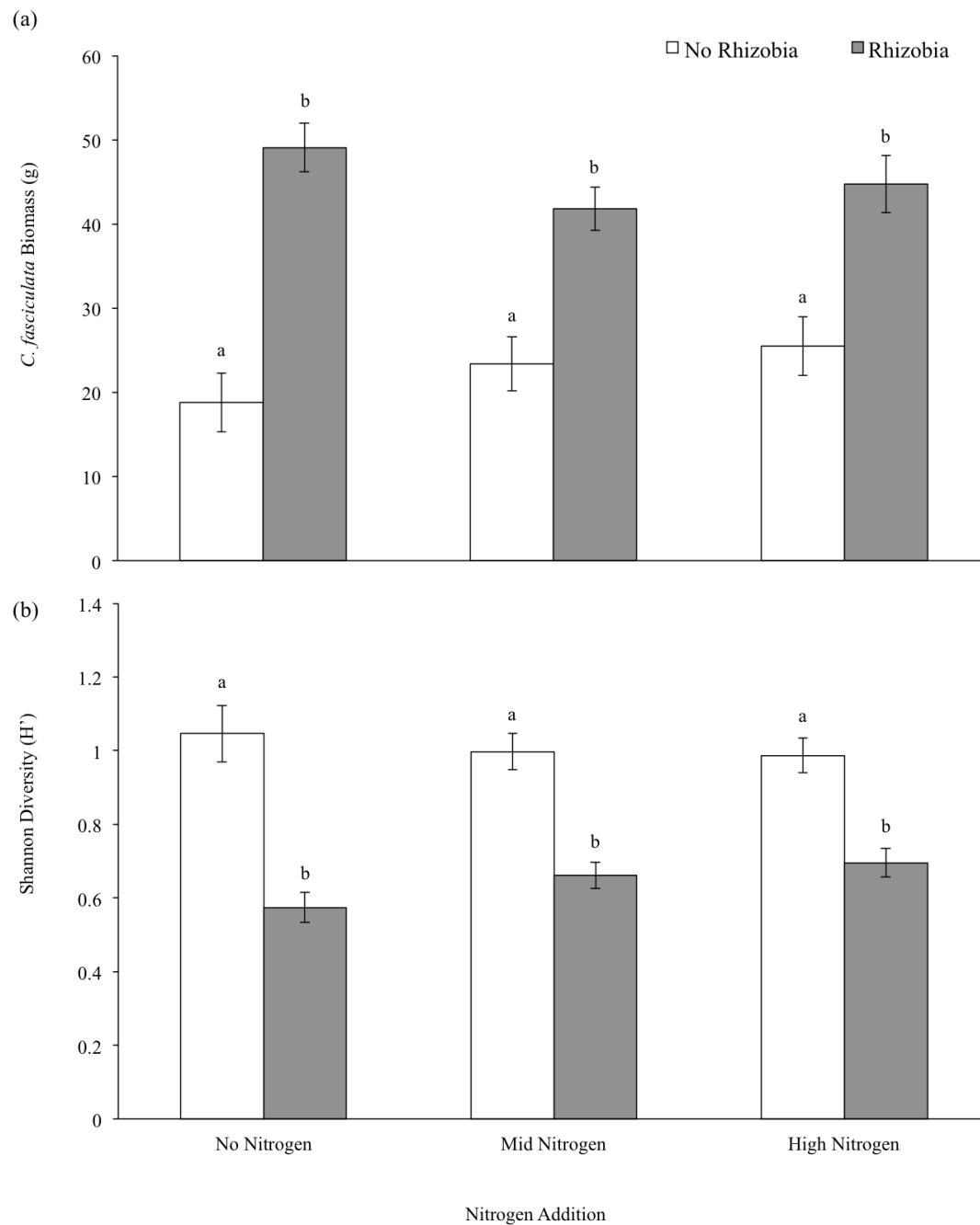
As predicted, the effects of rhizobia on diversity tended to be greater in low nitrogen treatments (Rhizobia $\times$ Nitrogen interaction: $F_{2,99} = 3.02$, $P = 0.053$, Figure 3.3B). In the absence of rhizobia, nitrogen addition did not affect total community diversity ($F_{2,51} = 0.54$, $P > 0.1$) but reduced subdominant community diversity ($F_{2,51} = 5.29$, $P < 0.01$). Yet, in the presence of rhizobia, nitrogen addition increased total community diversity ($F_{2,48} = 4.49$, $P < 0.05$), possibly by reducing *C. fasciculata* dominance ($F_{2,48} = 4.16$, $P < 0.05$). Many results based on subdominant community measures were qualitatively similar to that observed for the total community (Figures S3.3 and S4).

Community Composition Responses:

Rhizobia availability affected plant community composition, but the magnitude of rhizobia effect varied across *C. fasciculata* populations (PERMANOVA: Rhizobia $\times$ Population interaction $F_{5,99} = 3.58$, $P < 0.001$). Population identity of *C. fasciculata* drove changes in community composition in both the absence ($F_{5,51} = 5.12$, $P = 0.001$) and presence ($F_{5,48} = 4.97$,

Figure 3.3. Effects of rhizobia and nitrogen treatments on (a) *C. fasciculata* absolute aboveground biomass and (b) Shannon diversity index (bars represent means $\pm$ SE).

Different letters indicate statistical significance at $P < 0.05$ based on pairwise differences adjusted for multiple comparisons using a Tukey-Kramer correction.

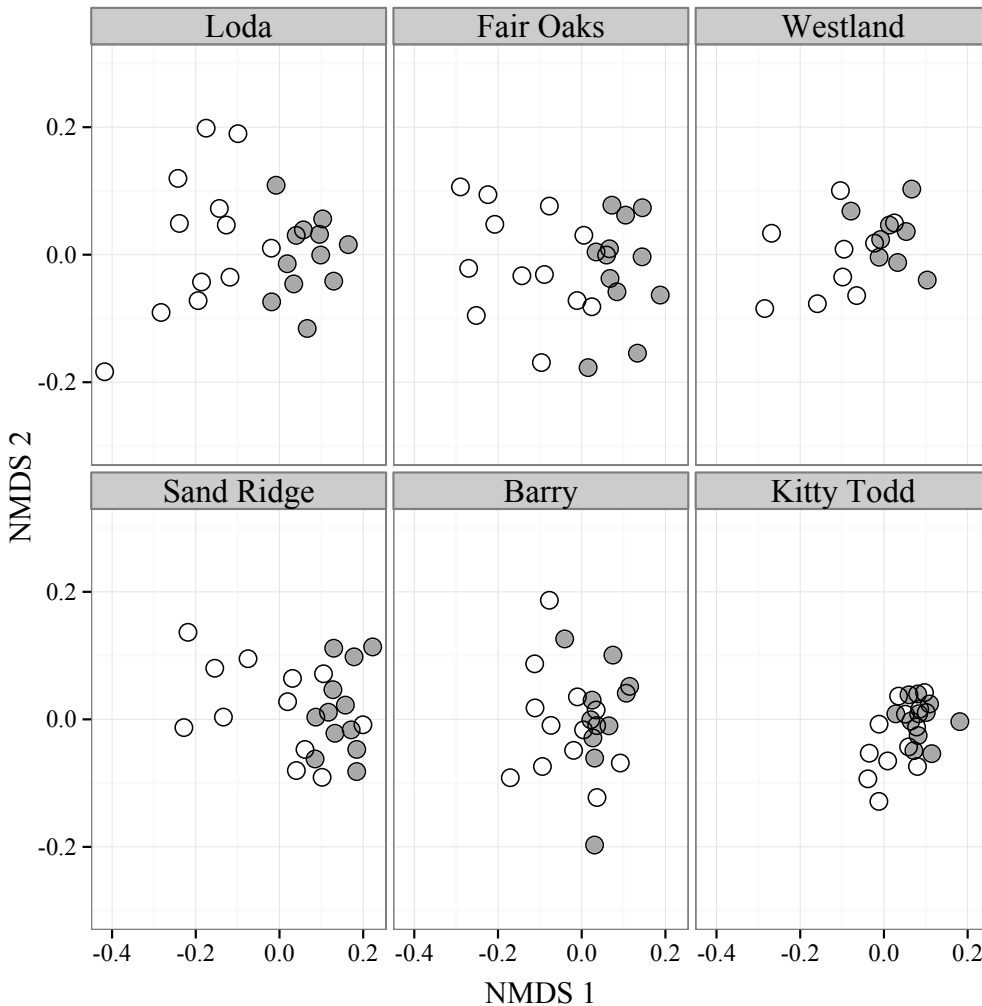


$P=0.001$) of rhizobia, but some populations such as Fair Oaks and Loda experienced greater shifts in community composition when rhizobia were present than others (Figure 3.4). Notably, these were also the populations that benefit most from rhizobia. Nitrogen availability also influenced community composition ($F_{2,99}=4.10$, $P<0.01$). Rhizobia influenced subdominant community composition, although these effects depended on population identity and nitrogen (Rhizobia $\times$ Population $\times$ Nitrogen: $F_{10,99}=1.59$, $P=0.017$). In no- and mid-nitrogen mesocosms, rhizobia consistently affected subdominant community composition (No-nitrogen: $F_{1,33}=3.11$, $P=0.022$; Mid-nitrogen: $F_{1,33}=3.62$, $P<0.01$), while in high-nitrogen mesocosms the effect of rhizobia varied across populations (Rhizobia $\times$ Population interaction: $F_{5,33}=1.89$, $P=0.03$).

Rhizobia also reduced β -diversity by 50% ($F_{1,133}=64.7$, $P<0.001$). For mesocosms inoculated with rhizobia, population identity did not affect β -diversity ($F_{5,60}=0.51$, $P>0.1$), likely because all populations had high *C. fasciculata* dominance and very low β -diversity. However, in the absence of rhizobia, β -diversity varied significantly between populations, indicating more stochastic assembly ($F_{5,63}=2.97$, $P=0.018$) with average β -diversity, represented by distance to the weighted mean of the multivariate community, ranging from 0.14 for Kitty Todd to 0.29 for Fair Oaks. As with many of the community responses, the population variation in β -diversity may be explained by population variation in dominance (correlation between β -diversity and the relative biomass of *C. fasciculata*, $r=-0.856$, $P=0.03$).

Both rhizobia and *C. fasciculata* population identity treatments had large effects on some species in the community. *O. biennis* grew larger in the absence of rhizobia ($F_{1,99}=32.24$, $P<0.001$) and with some *C. fasciculata* populations than others ($F_{5,99}=2.37$, $P=0.045$). *V. octaflora* also grew larger in the absence of rhizobia, although the magnitude of this effect varied

Figure 3.4. Effects of rhizobia on community composition for six *C. fasciculata* populations in experimental mesocosms. Plots are visualized with non-metric multidimensional scaling (NMDS) using the biomass of each species in a mesocosm (Stress = 0.14). Each point represents either a non-inoculated mesocosm (open circles) or inoculated mesocosm (grey circles).



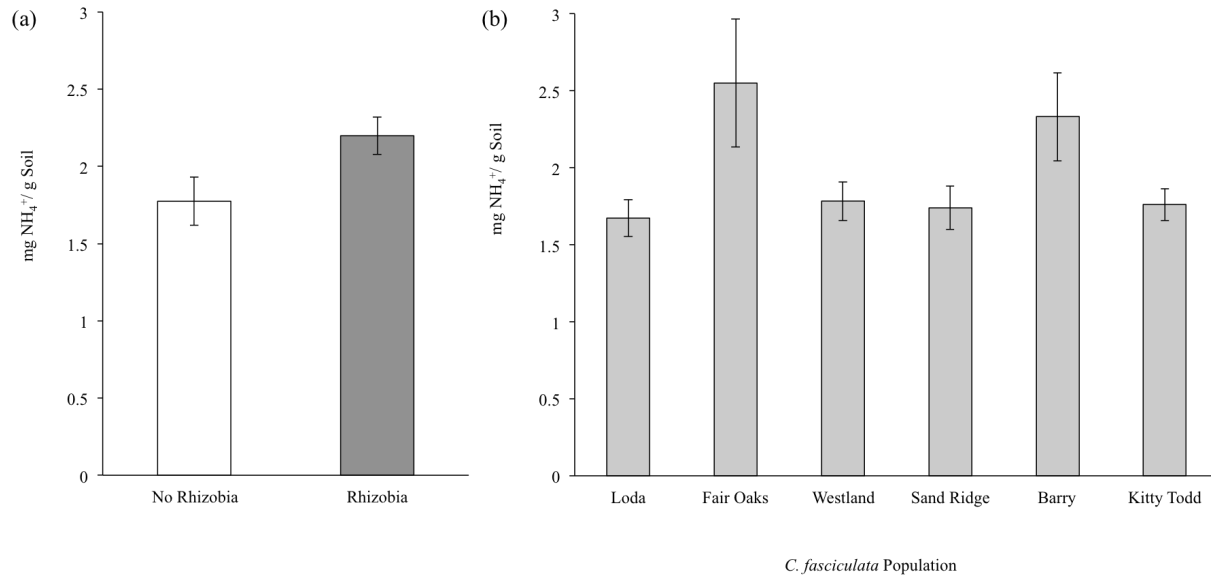
across *C. fasciculata* populations (Rhizobia $\times$ Population interaction: $F_{5,99}=3.33$, $P<0.01$). These effects likely resulted because populations vary in dominance and rhizobia increased *C. fasciculata* dominance, since both *O. biennis* ($r=-0.31$, $P<0.001$) and *V. octaflora* ($r=-0.24$, $P<0.01$) biomass decreased with increasing *C. fasciculata* biomass. *B. kalmii* growth varied

across *C. fasciculata* populations, with the amount of variation across populations depending on rhizobia and nitrogen treatments (Rhizobia $\times$ Population $\times$ Nitrogen interaction: $F_{10,99}=4.26$, $P<0.001$). For some populations, including Barry, Fair Oaks, and marginally in Westland, the magnitude of rhizobia effects on *B. kalmii* biomass varied across nitrogen fertilization treatments (rhizobia $\times$ nitrogen interactions: Barry: $F_{2,99}=5.5$, $P=0.01$; Fair Oaks: $F_{2,99}=4.08$, $P=0.035$; Westland: $F_{2,99}=3.12$, $P=0.08$). *M. punctata* was not influenced by population identity or rhizobia but did grow larger when fertilized ($F_{2,99}=17.11$, $P<0.001$). *P. arguta* and *D. spicata* were not affected by any treatments ($P>0.1$).

Productivity and Nutrient Responses:

Rhizobia increased total aboveground community productivity, but the magnitude of this effect depended on the *C. fasciculata* population (Rhizobia $\times$ Population interaction: $F_{5,99}=3.17$, $P=0.01$, Figure S3.5). Rhizobia also had the largest effects on aboveground productivity in nitrogen-limited mesocosms (Rhizobia $\times$ Nitrogen interaction: $F_{2,99}=4.24$, $P=0.015$). The productivity of the subdominant community increased with increasing nitrogen fertilization ($F_{2,99}=12.69$, $P<0.001$) but was not affected by rhizobia ($F_{1,99}=0.001$, $P>0.9$) or population treatments ($F_{5,99}=0.82$, $P>0.5$), suggesting that rhizobia primarily increased *C. fasciculata* productivity by providing an alternate nitrogen source with negligible negative consequences for the productivity of the rest of the community. In addition, rhizobia increased soil NH_4^+ concentrations ($F_{1,99}=4.25$, $P=0.04$, Figure 3.5A), and *C. fasciculata* population identity affected NH_4^+ availability ($F_{5,99}=2.45$, $P=0.04$, Figure 3.5B).

Figure 3.5. Ammonium nitrate (NH_4^+) concentrations at time of harvest for (a) rhizobia treatments and (b) *C. fasciculata* population identity treatments in experimental mesocosms (bars represent means $\pm$ SE).



Discussion

Rhizobia effects on plant diversity and community composition are context-dependent, varying across both abiotic and biotic environments. The effects of rhizobia on community and ecosystem properties were more pronounced when nitrogen was most limiting and also depended on intraspecific variation in the legume host, likely because of *C. fasciculata* variation in traits related to competitive ability and mutualistic interactions with rhizobia. Consistent with our predictions based on simple mutualism theory and observed patterns of context dependency in resource mutualisms, the presence of rhizobia and intraspecific variation in a dominant legume host were most likely to influence communities and ecosystems in low nitrogen conditions.

Genetic variation in legume host plants mediates rhizobia effects on communities and ecosystems

Mutualistic interactions, and therefore the effects of mutualisms on communities and ecosystems, may depend on the genetic identity of species involved in the interaction. For example, *O. biennis* plant genotypes can influence an ant-aphid mutualism because different plant genotypes support different population densities of aphids and aphid-tending ants (Johnson 2008). In the tall fescue–endophyte symbiosis, the genotype of both mutualist partners affected the symbiotic relationship and altered plant community composition through variation in ergot alkaloid traits that affect herbivory (Rudgers et al. 2010a). While rhizobia have been shown to alter diversity and community composition (van der Heijden et al. 2006a, Keller 2014), here we show that rhizobia effects depend on the genetic identity of the legume partner. This may result from genetic variation in mutualism-related traits such as nodulation or mutualism benefit. In particular, rhizobia effects on *C. fasciculata* dominance, varied across populations. Some populations such as Fair Oaks, Loda, and Sand Ridge received a greater growth benefit from rhizobia, whereas others such as Barry and Kitty Todd did not significantly benefit from rhizobia, but are highly dominant even in the absence of rhizobia. *C. fasciculata* populations varied substantially in their effects on diversity, community composition, and community convergence (β -diversity) in the absence of rhizobia, likely because substantial variation in dominance among populations was observed in the absence of rhizobia. In the presence of rhizobia, however, all mesocosms had low diversity and converged on similar community compositions regardless of legume population because all populations were able to reach high dominance when rhizobia were present.

Variation among populations in dominance may be a common mechanism explaining both variation among genotypes in community or ecosystem effects and also explaining how

mutualisms scale-up to influence communities and ecosystems. For example, variation in dominance of establishing *Solidago altissima* genotypes can influence the establishment of competing plants (Crutsinger et al. 2008) and in this system, the diversity-reducing effects of rhizobia are correlated with increased host plant dominance (Keller 2014). Similarly, in the tall fescue-endophyte mutualism, endophytes decrease β -diversity by increasing the dominance of tall fescue (Keller, Rudgers, Chase, and Clay *unpublished manuscript*). In both the fescue-endophyte defense mutualism and the legume-rhizobium resource mutualism described here, microbial mutualists increased the dominance of their plant hosts, and this increased dominance was associated with reduced β -diversity. These results illustrate that plant-microbe mutualisms may be just as important drivers of β -diversity as other factors, including competition and predation (Chase et al. 2009, Segre et al. 2014), habitat connectivity and dispersal (Cottenie et al. 2003, Cadotte 2006, Soininen et al. 2007), productivity (Chase 2010), environmental heterogeneity (De Cáceres et al. 2012), assembly history (Fukami et al. 2010, 2013, Dickie et al. 2012), and disturbance (Vanschoenwinkel et al. 2013). Given intraspecific variation in dominance in this system and the fact that rhizobia results in high legume dominance regardless of population, community composition was less predictable across populations in the absence of rhizobia than the presence of rhizobia. As a result, effects of legume genetic variation on community assembly were much greater in the absence of rhizobium mutualists.

Just as rhizobia influence community properties by altering legume dominance, rhizobia also can influence nutrient availability through nitrogen-fixation. In this study, rhizobia increased soil ammonium concentrations, possibly through reduced usage of existing nitrogen by the legume or increased nitrogen availability through nodule and plant senescence, nitrogen leaking, and root exudates (Vandermeer 1989, Halvorson et al. 1991, Fustec et al. 2010). Moreover, not

all individuals of the same species may affect nutrient dynamics in the same way. Genotypes of focal species have also been shown to differentially alter nutrient availability and ecosystem functions (Madritch and Hunter 2002, Schweitzer et al. 2004, Silfver et al. 2007). In our study, population identity affects soil ammonium availability, indicating that populations varied in either their utilization of existing nitrogen or their association with nitrogen-fixing rhizobia. Surprisingly and in contrast to the community responses we observed, both rhizobium and population effects on nitrogen availability were consistent across nitrogen treatments.

Soil nitrogen availability mediates rhizobium effects on communities and ecosystems

A recent meta-analysis found that abiotic conditions generate the greatest context dependency in mutualisms (Chamberlain et al. 2014). Here, rhizobia effects on diversity, community composition and community similarity were greatest in low nitrogen environments. This finding is consistent with our predictions based on basic resource mutualism theory. In nutrient-poor communities, rhizobia increase the competitive ability of legumes relative to non-leguminous species (Halvorson et al. 1991, Maron and Connors 1996, del Moral and Rozzell 2005). In addition, the input of nitrogen into the soil from legumes is proportionally less in nutrient-rich environments. Therefore, the effects of legumes and rhizobia on community patterns should be more pronounced in nitrogen-limited habitats compared to nitrogen-rich habitats. Over longer time-scales, the duration and intensity of conditional effects of rhizobia on communities may also depend on how rapidly the presence of the legume-rhizobium mutualism elevates available nitrogen.

Nitrogen and intraspecific variation interactively influence rhizobium effects on communities and ecosystems

Abiotic environmental conditions may affect the importance of intraspecific variation to communities (Gibson et al. 2012). For example, in experimental communities of a sedge, grass, and forb, genotype effects on coexistence between competing plant species varied across two environments that differed in nutrient and light availability (Fridley et al. 2007). Similarly, Burkle and coauthors found that the effects of *S. altissima* genetic diversity on floral visitation depended on soil fertility perhaps because of changes in floral rewards with nutrient enrichment (2013). In the case of resource mutualisms, like the legume-rhizobium symbiosis, nutrient availability may influence the magnitude of intraspecific variation in mutualism outcome and the resulting effects on communities and ecosystems. Here, we found that *C. fasciculata* populations varied in their effects on subdominant species composition when rhizobia were absent (but not when present) in high-nitrogen conditions, but that population identity did not influence the surrounding community in no- and mid-nitrogen conditions in either the presence or absence of rhizobia. The subdominant community had higher biomass with the high nitrogen fertilization, perhaps leading to greater potential variation in community structure compared to when nitrogen was more limiting and all subdominant species constituted a small proportion of the mesocosms.

Caveats

Recent theoretical work shows that even if a species is not present in the contemporary community, its presence and establishment during transient stages can influence subsequent community assembly (Miller et al. 2009). Therefore, while *C. fasciculata* is an early successional species not often found in high density in later successional grasslands, the strong effects on

early assembly processes demonstrated in this short-term greenhouse experiment may influence long-term dynamics. Multi-year field experiments would be necessary test how the abiotic and biotic factors explored here influence longer-term successional dynamics.

Understanding how particular traits drive the effects of intraspecific variation on species interactions and community patterns remains an important question (Hersch-Green et al. 2011). Here we suspect that traits related to mutualistic interactions with rhizobia may explain some of the variation (or lack of variation) in legume population effects on community and ecosystems. While we only include six populations limiting our power to detect significant associations between population mean traits and community or ecosystem outcomes, variation in rhizobium benefit across populations predicted the effects of rhizobia on community and some ecosystem properties. In addition, increased mean leaf number (as measured in the common garden) also reduced subdominant community diversity ($r=-0.90$, $P=0.01$), although this effect was only observed in the presence of rhizobia. These effects of plant traits on community responses likely result from variation in shading caused by different populations.

Finally, here we explore how existing intraspecific variation influences interactions within the community; this study does not address how this variation may be maintained. Future studies could consider how feedbacks from changes in diversity and community composition may increase or decrease the amount of genetic variation within and across legume populations.

Conclusions

By associating with a potentially dominant early successional species, rhizobia can drive substantial changes in community and ecosystem processes, but the importance of this mutualism to community and ecosystem processes varies across both biotic and abiotic

environments. Just as outcomes of pairwise mutualistic interactions are often context-dependent, we have shown that effects of the legume-rhizobium resource mutualism on communities and ecosystems vary with the genetic identity of the legume host and soil nutrient availability. In our study system, the effects of the legume-rhizobium mutualism on diversity, community composition, and ecosystem processes appear to be influenced by the magnitude of growth benefit that the host legume population receives from rhizobia, which is greater in lower nitrogen environments. Moreover, intraspecific variation across legume populations is most important to the surrounding community when rhizobia are absent and soil nitrogen availability is high. We suggest that plant community dynamics can be better understood by considering how the effects of positive symbiotic interactions and intraspecific genetic variation in dominant species vary across abiotic and biotic environments.

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APPENDIX

Figure S3.1. Common garden *C. fasciculata* population differences in (a) aboveground biomass, (b) number of leaves (c) per nodule fitness benefit, and (d) number of branches
(bars represent means $\pm$ SE).

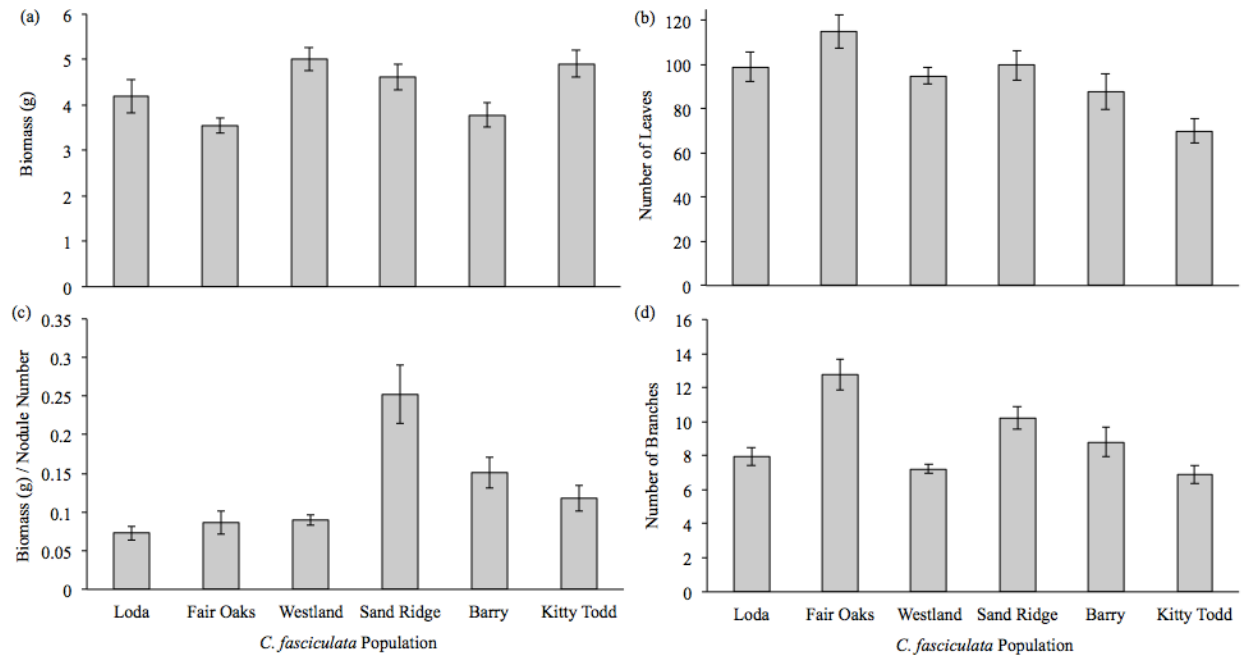


Figure S3.2. Total aboveground biomass of *C. fasciculata* in each population treatment with and without rhizobia (bars represent means $\pm$ SE).

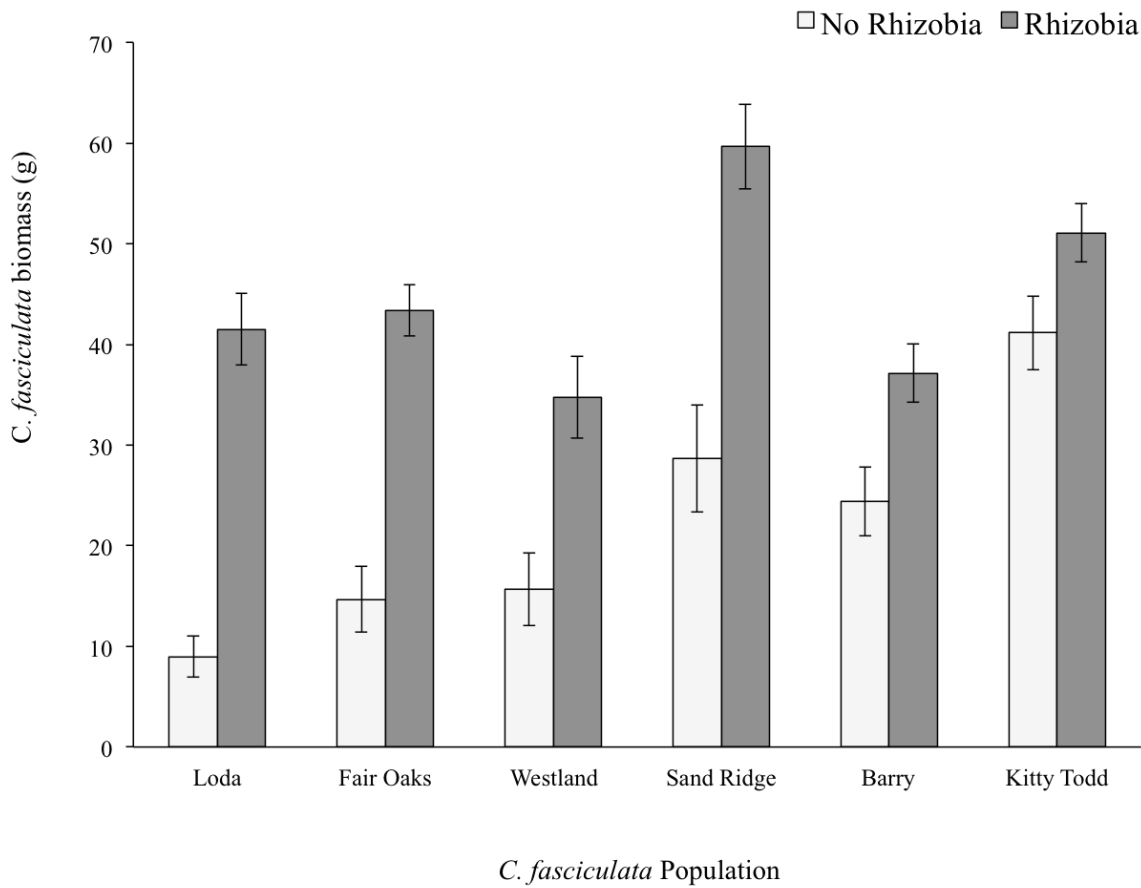


Figure S3.3. Effects of *C. fasciculata* population and rhizobia treatments on the Shannon diversity index of the subdominant community (bars represent means $\pm$ SE).

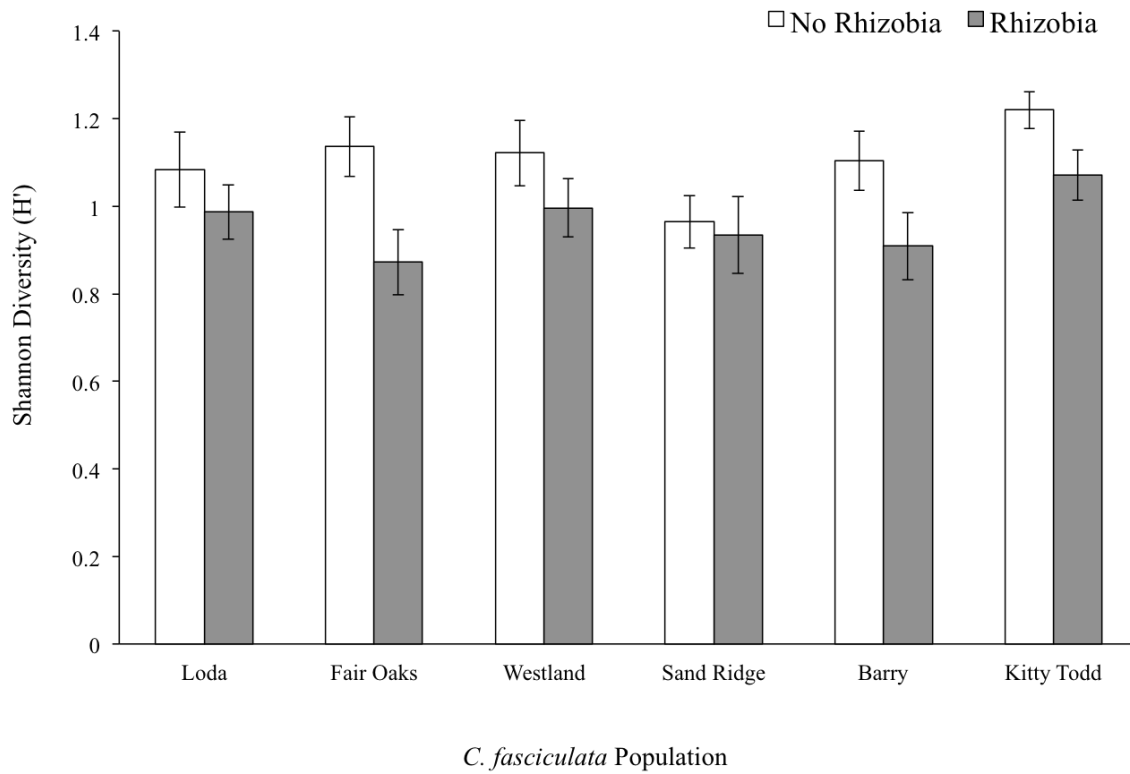


Figure S3.4. Effects of rhizobia and nitrogen treatments on the Shannon diversity index of the subdominant community (bars represent means $\pm$ SE).

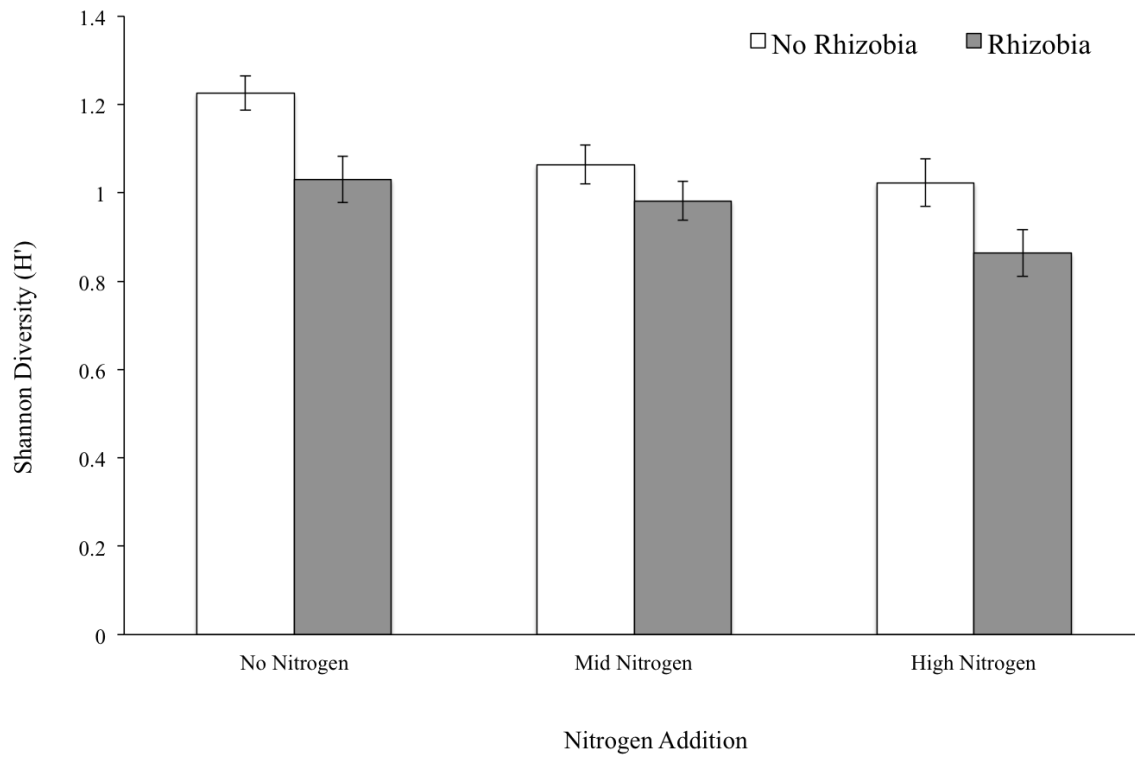
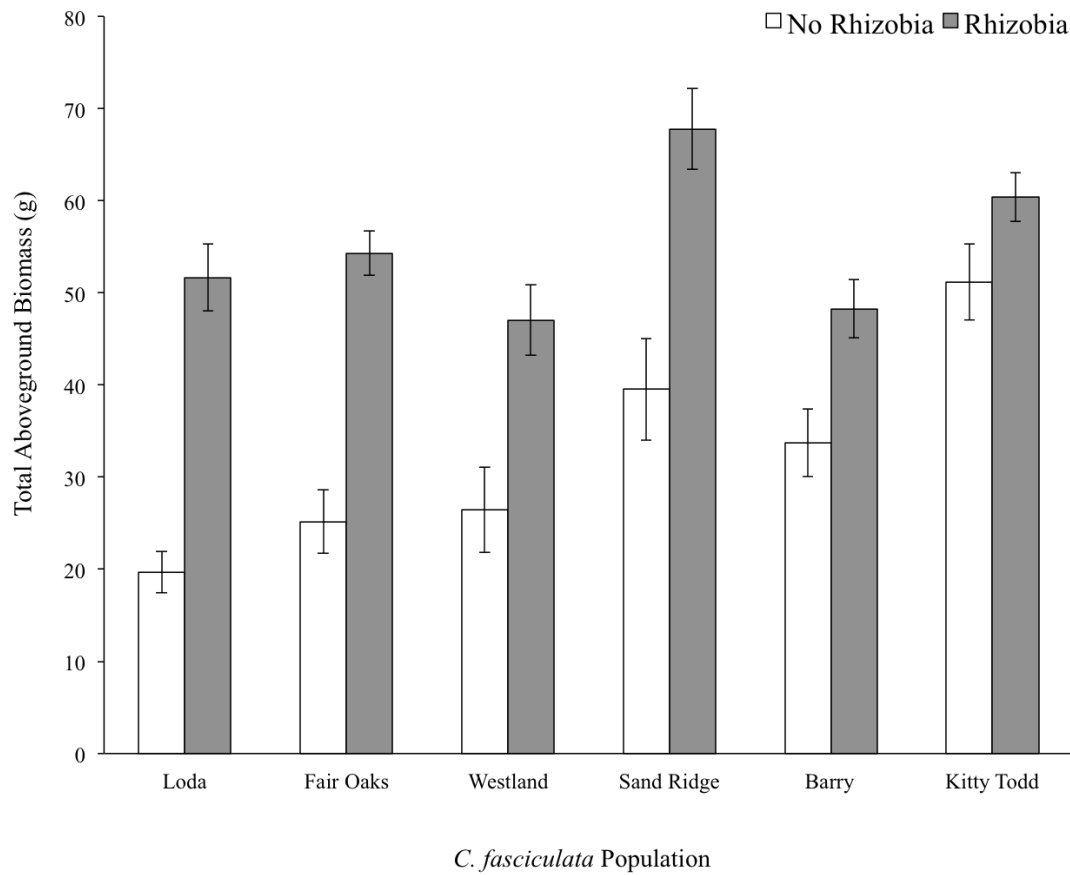


Figure S3.5. Total aboveground biomass of all species for each *C. fasciculata* population treatment with and without rhizobia (bars represent means $\pm$ SE).



CHAPTER FOUR

RHIZOBIUM MUTUALISMS ALTER COMPETITIVE INTERACTIONS

Abstract

Resource mutualisms, such as the interaction between legumes and rhizobia, have the potential to either inhibit or facilitate other species in the community by altering competitive interactions or resource availability. For example, rhizobia can provide their legume host a competitive advantage over other species by increasing legume growth. Alternatively, by increasing nitrogen availability in the community, the legume-rhizobium mutualism could facilitate the growth of other species by reducing competition for a limiting resource. Here, I explore how rhizobia alter the competitive interactions between the legume *Chamaecrista fasciculata* and non-leguminous species in two separate experiments. First, I explore how rhizobia alter pairwise competitive outcomes between *C. fasciculata* and other species; second I use a response surface design to test how rhizobia-mediated effects on competition may be density-dependent. I find that *C. fasciculata* inhibited growth of non-leguminous forbs, but rhizobia ameliorated these effects by promoting increased growth of non-legume species even in the face of increased *C. fasciculata* biomass. I also find that the effects of rhizobia on competitive interactions are density-dependent and vary across competitor species. These results provide insight into the variable responses of co-occurring non-leguminous species to the presence of rhizobia in previous experiments.

Introduction

Mutualistic interactions can strongly affect the species involved in the partnership, but also may affect other species in the community, either positively or negatively. For example, fig wasps may serve as keystone pollinators by increasing food availability for numerous other species in the community; their loss can lead to drastic changes in community composition (Terborgh 1986). Conversely, other mutualists such as foliar endophytes associated with tall fescue may make their host partner more competitive, alter herbivory, and ultimately lead to reduced plant diversity (Clay and Holah 1999, Rudgers et al. 2010a). Mutualisms that generate habitat for other species, such as coral reefs or ant-dispersed bromeliads increase habitat availability (Stachowicz 2001, Céréghino et al. 2010), may promote coexistence between species and provide refuge against predators (Bruno and Bertness 2001). In general, the effects of mutualisms on co-occurring species in the community may depend on the relative benefit that the mutualism provides the host(s), the potential for these benefits to extend to other species in the community, the specificity of the mutualisms, or on the abiotic and biotic factors that the mutualism affects.

Resource mutualisms have the potential to have cascading effects on other community members by decreasing nutrient limitation as nutrients are returned to the community during senescence or through inefficient nutrient transfer (Fustec et al. 2009). They may also negatively affect other species in the community if the mutualist increases its hosts competitive ability (Morris and Wood 1989, Maron and Connors 1996, Keller 2014). For example, mycorrhizae increase the competitive ability of their hosts over non-mycorrhizal species in grassland environments; excluding mycorrhizae prevents this competitive dominance (Moora and Zobel 1996, Hart et al. 2003). In contrast, the presence of arbuscular mycorrhizae fungi may mediate

coexistence between species by allowing competitively inferior mycorrhizal plant species to persist in the presence of superior competitors that are less mycorrhizal dependent (Grime et al. 1987). These examples illustrate that the outcomes of resource mutualist-mediated interactions on other community members may vary, potentially depending on the identity of particular mutualists (van der Heijden et al. 2003), environmental variation (Collins and Foster 2009), or the density of competing species (Schroeder-Moreno and Janos 2008).

The legume-rhizobium mutualism, in which rhizobia bacteria fix atmospheric nitrogen in exchange for carbohydrates from the plant, has been shown to both facilitate and inhibit other species within communities (Morris and Wood 1989, Maron and Connors 1996, Keller 2014). For example, although lupine promoted larger growth of the few individuals of other species that successfully colonized the nutrient deficient Mount St. Helens following the eruption in 1983, lupines also prevented most seedling establishment, likely due to competitive inhibition (Morris and Wood 1989). Legume-rhizobium mutualisms have the potential to provide the legume with a competitive advantage over non-leguminous species in nitrogen-limited environments.

Conversely, by providing the legume access to atmospheric nitrogen, rhizobia also have the potential to reduce competition between leguminous and non-leguminous species in two ways. First, they may reduce niche overlap between species through reduced competition for soil nitrogen since legumes are accessing a distinct pool of nitrogen unavailable to their competitors. Second, greater complementarity between legumes and non-leguminous species can occur when fixed nitrogen becomes available to non-leguminous species following the senescence of the legumes or rhizobia, leaking from rhizosphere, and from root exudates (Vandermeer 1989, Halvorson et al. 1991, Spehn et al. 2002, Fustec et al. 2010). Both mechanisms may be important: a study exploring competition between a grass and legume, using stable isotopes,

found that the grass species had higher biomass when grown with the legume, due to increased soil nitrogen available to the grass rather than transfer of rhizobial-fixed nitrogen (Vallis et al. 1967); while another study found that 8-39% of the nitrogen found in some non-leguminous species was transferred from co-occurring legumes (Spehn et al. 2002).

By affecting competitive interactions between legumes and other species, rhizobia have the potential to alter the diversity and composition of communities. While some studies have found that rhizobia reduce diversity by increasing legume competitive dominance (Keller 2014, Keller and Lau In Review), other work has shown that rhizobia can increase the evenness of the community by allowing subordinate legumes to coexist with competitively superior non-leguminous species (van der Heijden et al. 2006a), or have no affect on community patterns (Bauer et al. 2012). Differences in densities and dominance of focal legumes may explain some of the variation in these results. Rhizobia may provide legumes with the opportunity to avoid competitive exclusion when they are at low densities, while also potentially promoting other species through rhizobia driven niche differentiation. In contrast, when the legume is already highly abundant or when the non-leguminous species are less abundant in the community, the benefits that the legume receives from rhizobia may promote a greater competitive ability for light or other resources. In my previous work exploring the effects of rhizobia mutualists in early successional mixed species mesocosm communities sown with a high density of legumes, I found some competing species to benefit from rhizobia while others are inhibited (Keller 2014, Keller and Lau In Review). Understanding how rhizobia alter competition could provide more insight into the community level effects of the legume-rhizobium mutualism. Here, I explore how rhizobia alter the competitive interactions between a focal legume and co-occurring non-leguminous forbs and grasses. I first test how rhizobia alter the competitive response of non-

legumes in pairwise interactions. I then use a response surface competition design to test how rhizobia alter density-dependent competitive responses.

Methods

Study System

Chamaecrista fasciculata is an early successional annual legume found from the Great Plains to the Eastern United States (Kelly 1992, Fenster 1997, Galloway and Fenster 2000).

Chamaecrista fasciculata can become highly dominant in some locations with densities exceeding 100 plants per m² (Keller pers. obs.). As such, it can have large effects on the diversity and composition of the surrounding plant community, especially when grown in the presence of nitrogen-fixing rhizobium symbionts (Keller 2014, Keller and Lau In Review).

Pairwise Competition Experiment

I conducted a pairwise competition experiment to test how rhizobia availability alters the competitive dynamics between *C. fasciculata*, and seven other species. The experiment included interspecific and intraspecific competition treatments of *C. fasciculata* with each of the seven other species found to commonly co-occur with *C. fasciculata* in natural systems (*Andropogon gerardii*, *Bromus kalmii*, *Danthonia spicata*, *Monarda punctata*, *Oenothera biennis*, *Schizachyrium scoparium*, and *Solidago rigida*). In June 2013, seeds of all eight species were germinated in flats, then transplanted 7 days later into 656mL containers (D40 Deepots, Stuewe and Sons, Inc., Tangent OR) filled with potting soil (LP5, SunGro Horticulture, Agawam MA). Each pot was planted with two individuals spaced 2cm apart in the center of the pot, either with two individuals of the same species for the intraspecific competition treatments or with one *C.*

fasciculata plant and one plant of one of the seven other species for the interspecific competition treatments. Half of the pots were inoculated by applying 1mL of *B. elkanii* rhizobia strain 6437 (density of 5.7×10^8 cells) cultured in TY media to each individual in the pot while the non-inoculated pots received 1mL of sterile TY media. Competition treatments were replicated 14 times per treatment (N=420 pots). However, the final sample size was 403 pots due to some early establishment mortality.

Pots were harvested in early November 2013, after 6 months of growth. For each individual, I measured plant height, sorted aboveground and belowground biomass, and counted rhizobia-housing nodules formed on the plant roots of each *C. fasciculata* plant. Biomass was dried at 60°C for >48 hours then weighed.

To explore *C. fasciculata* competitive effects on each species, I performed separate ANOVAs for each species on competitor aboveground biomass, belowground biomass, total biomass, height, and root:shoot ratio using competition treatment, rhizobia treatment, and the competition $\times$ rhizobia interaction as fixed predictor variables. To test how rhizobia influenced *C. fasciculata* competitive response, I performed ANOVAs on *C. fasciculata* aboveground biomass, belowground biomass, total biomass, plant height, and root:shoot ratio with rhizobia treatment and competitor species as predictor variables. I performed Pearson correlation tests to explore how *C. fasciculata* growth traits are related to nodule number for inoculated plants. I also assessed competition through multiple commonly used indices: relative total yield, which provides insight into potential niche differentiation between species (Weigelt and Jolliffe 2003); relative neighbor effect, which provides a measure of competition intensity (Weigelt and Jolliffe 2003); and the corrected index of relative competition intensity, which accounts for potential biases caused by having upper and lower bounds fixed (Oksanen et al. 2006).

First, I calculated relative yield total as:

$$\text{Eqn. 1: RYT} = Y_{i(j)} / \hat{Y}_{ii} + Y_{j(i)} / \hat{Y}_{jj}$$

where $Y_{i(j)}$ is the biomass of species i in mixture with species j , $Y_{j(i)}$ is the biomass of species j in mixture with species i , and $\hat{Y}_{ii}$ and $\hat{Y}_{jj}$ are the means of each species in monoculture. Greater RYT values indicate that species are making different demands on resources than their competitor (higher niche differentiation). I also calculated relative neighbor effect as:

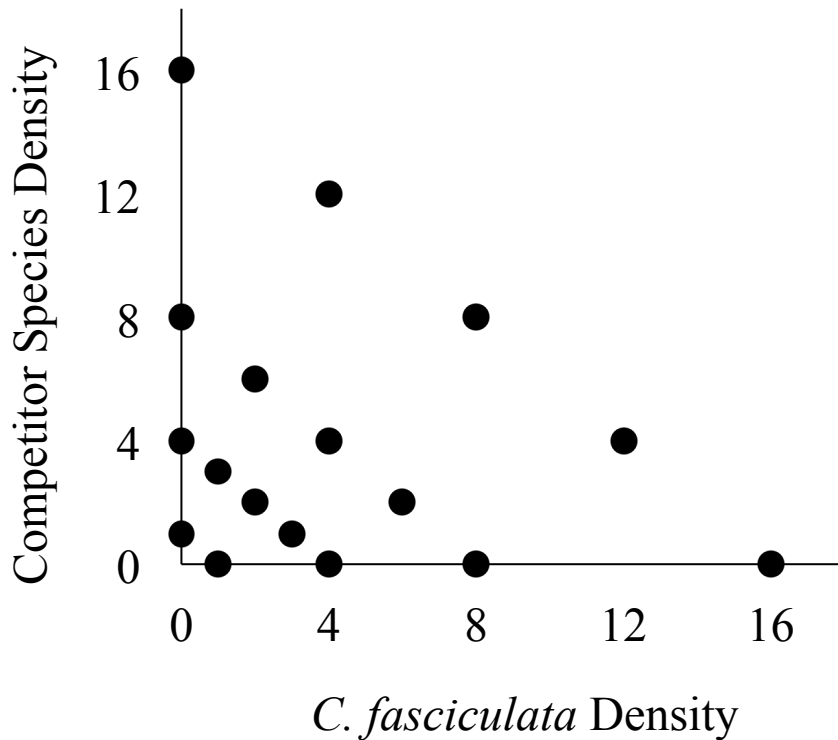
$$\text{Eqn. 2: RNE} = (X_r - X_c) / (\text{Max } X_r, X_c)$$

and the corrected index of relative competition intensity (sensu Oksanen et al. 2006) as:

$$\text{Eqn. 3: CRCI} = \arcsin \text{RNE}$$

where X_r is the plant biomass in interspecific competition and X_c is plant biomass in intraspecific competition). RNE and CRCI were calculated on randomly assigned pairs of interspecific and intraspecific pots of the same species and rhizobia treatments. For each index, I conducted separate one-way ANOVA analyses for each species using rhizobia treatment as a predictor variable. Data were log or square root transformed where appropriate to meet assumptions of normality. All analyses were conducted in R (R Development Core Team 2015).

Figure 4.1. Response surface experimental design.



Response Surface Competition Experiment

To test how rhizobia alter the competitive interactions between *C. fasciculata* and commonly co-occurring competitors, I performed a second competition experiment using a response surface design that manipulated the densities of both *C. fasciculata* and a competing species (*Monarda punctata*, *Rudbeckia hirta*, and *Solidago rigida*). I established 17 different density combinations that totaled 1, 4, 8, or 16 plants per pot (Figure 4.1) in 6 liter pots (Nursery Supplies 600) filled with potting soil (LP5, SunGro Horticulture, Agawam MA). In September 2014, I transplanted one-week old seedlings into the pots using the same spatial arrangement for each treatment. Half of the pots were inoculated with rhizobia by applying 1mL of *B. elkanii* rhizobia strain 6437 (density of 5.7×10^8 cells) to each individual in the pot. Each treatment was

replicated 4 times for a total of 344 pots, located in a greenhouse at the W.K. Kellogg Biological Station.

After 5 months of growth, I clipped and sorted aboveground biomass for each species, dried the biomass at 60°C for >48 hours, and then weighed it. I recorded total biomass per species per pot and calculated average individual biomass per species per pot by dividing the total biomass by the number of surviving individuals. Although I was unable to separate belowground biomass due to dense root growth, I examined roots from each pot to confirm the success of rhizobia treatments based on the presence of nodules on the legumes and to check for contamination of non-inoculated plants.

To test the effects of increasing *C. fasciculata* density, increasing competitor density, rhizobia availability, and all interactions on average individual competitor biomass per pot (total biomass/ number of surviving individuals) and total competitor biomass per pot, I performed separate ANCOVAs for the three focal competitor species. I also tested how rhizobia and competitor densities influence *C. fasciculata* competitive response for each competitor species with ANCOVAs including average individual biomass and total biomass of *C. fasciculata* per pot as response variables and rhizobia treatment, intraspecific density, and interspecific density and all interactions as predictor variables. Data was log transformed where appropriate to meet assumptions of normality.

This response surface competition design also allows me to explore how rhizobia-mediated effects on competition may be dependent on competitor density through multiple regression based approaches treating density as continuous variables. I constructed non-linear reciprocal yield multiple regression models with second order polynomials (sensu Box and

Wilson 1951, Spitters 1983) to estimate the intraspecific and interspecific competition coefficients (eqn. 4).

Eqn. 4:
$$1/W_x = b_{x0} + b_i N_x + b_j N_y + \gamma_i N_x^2 + \gamma_j N_y^2 + N_x N_y$$

In this equation: b_i and b_j represent the intraspecific and interspecific competition coefficients, respectively; b_{x0} is the intercept; γ_i and γ_j represent the quadratic curvature associated with intraspecific and interspecific competition, respectively; N_x and N_y are the densities of intraspecific and interspecific competition, respectively; and W_x is the average individual biomass of species x per pot. This quadratic, non-linear, function is most appropriate because it allows me to properly capture the curvature and asymptotic relationship with increasing plant densities. I fit these multiple regression models separately for each species with and without rhizobia. From the partial regression coefficients, I obtained the competition coefficients b_i and b_j which represent the strength of intra- and interspecific competition, respectively.

I then tested whether rhizobia altered the competitive interactions between the two species by testing whether regression outcomes differed depending on rhizobium presence with an F-test (sensu Zar 2010, Thompson et al. 2015).

Eqn. 5:
$$F = ((SS_t - SS_p) / (m+1)(k-1)) / (SS_p/DF_p)$$

Here: SS_t represents the total residual sum of squares; SS_p is the pooled residual sum of squares; m is the number of independent factors in the regressions; k is the number of regressions; and DF_p is the pooled residual degrees of freedom. A significant F-statistic would indicate that

rhizobia alter the density-dependent competitive relationship between *C. fasciculata* and the competitor species. All analyses were conducted in R (R Development Core Team 2015).

Results

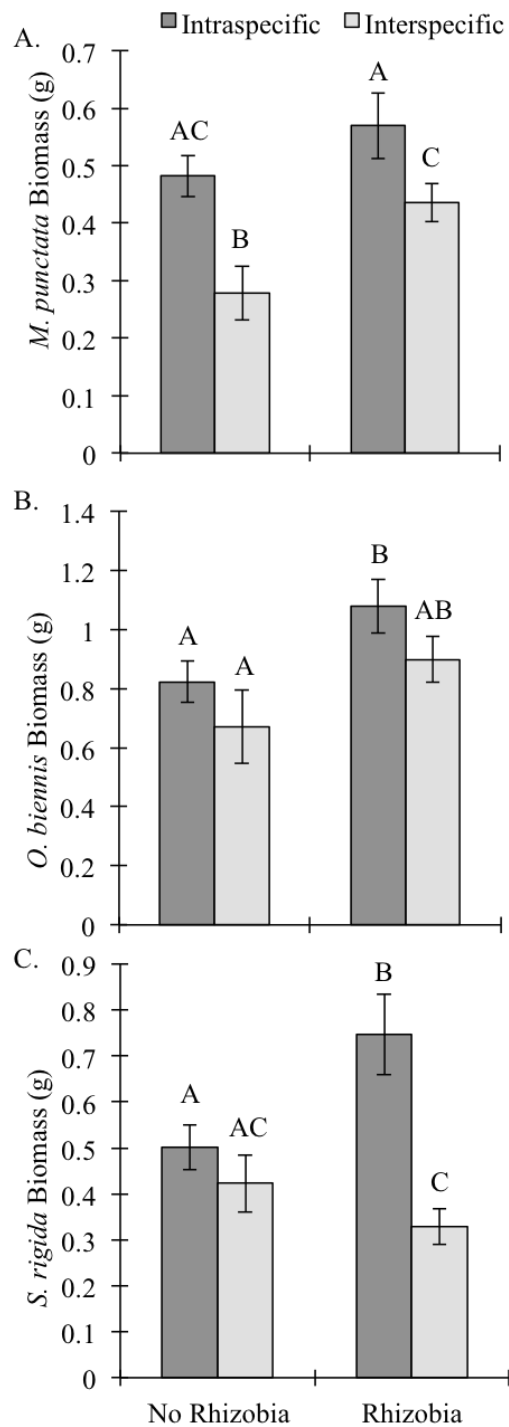
Pairwise Competition

Rhizobia increased *C. fasciculata* aboveground biomass by 87.8% ($F_{1,198}=47.7$, $P<0.0001$) and reduced root:shoot ratios by 21% ($F_{1,198}=21.27$, $P<0.0001$). Rhizobia also increased *C. fasciculata* belowground and total biomass, but the magnitude of that increase depended on the identity of the competing species (rhizobia $\times$ species interaction: Belowground: $F_{7,198}=2.24$, $P=0.03$; Total: $F_{7,198}=2.13$, $P=0.04$; and marginally for aboveground: $F_{7,198}=1.9$, $P=0.07$). *Chamaecrista fasciculata* biomass (aboveground, belowground, and total) and height increased with increasing nodule number (all $r>0.47$, $P<0.0001$).

Chamaecrista fasciculata competition reduced *Monarda punctata* biomass (aboveground, belowground, and total all $F_{1,52}>9.39$, $P<0.01$; Figure 4.2A), and tended to reduce *O. biennis* biomass (Aboveground: $F_{1,51}=2.85$, $P=0.097$; Belowground: $F_{1,51}=5.29$, $P<0.05$; Total: $F_{1,51}=3.11$, $P=0.083$; Figure 4.2B). A rhizobia main effect increased total and belowground *M. punctata* biomass (all $F_{1,52}>6.73$, $P<0.05$) and all measures of *O. biennis* biomass (all $F_{1,51}>5.76$, $P<0.05$). The relative yield total of *M. punctata* and *C. fasciculata* increased with rhizobia present, indicating that the two species may be utilizing different resource pools when rhizobia are present ($F_{1,26}=14.15$, $P<0.001$).

For *S. rigida*, the competitive effects of *C. fasciculata* were greater when rhizobia were present (Competition $\times$ Rhizobia for each of the three biomass analyses: all $F_{1,51}>4.52$, $P<0.05$; Figure 4.2C). *Solidago rigida* plants were also taller when competing with conspecifics and the

Figure 4.2. Effects of pairwise competition and rhizobia on the biomass of A) *M. punctata*, B) *O. biennis*, and C) *S. rigida*. Different letters indicate statistical significance at $P < 0.05$ based on pairwise differences adjusted for multiple comparisons using a Tukey-Kramer correction.



reduction in their height from interspecific competition was greatest when rhizobia were present (Competition $\times$ Rhizobia: $F_{1,52}=4.80$, $P<0.05$). Relative neighbor effect (RNE) and corrected relative competition intensity (CRCI) analyses also indicate that *C. fasciculata* competition on *S. rigida* tended to be marginally stronger in the presence of rhizobia (all $F_{1,24}>3.01$, $P<0.1$).

All four grass species included in the pairwise competition study had weaker responses to *C. fasciculata* competition and rhizobia availability. Neither *A. gerardii*, *B. kalmii*, *D. spicata*, or *S. scoparium* were significantly affected by competition or rhizobia treatments ($P>0.1$).

Table 4.1. F-test results from response surface analyses (sensu Zar 2010) examining how rhizobia alter the competitive responses of focal species to densities of intraspecific and interspecific competitors. Species in parentheses indicate the species that *C. fasciculata* is competing with for the particular analysis.

Focal Species	F-stat	DF	P-value
<i>Monarda punctata</i>	3.082	3,102	0.0307
<i>Chamaecrista fasciculata</i> (x Mon)	100.070	3,102	<0.0001
<i>Solidago rigida</i>	0.803	3,102	0.495
<i>Chamaecrista fasciculata</i> (x Sol)	184.508	3,102	<0.0001
<i>Rudbeckia hirta</i>	2.640	3,102	0.0535
<i>Chamaecrista fasciculata</i> (x Rud)	79.063	3,102	<0.0001

Response Surface Competition

Individual and total *C. fasciculata* biomass increased when rhizobia were available regardless of the competing species identity (All: $F_{1,96} > 37.37$, $P < 0.0001$), but individual *C. fasciculata* biomass decreased with increasing *C. fasciculata* density (competing with: *S. rigida*: $F_{1,96} = 48.13$, $P < 0.0001$; *M. punctata*: $F_{1,96} = 32.38$, $P < 0.0001$; *R. hirta*: $F_{1,96} = 24.57$, $P < 0.0001$). Total *C. fasciculata* biomass increased with increasing intraspecific density ($F_{1,96} = 28.34$, $P < 0.0001$) but was not affected by the density of interspecific competitors ($P > 0.1$).

Increasing *C. fasciculata* density decreased the individual plant size of *S. rigida* and *R. hirta* competitors (*S. rigida*: $F_{1,96} = 20.08$, $P < 0.0001$; *R. hirta*: $F_{1,96} = 29.71$, $P < 0.0001$). Increasing intraspecific density of each competitor species also decreased individual plant size (*S. rigida*: $F_{1,96} = 13.72$, $P < 0.001$; *R. hirta*: $F_{1,96} = 27.17$, $P < 0.0001$). Rhizobia availability did not affect *S. rigida* or *R. hirta* biomass in the ANCOVA (*S. rigida*: $F_{1,96} = 0.63$, $P > 0.1$; *R. hirta*: $F_{1,96} = 0.005$, $P > 0.1$). Effects of *C. fasciculata* competition on individual *M. punctata* biomass varied depending on rhizobia availability (Rhizobia $\times$ Interspecific Density: $F_{1,96} = 4.84$, $P = 0.03$) and the density of *M. punctata* (Intraspecific Density $\times$ Interspecific Density: $F_{1,96} = 4.31$, $P = 0.04$).

Total *S. rigida* biomass per pot decreased with increasing *C. fasciculata* density ($F_{1,96} = 13.29$, $P < 0.001$) and increased with increasing *S. rigida* density ($F_{1,96} = 22.19$, $P < 0.0001$), but was not affected by rhizobia. Total *R. hirta* biomass per pot also decreased with increasing *C. fasciculata* density ($F_{1,96} = 28.86$, $P < 0.001$) and tended to increase with increasing *R. hirta* density ($F_{1,96} = 3.52$, $P = 0.063$), but also was not affected by the rhizobia availability ($P > 0.1$). Intraspecific competition, interspecific competition, and rhizobia presence interacted to affect *M. punctata* total biomass per pot (3-way interaction: $F_{1,96} = 4.86$, $P < 0.05$). When *C. fasciculata* was present, rhizobia differentially increased total *M. punctata* biomass depending on intraspecific density

Table 4.2. Results from the multiple regressions for each species included in the response surface competition experiment. The intercept is b_0 . Intra- b_i is the intraspecific competition partial regression coefficient; Inter- b_j is the interspecific competition partial regression coefficient; Ratio represents the ratio of the intraspecific competition coefficient to the interspecific competition coefficient. Intra- γ_i is the intraspecific competition curvature and Inter- γ_j represents the interspecific competition curvature. * indicates significant at $P < 0.05$, ^ indicates marginal $P < 0.1$.

Species	Rhizobia Treatment	b_0	Intra- b_i	Inter- b_j	b_i/b_j Ratio	Intra- γ_i	Inter- γ_j	Adj r^2	P-value
<i>Monarda punctata</i>	No Rhizobia	0.275	0.261*	0.346*	0.754	-0.011^	-0.019*	0.524	<0.0001
	Rhizobia	-0.382	0.374*	0.635*	0.589	-0.016*	-0.027*	0.705	<0.0001
<i>Chamaecrista fasciculata</i> (x Mon)	No Rhizobia	0.381	0.111*	0.057	1.947	-0.002	-0.002	0.609	<0.0001
	Rhizobia	-1.00	0.214*	0.141*	1.518	-0.008*	-0.008^	0.452	<0.0001
<i>Solidago rigida</i>	No Rhizobia	0.758	0.160*	0.263*	0.608	-0.005	-0.006	0.619	<0.0001
	Rhizobia	0.903	0.123^	0.274*	0.449	-0.004	-0.008	0.579	<0.0001
<i>Chamaecrista fasciculata</i> (x Sol)	No Rhizobia	0.352	0.134*	-0.050	-2.68	-0.003	0.006*	0.6927	<0.0001
	Rhizobia	-1.098	0.218*	0.092^	2.370	-0.008*	-0.004	0.601	<0.0001
<i>Rudbeckia hirta</i>	No Rhizobia	-1.316	0.357*	0.653*	0.547	-0.012*	-0.027*	0.777	<0.0001
	Rhizobia	-1.122	0.297*	0.517*	0.574	-0.010*	-0.023*	0.804	<0.0001
<i>Chamaecrista fasciculata</i> (x Rud)	No Rhizobia	0.128	0.194*	0.072	2.694	-0.007*	-0.001	0.484	<0.0001
	Rhizobia	-0.982	0.176*	0.096	1.833	-0.006	-0.002	0.3967	<0.0001

($F_{1,68}=3.95$, $P=0.05$). In the absence of interspecific competition, increasing *M. punctata* density increased *M. punctata* total biomass ($F_{1,28}=11.74$, $P<0.01$), but rhizobia did not affect this increase ($P>0.1$).

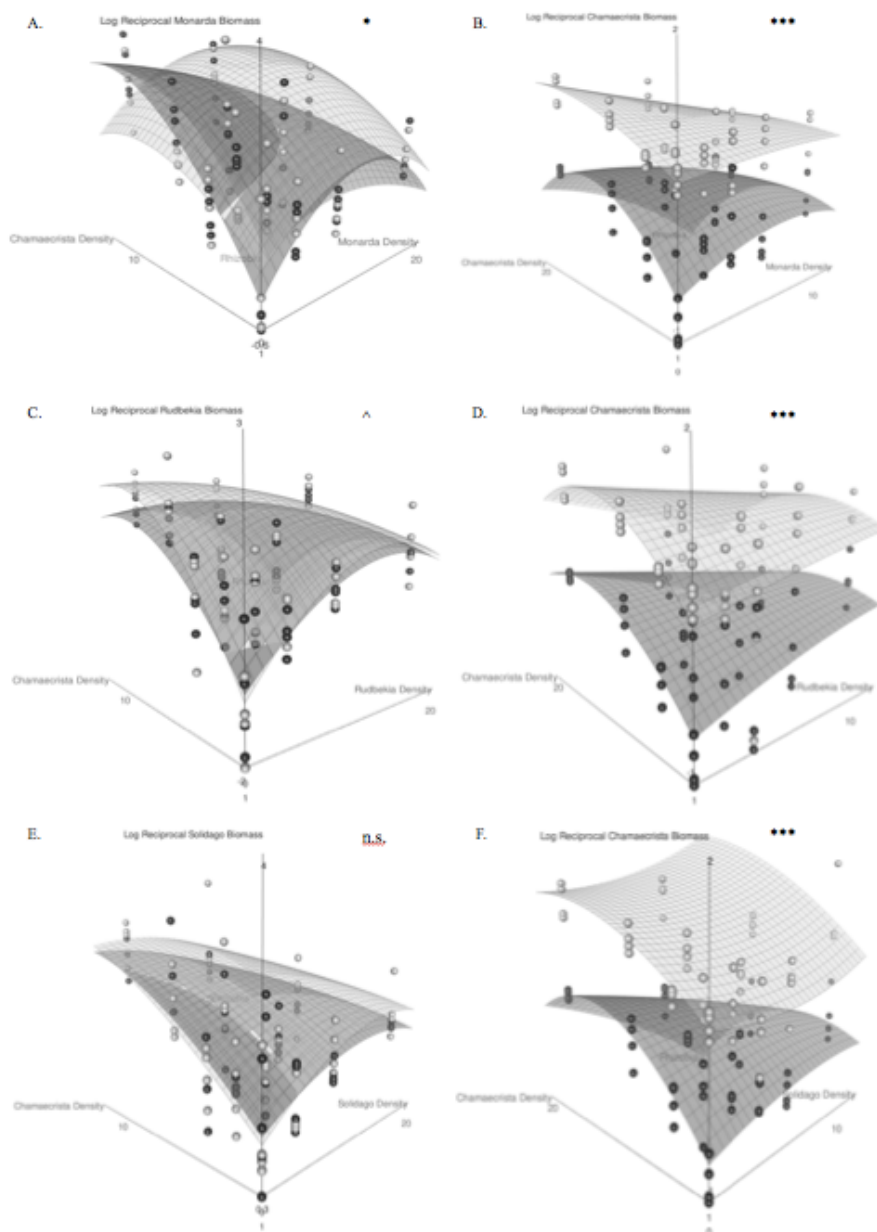
When testing how rhizobia alter density-dependent *C. fasciculata* competitive effects and responses through a response surface analysis, I found that rhizobia differentially altered how increasing interspecific and intraspecific competition affect *M. punctata* and *R. hirta* biomass (Table 4.1, Figure 4.3). In particular, rhizobia increased *M. punctata* biomass, except at higher *C. fasciculata* densities (Figure 4.3A). Rhizobia also increased *R. hirta* biomass, but to a greater degree with increasing intra- and inter-specific densities (Figure 4.3C). The competitive response of *C. fasciculata* biomass across intraspecific and interspecific densities of the three competing species also varied depending on rhizobia availability (Table 4.1, Figure 4.3). Interspecific competition tended to be stronger for the three forb competitors; intraspecific competition was stronger on *C. fasciculata* biomass (Table 4.2).

Discussion

Rhizobia have the potential to substantially alter interspecific competition in plant communities, either through facilitation or promoting competitive inhibition. Through a series of greenhouse experiments explicitly testing the competitive interactions between the legume *C. fasciculata* and other co-occurring species, I found evidence for both processes occurring. The magnitude of effects depended on both intraspecific and interspecific competitor densities. In pairwise competition with *C. fasciculata*, *M. punctata* and *O. biennis* were facilitated by rhizobia, while *S. rigida* was negatively affected. Results from the regression based response surface design provide further evidence that *C. fasciculata* competitive effects are mediated by

Figure 4.3. 3D Response surfaces for competition between: *M. punctata* and *C. fasciculata* (A and B); *R. hirta* and *C. fasciculata* (C and D); and *S. rigida* and *C. fasciculata* (E and F).

Counter-intuitively, due to the reciprocal biomass response, higher values represent smaller individual plants. Light gray surfaces/ points represent rhizobia absent treatments, while dark gray represent rhizobia present treatments. ^ indicates $P<0.1$, * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.0001$.



rhizobia, and illustrated that the magnitude and direction of rhizobia effects (facilitation or inhibition) depend on density. At some intra- and interspecific density combinations, the competing species were facilitated by rhizobia presence, while competitive suppression occurred at other density combinations. Overall, this response surface design provides evidence that there are density-dependent mechanisms by which rhizobia affects *C. fasciculata* interactions with other species, and may explain some of the contradictory results observed across studies.

Competition for limited resources has been shown repeatedly to vary with plant densities (e.g. Shaw and Antonovics 1986, Goldberg and Fleetwood 1987, Miller and Werner 1987, Goldberg and Barton 1992, Adler et al. 2006). The results of this study are consistent with previous theory and experimental findings. However, the shape of the relationship between individual plant biomass and intra- and inter-specific density depends on competitor species identity, with some competing species experiencing stronger competitive effects at increasing intraspecific and interspecific densities than others. Resource competition likely contributes to the greater competitive effect with increasing density. In particular, while total biomass increases with increasing intraspecific density, consistent with resource competition expectations, individual plant size decreases due to decreased nutrient availability for each individual (Silvertown and Charlesworth 2001).

In previous work, I have shown that *C. fasciculata* can become competitively dominant over other species in the community (Keller 2014, Keller and Lau In Review). In the work presented here, I also find that *C. fasciculata* is a stronger competitor than any of the competing species, causing a greater interspecific competitive effect on each of the other species than intraspecific competitive effects. In addition, *C. fasciculata* growth is more limited by intraspecific competition than interspecific competition. Over time, *C. fasciculata* competitive

superiority could lead to exclusion of the inferior competitors. Importantly however, although competitive effects increase with increasing density, these patterns are mediated by rhizobia availability. Interestingly, based on the ratio of intraspecific and interspecific competition coefficients, competitive exclusion may occur faster in the absence of rhizobia than when rhizobia are available.

Rhizobia have the potential to promote niche differentiation between legumes and non-leguminous species by providing legumes access to different nitrogen pools. They may also increase legume competitive dominance by providing access to a limiting resource over other species in the community. These mechanisms may either promote or reduce coexistence by altering the mean fitness differences between species (Chesson 2000). Generally, except at lower densities all three non-leguminous species (*M. punctata*, *R. hirta*, and *S. rigida*) grew larger in the presence of rhizobia when competing with *C. fasciculata*. This indicates that upon reaching a threshold density to tolerate intense competition from *C. fasciculata*, coexistence may be more likely to occur when rhizobia are present, likely due to greater niche differentiation. This may also provide greater insight into the different patterns observed between three recent studies exploring rhizobia effects on plant communities in which rhizobia was found to: increase evenness by facilitating legume coexistence with intense competitors (van der Heijden et al. 2006a); not affect patterns of diversity, but promoting legume biomass (Bauer et al. 2012); and increasing dominance of the focal legume and reducing diversity (Keller 2014). Initial legume density was 19.3%, 25%, and 33% for the three studies, respectively; perhaps a reduced density of subordinate species makes it more difficult for these species to reach that threshold of density or size to overcome competitive suppression from legumes. These differences may be further amplified by the lower density studies (van der Heijden et al. 2006a, Bauer et al. 2012)

manipulating multiple legumes per pot, thereby further reducing each legume's individual density, while Keller (2014) manipulated one focal legume known to establish disturbed sites at high densities. In the absence of rhizobia, legumes and non-leguminous species are competing for the same nitrogen pools; however, rhizobia may ameliorate the effects of increased plant densities on resource competition thereby facilitating greater competitor biomass due to differential resource allocation between the species. Increasing competition for limited resources associated with higher plant density may increase the probability that rhizobia drive facilitation with increasing plant density by alleviating these stresses through niche differentiation (Tilman 1988). Accordingly, while there is a still negative density-dependence on plant growth observed here, rhizobia reduce competitive effects of *C. fasciculata* on *M. punctata* and *R. hirta* to a greater degree with increasing inter- and intraspecific densities.

This study illustrates the range of ways that rhizobia may alter competitive interactions in plant communities. While there has been a large body of work with arbuscular mycorrhizal fungi demonstrating a range of consequences on competition and community patterns (e.g. Hetrick et al. 1994, Moora and Zobel 1996, Hartnett and Wilson 1999, Schroeder-Moreno and Janos 2008, Collins and Foster 2009, Danieli-Silva et al. 2010 among others), the literature on the effects of rhizobia is limited with minimal focus on underlying mechanisms (e.g. Thompson et al. 1990, van der Heijden et al. 2006, Bauer et al. 2012, Keller 2014, Keller and Lau In Review). Yet, the mechanisms may be similar between these two resource mutualisms. For example, mycorrhizae have been shown to alter competitive interactions by increasing the competitive dominance of select mycorrhizal species over non-mycorrhizal species (Hetrick et al. 1994), promoting coexistence through higher intraspecific competition relative to interspecific competition (Moora and Zobel 1996), and altering the resource distribution between species (Hartnett and Wilson

1999). Mycorrhizal mediated competitive effects may also be determined by the density of each competitor, with greater facilitation observed at higher interspecific density, but greater inhibition at higher intraspecific densities and at higher interspecific density when intraspecific density is low (Schroeder-Moreno and Janos 2008), similar to the results presented here.

Ecologists are just beginning to understand the effects rhizobia have on other community members. I have found even in the absence of the legume, rhizobia have positive effects on *O. biennis* and *S. rigida* biomass. While rhizobia have been shown to colonize root tissues of non-leguminous species (Chabot et al. 1996, Yanni et al. 2001, Perrine-Walker et al. 2007), more research into potential non-target effects of rhizobia is needed to better understand the underlying mechanisms driving these benefits. Symbiotic interactions, such as the legume-rhizobia mutualism, continue to be shown as important drivers of community processes such as competitive exclusion or facilitation of co-occurring species. While *C. fasciculata* presence and increasing intraspecific and interspecific density results in decreased plant performance in experimental conditions, rhizobia have the potential to ameliorate the negative effects of competitive suppression from a potentially dominant species.

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CHAPTER FIVE

EFFECTS OF MULTIPLE MUTUALISTS ON PLANTS AND THEIR ASSOCIATED ARTHROPOD COMMUNITIES

Abstract

Although most studies of mutualisms typically focus on a single partner at a time, host species often associate with multiple mutualist partners simultaneously. Because of potential interactions between mutualists, focusing on only a single type of mutualism could lead to a biased perspective of mutualism benefit and how mutualisms may scale-up to affect communities and ecosystems. The legume *Chamaecrista fasciculata* engages in a resource mutualism with nitrogen-fixing rhizobia and also forms symbiotic interactions with ants by providing ants with nectar in exchange for defense against herbivores. Although they provide very different benefits to the plant, both mutualists receive carbon resources from the plant. As a result, these two mutualisms may be likely to interact, potentially competing for carbon resources. In a full-factorial field experiment, we explored how rhizobia and ants independently and interactively influence *C. fasciculata* fitness and the arthropod community associating with *C. fasciculata*. *Chamaecrista fasciculata* received substantial fitness benefits from rhizobia, but there was a cost of associating with ants. Interestingly, ants and rhizobia influenced each other: ants reduced plant allocation to rhizobia, but ants also increased rhizobia contamination of uninoculated plants, suggesting that ants may disperse rhizobia. In turn, rhizobia increased ant abundances, with ants preferentially tending plants with rhizobia. Additionally, rhizobia and ants interacted to influence the abundance of other arthropods found on the plants. Rhizobia increased arthropod abundances but ants negated these increases. As these results illustrate, multiple mutualists may interact, influencing each others' abundance or fitness and the abundance of other community members.

Introduction

In natural systems, many species associate with more than one mutualist partner at a time, even though most studies focus primarily on pairwise interactions (but see Barillas et al. 2007, Mack and Rudgers 2008, Ohm and Miller 2014 among others). Because multiple mutualists may influence each other and interact to influence their hosts, studying a single mutualism could lead to a biased assessment of the fitness consequences of mutualism. For example, *Chondus crispus* seaweed experienced positive growth in the presence of two snail species that provide complementary protective benefits but experienced heavy fouling from gastropods and decreased growth in the presence of only one of the species (Stachowicz and Whitlatch 2005). Examining the relationship between a single snail species and the seaweed may not have revealed a mutualistic interaction.

Multiple mutualists that share a common host may directly interact and / or indirectly influence each other through their shared hosts. Positive interactions may occur if the presence of one mutualist ameliorates abiotic or biotic conditions that would otherwise limit the other mutualist. For example, the presence of arbuscular mycorrhizae increased pollinator resources (flower number, inflorescence size, and nectar availability) and pollinator visitation (Gange and Smith 2005). In contrast, multiple mutualists may be more likely to negatively interact if they overlap in the function they provide to the host or compete for space or resources from the host species. For example, *Vicia faba* plants associating with arbuscular mycorrhizae fungi (AMF) produced less extrafloral nectar (EFN), the key resource attracting ant defenders because of carbon limitation (Laird and Addicott 2007). In essence, mycorrhizae and ants were competing for the same limiting resource.

Interactions that are commonly considered to be mutualistic symbiotic relationships

frequently range from mutualism to parasitism, depending on environmental conditions, including the presence of other species in the community (Bronstein 2009). Interactions with multiple mutualists may explain some of this context-dependency, both because of the potential for one mutualist to influence the presence or abundance of another mutualist and also because mutualists may interact to influence host fitness. Afkhami and coauthors (2014) recently outlined the range of multiple mutualist effects, from enhanced positive effects to reduced fitness from antagonistic direct or indirect interactions between mutualists. Positive effects could arise via additive or partially additive effects between mutualists, or through complementary effects where the benefit is greater than would be predicted assuming additive benefits (Afkhami et al. 2014). Multiple mutualists may also buffer against temporal or spatial variability (Thompson 2005, Afkhami et al. 2014). Negative effects of multiple mutualists arise when the mutualists directly compete for host resources, or when reduced host allocation of rewards to multiple mutualists results in reduced efficiency of the antagonistic abiotic and biotic factors that each mutualist mitigates (Afkhami et al. 2014). Whether multiple mutualists provide enhanced or reduced fitness benefits may also depend on the degree of overlap in benefits provided to the host and benefits received from the host (Afkhami et al. 2014). When mutualists provide different benefits to the host, such as resource mutualisms and defense mutualisms, they may be more likely to synergistically increase host fitness. However, if both species utilize the same traded resource from the host, there may be direct competition for this resource or indirect competition through changes in host allocation, potentially leading to reduced benefits to the host than would be predicted from additive models.

Just as multiple mutualists may influence the host, these effects may scale-up to also influence higher trophic levels within the community. Community-level responses to mutualists

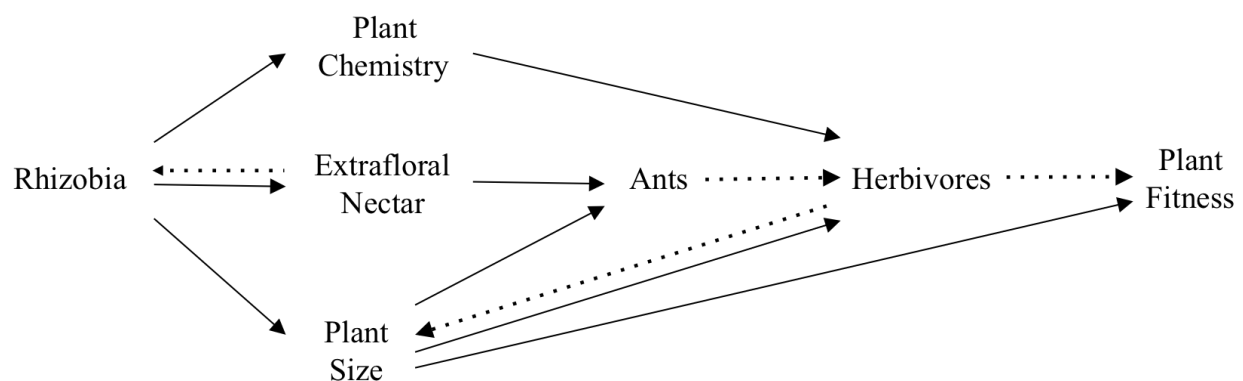
may be highly dependent on the ecological factors that are affected by the mutualists. For example, both resource mutualists and defense mutualists may alter plant chemistry (Vance 2001), while defense mutualists may alter herbivory (Chamberlain and Holland 2009). Rhizobia, in particular, may alter plant chemistry in ways that increase attractiveness to herbivores and may improve nectar quality, thereby increasing attractiveness to nectar-tending ant defenders. In this case, the presence of rhizobia may make the plant more attractive to ant defenders and may make ant defenders even more valued mutualists because of the increased plant attractiveness to herbivores (Ballhorn et al. 2013). If one mutualist promotes or inhibits another mutualist, the community level consequences of changes in allocation between mutualists may even scale-up to affect competitive dynamics or other trophic levels.

While many species host numerous mutualists, there is still a very limited appreciation for the interactions between even a subset of mutualists associating with a single host. For example, the North American legume *Chamaecrista fasciculata* associates with rhizobia, mycorrhizae fungi, ants, bees, and possibly with endophytes. Each of these mutualisms provides a unique benefit to the host plant, such as different types of resource acquisition, defense, and pollination; yet, all are rewarded with plant carbon. Because these mutualists provide different services, we may expect additive or synergistic interactions between different mutualists on plant fitness. Alternatively, because they all compete for the same carbon resource, we may expect subadditive effects of multiple mutualists on plant fitness. Only experimental manipulation of multiple mutualists can identify the relative importance of these two mechanisms and, therefore, the net effects of multiple mutualists on plant fitness. Here we factorially manipulate the presence of rhizobia, which convert atmospheric nitrogen into ammonium in exchange for carbon fixed through photosynthesis, and ant defenders, which regularly visit *Chamaecrista*

fasciculata extrafloral nectaries and remove sugar and amino-acid rich nectar (EFN) in exchange for defending the plant against herbivores. Rhizobia have been shown to increase *C. fasciculata* growth and fitness, alter plant morphological traits, and even influence competitive interactions (Keller 2014, Keller and Lau in review). By altering these factors, rhizobia may be especially likely to affect the way other mutualists, such as ant defenders, interact with the host (Figure 5.1). Nectar-tending ants may reduce herbivory and therefore increase plant fitness, but these benefits likely depend on the strength of herbivory (Frederickson et al. 2012). The effects of ants on host plants can also vary greatly with the region the plants are found in, whether the relationship are obligate or facultative, and even the number of ant species found on a plant (Rosumek et al. 2009). Because EFN can be costly for the plant to produce (Heil 2011), we expect production of EFN to increase in the presence of rhizobia due to increased plant size and nutrient availability. However, EFN production could decrease due to overlap in demands between ants and rhizobia for carbon allocation from the host plant. Ant and rhizobia mutualists could act synergistically resulting in greater plant fitness than predicted from their additive effects if ants become even more beneficial in the presence of rhizobia by defending higher nutrient and more herbivore-susceptible plant tissue, or if rhizobia increase nectar production through greater photosynthetic capacity and nutrient availability. Alternatively, the presence of both ants and rhizobia could reduce the fitness benefits predicted based on additive effects of ants and rhizobia, if ants and rhizobia compete for limited carbon resources. In addition, we expect that herbivores will prefer plants with rhizobia due to higher tissue quality, but that greater ant pressure will reduce abundance of herbivores and other arthropods.

Figure 5.1. Diagram illustrating the possible interactions and effects of multiple mutualists

(ants and rhizobia) on plant traits, herbivores, and plant fitness. Rhizobia (e.g., nodule number) may be correlated with extrafloral nectar production, either because of trade-offs in carbon allocation to EFN vs. nodules or because rhizobia increase plant growth and nitrogen availability, thereby increasing EFN quantity or quality. Rhizobia are predicted to increase plant size, and increased plant size can increase herbivore densities, ant abundance and fitness. Rhizobia are also predicted to affect plant chemistry through changes in carbon to nitrogen ratios, and lower carbon:nitrogen can increase herbivory. Extrafloral nectar can increase ant abundances, which should reduce herbivory. Herbivory can reduce plant size and plant fitness. Solid lines indicate positive effects, and dotted lines indicate negative effects.



Methods

Study System

Chamaecrista fasciculata is an annual legume native to North America that occurs in both highly disturbed grasslands and high quality prairies of the Midwestern and Eastern United States (Irwin and Barneby 1982, Galloway and Fenster 2000). *Chamaecrista fasciculata* maintains multiple mutualistic interactions: it forms mutualistic interactions with rhizobia,

Bradyrhizobium sp., which provides the plant with nitrogen in exchange for carbohydrates (Keller 2014); it produces extrafloral nectar that it exchanges with ants for defense (Barton 1986, Kelly 1986, Rios et al. 2008); and it is buzz-pollinated and predominately outcrossing (Fenster and Galloway 2000).

Experimental Design

To explore how multiple mutualists independently and interactively affect traits, arthropod densities, and host fitness, we conducted a full-factorial experiment manipulating the presence of nitrogen-fixing rhizobia and ant defenders on *C. fasciculata* plants in a field experiment at the W.K. Kellogg Biological Station (n = 20 replicates per treatment; N = 80 plants total). To establish the experiment, we partially buried 80- 2.75 liter pots (Nursery Supplies 300) filled with potting soil (LP5, SunGro Horticulture, Agawam MA) to the pot rim, placed 1-meter apart in a disturbed, old-field community with neighboring vegetation clipped back regularly throughout the season. Prior to planting, we surface sterilized *C. fasciculata* seeds with 95% ethanol for 2 minutes and 10% bleach for 2 minutes. We transplanted one-week old seedlings into each pot in early June 2015 and randomly applied rhizobia and ant treatments. We manipulated the presence of rhizobia, *B. elkanii*, by applying 5mL of rhizobia inoculant ($\sim 2.1 \times 10^6$ cells based on OD670, strain 6437 from Minnesota) cultured in TY media to the base of each *C. fasciculata* seedling in half of all pots. Non-inoculated pots received 5mL of sterile TY media without rhizobia as a control. Ant presence was manipulated by applying Tanglefoot (Grand Rapids, MI), a non-toxic sticky substance to prevent ant movement up plants, to the base of the stem of the plant for half of all pots. This has been previously shown to be an effective way to eliminate ant presence on *C. fasciculata* plants (Rios et al. 2008). Tanglefoot was reapplied as

needed over the course of the experiment to ensure a constant barrier to ant movement onto the plants. There was some establishment mortality of plants in the first two weeks of the experiment. These individuals were not replaced to prevent size differences between individuals as time elapsed, so the final sample sizes were: $n = 17$ rhizobia absent/ants absent; $n = 19$ rhizobia absent/ants present; $n = 19$ rhizobia present/ants absent; and $n = 14$ rhizobia present/ants present. The experimental area was surrounded with 7-foot tall fencing to prevent deer herbivory.

Field Sampling

Plants were censused for ants and other arthropods every three weeks from July to September 2014. At each census, we recorded the density of ants and arthropod herbivores visiting each plant during two-minute observation periods conducted four times throughout the day (morning, twice afternoon, and evening). It took approximately 10 hours to complete each census (2.5 hours to census all plants during each of the four census periods). Arthropods counted only included putative herbivores with predators excluded. However, due to the lack of arthropod collection and identification, we conservatively refer to these as arthropods. Ants were counted separately. We also counted the number of extrafloral nectaries, leaves, and branches, and measured plant height in July. Seed pods were harvested as they ripened to prevent losing seeds through ballistic dispersal. Plants were harvested at 18 weeks after planting, after all seed pods matured. At harvest, we counted the number of rhizobia-housing nodules on the roots and the total number of pods and seeds produced. Aborted seeds were not included in the total seed number. Aboveground and belowground biomass was dried for >24 hours at 65°C then weighed.

Statistical Analyses

To test the effects of rhizobia and ant presence on plant fitness (seed and pod number), plant biomass, plant traits, arthropod density, and ant visitation, we performed general and generalized linear models with rhizobia presence, ant presence, and the rhizobia $\times$ ant interaction as fixed factors. We also performed ANCOVA to determine if the relationship between treatments and the aforementioned response variables were affected by variation in particular plant traits potentially mediating interactions with mutualists, such as biomass, nectary number, and nodule number. Repeated measures ANOVA showed no change in treatment effects on ant and arthropod densities over time, so here we present only the analyses based on data pooled across censuses. Data was log-transformed or square root transformed where appropriate to meet assumptions of normality. In instances where normality was not met from transformations (nodule number and arthropod number), we modeled the data with a negative binomial distribution (chosen by Akaike Information Criteria) with log link function and then conducted likelihood ratio tests. To test whether ants influenced contamination of no rhizobia treatments by dispersing rhizobia, we included contamination as a binomial response and ant presence as a fixed predictor variable in a generalized linear model. Pearson correlations were conducted to test relationships between continuous variables, such as aphid abundance and ant abundance, and rhizobia number and plant fitness. All analyses were performed in R using the car and lme4 packages and SAS using Proc Genmod using type III SS.

Results

Rhizobia effects on ants

Tanglefoot successfully prevented ant movement onto plants (average of 1.14 ants per plant when excluded and 27.18 when present; $\chi^2=40.9$, $P<0.0001$). Rhizobia increased ant

Table 5.1. Treatment effects of rhizobia and ants on ant abundance, plant fitness (seeds and pods), and aboveground biomass analyzed with two-way ANOVA.

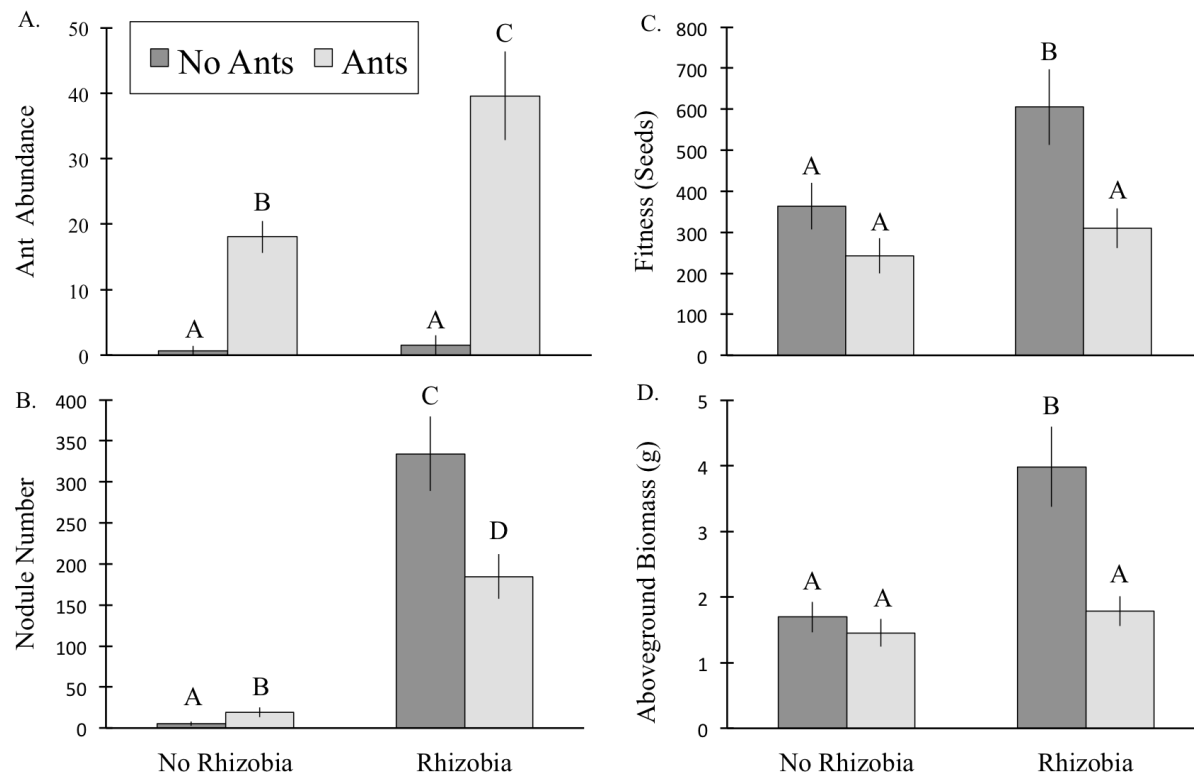
	Ant Abundance		Plant Fitness (Seeds)		Plant Fitness (Pods)		Aboveground Biomass	
Factor	<i>F</i>	<i>P-value</i>	<i>F</i>	<i>P-value</i>	<i>F</i>	<i>P-value</i>	<i>F</i>	<i>P-value</i>
Rhizobia	4.41	0.036	5.91	0.018	10.37	0.002	12.18	0.0009
Ants	40.90	<0.001	9.58	0.003	11.84	0.001	7.39	0.008
Rhizobia × Ants	0.00	0.985	0.05	0.826	0.12	0.734	3.13	0.082

density by 219.2% (average of 18.05 ants per plant for non-inoculated plants and 39.57 ants per plant when inoculated with rhizobia; $F_{1,65}=4.41$, $P<0.05$, Table 5.1, Figure 5.2A). This pattern was largely driven by rhizobia increasing plant size and nectary numbers (effect of size on ants: $F_{1,29}=9.42$, $P<0.01$; effect of nectary number on ants: $F_{1,29}=4.39$, $P=0.04$; Table S5.1); however rhizobia-inoculated plants tended to have more ants even after accounting for these two plant traits (Table S5.1).

Ant effects on rhizobia

Ants reduced nodule number of plants inoculated with rhizobia ($\chi^2=5.68$, $P=0.017$); however, ants increased nodule numbers of uninoculated plants because ants significantly increased the likelihood of contamination ($\chi^2=6.04$, $P=0.014$) ($\chi^2=6.6$, $P=0.01$; Table 5.2, Figure 5.2B). 68.4% of pots were contaminated when ants were present, and these contaminated pots produced an average of 28.0 nodules, which is significantly less than the average of 184.7 nodules in rhizobia inoculated pots. Only 29.4% of pots were contaminated when ants were

Figure 5.2. Effects of rhizobia and ants on A) average number of ants visiting the plants; B) average number of nodules formed on plant roots; C) total seed set, and D) *C. fasciculata* aboveground biomass (means $\pm$ SE). Different letters indicate statistical significance at $P < 0.05$ based on pairwise differences adjusted for multiple comparisons with a Tukey HSD correction.



excluded; producing an average of 18.8 nodules in contaminated pots, which is also significantly reduced from the average of 334.4 nodules in rhizobia inoculated pots.

Rhizobia and ants effects on plant traits and fitness

Rhizobia increased seed set ($F_{1,65}=5.91$, $P=0.018$, Figure 5.2C), the number of pods

produced ($F_{1,65}=10.37$, $P<0.01$), and plant biomass ($F_{1,65}=12.18$, $P<0.001$) (Table 5.1). In contrast, ants reduced plant fitness and biomass (Seeds: $F_{1,65}=9.58$, $P<0.001$; Pods: $F_{1,65}=11.84$, $P=0.001$; Biomass: $F_{1,65}=7.39$, $P<0.01$; Table 5.1, Figure 5.2). No significant rhizobia $\times$ ant interactions were detected on seed or pod number ($P>0.1$), but rhizobia tended to only increase biomass in the absence of ants (Rhizobia $\times$ Ant: $F_{1,65}=3.13$, $P=0.082$, Figure 5.2D). Although increased size may be contributing to greater fitness, rhizobia and ant main effects on fitness go beyond simply increasing plant size, with the relationships between size and fitness varying depending on the presence of rhizobia or ants (Table S5.1). Nodule numbers were positively correlated with plant fitness (Seeds: $r=0.58$, $P<0.0001$; Pods: $r=0.69$, $P<0.0001$). Rhizobia also increased the number of extrafloral nectaries ($F_{1,65}=16.70$, $P<0.001$). Although rhizobia increased plant size ($F_{1,65}=13.26$, $P<0.001$) and increased plant size is associated with increased EFN number ($F_{1,61}=10.31$, $P<0.01$), plant size effects alone tend to not fully explain rhizobia effects on EFN number (marginal main effect of rhizobia even after including plant size as a covariate: $F_{1,61}=3.54$, $P=0.06$).

Table 5.2. Likelihood ratio tests of the effects of rhizobia and ant treatments on nodule number, arthropod abundance, and aphid abundance.

	Nodule Number		Arthropod Abundance		Aphid Abundance	
Factor	χ^2	<i>P</i> -value	χ^2	<i>P</i> -value	χ^2	<i>P</i> -value
Rhizobia	47.05	<0.0001	13.50	0.0002	6.37	0.012
Ants	0.92	0.337	19.32	<0.0001	0.14	0.706
Rhizobia $\times$ Ants	6.60	0.010	8.58	0.0034	0.09	0.765

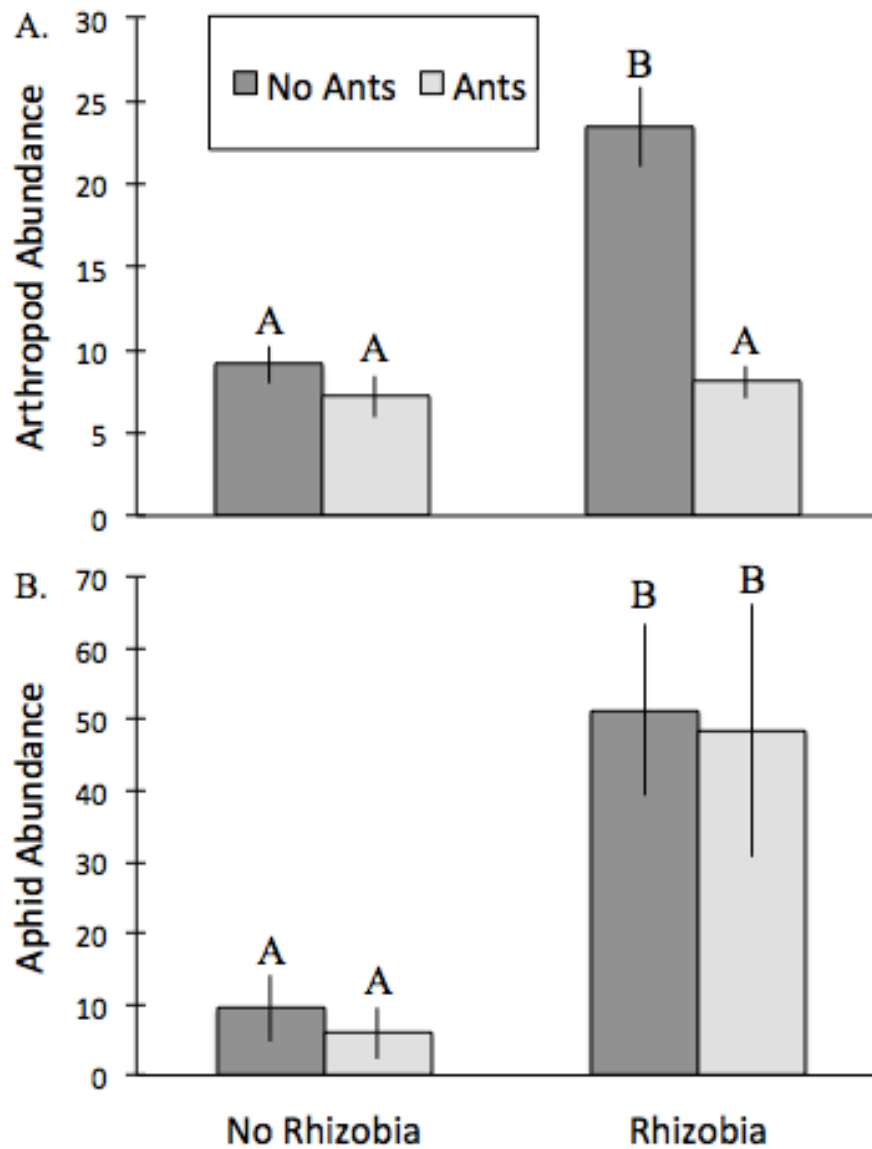
Rhizobia and ant effects on non-ant arthropods

Rhizobia increased the density of non-ant arthropods on *C. fasciculata* plants, but only in the absence of ants (Rhizobia $\times$ Ant interaction: $\chi^2=8.58$, $P<0.01$). Ants strongly reduced arthropod density of inoculated plants such that arthropod numbers were very similar to those observed on uninoculated plants (Table 5.2, Figure 5.3A). Much of the effect of rhizobia on arthropod resulted from rhizobia increasing plant size and EFN number; however, rhizobia still marginally tended to increase arthropod density after included these traits as covariates (Table S5.2) and these rhizobia effects were even stronger for larger plants (Rhizobia $\times$ Aboveground Biomass: $\chi^2=7.34$, $P<0.01$). Additionally, increased nodule number was positively correlated with arthropod density, even when accounting for variability in plant size ($\chi^2=3.99$, $P<0.05$). While we excluded aphids from initial analyses due to large disparities in densities between aphids and other arthropods, rhizobia also increased aphid abundance ($\chi^2=6.37$, $P=0.012$, Table 5.2, Figure 5.3B). Although we observed some aphid tending by ants, it does not appear that ants are tending aphids enough to lead to increased aphid abundances in the presence of ants ($P>0.1$). However, there is a trend to higher aphid abundance on plants with increasing ant abundance ($r=0.34$, $P=0.052$).

Discussion

Multiple symbionts associating with *Chamaecrista fasciculata* have very different fitness effects on the host. As predicted, nitrogen-fixing rhizobia provided a substantial fitness benefit to their host. Surprisingly, however, ant visitation reduced plant fitness. Ants negated the fitness gains provided by rhizobium mutualists, with the fitness of plants possessing both mutualists not differing significantly from plants lacking rhizobia. Because ants reduced nodule number, competition for carbon rewards between rhizobia and ants is likely contributing to the lack of

Figure 5.3. Treatment effects of rhizobia and ants on A) arthropod and B) aphid abundances (means $\pm$ SE). Different letters indicate statistical significance at $P < 0.05$ based on post-hoc pairwise contrasts after adjustment for multiple comparisons.



rhizobia fitness benefit in the presence of ants. While nectar-tending ants are typically considered to be mutualistic, this study demonstrates a substantial fitness cost to supporting ants despite ecological benefits of reduced arthropod load. Moreover since ants reduced nodule number, competition for carbon rewards between rhizobia and ants mediated through extrafloral nectar production is likely contributing to the lack of rhizobia fitness benefit in the presence of ants. In short, hosts supporting multiple mutualistic interactions may experience variation in fitness effects depending on the presence or abundance of these multiple interacting species.

Interactions between mutualists

When engaging in multiple mutualisms, host plants may experience trade-offs due to limited resource allocation to partner species. For example, higher investment to foliar endophytes by *Lolium multiflorum* led to less root colonization by arbuscular mycorrhizae fungi (Omacini et al. 2006). Similarly, we find that plants produced more rhizobia-housing nodules in the absence of ants. Ant presence increases nectar production through source-sink dynamics and the jasmonic acid induced pathway (Bixenmann et al. 2011, Heil 2011), potentially decreasing available carbon to allocate to rhizobia. Interestingly, our mutualists had asymmetric effects on each other. Although ants reduced allocation to rhizobia, rhizobia increased ant abundance, likely because rhizobia altered traits potentially influencing plant attractiveness to ants. Specifically rhizobia increased extrafloral nectary number and plant size which provide resources and habitat for increased ant abundances and also increased the density of arthropod prey. Rhizobia also may influence EFN production and plant protein content (Godschalx et al. 2015). While we observed increasing ant numbers with increasing plant size, rhizobia significantly increased ant abundance even after controlling for size, suggesting that rhizobium

effects may be mediated by other plant traits such as nectar quality. Our results differ from a recent study by Godschalx et al. (2015) exploring how rhizobia affect the chemical composition of *Vicia fabia* (lima bean) plants and nectar as well as ant visitation. They demonstrate that rhizobia increased the protein content of the plant but decreased EFN production and ant visitation. However, lima bean plants associating with rhizobia also produced higher concentrations of cyanogenic compounds (HCNp) which serve as an alternative form of plant defense that may deter herbivores and prevent EFN production from being induced in this facultative ant-plant mutualisms (Godschalx et al. 2015). Our differences may be a result of the lack of alternative forms of chemical defenses but increased need for EFN-mediated defense from higher tissue quality in the presence of rhizobia.

Effects of mutualists on plant fitness

While rhizobia significantly increased plant fitness, ants negated these fitness benefits from rhizobia. These symbionts effectively counteract each other, such that plant fitness in the presence of both ants and rhizobia does not differ significantly from uninoculated plants growing in the absence of ants. The fitness benefit from rhizobia could be counteracted by carbon costs of supporting ants and reduced allocation to rhizobia when ants are present. Trade-offs in host benefits from allocated carbon and asymmetric interactions between the mutualists may drive complex interactions between multiple mutualists associating with host species, emphasizing the need to continue to move beyond pairwise studies of host-symbiont interactions. In particular, the benefits of ants might be higher in presence of rhizobia due to higher ant abundances, and potentially a greater need for more ants due to greater palatability to herbivores from higher tissue quality (Katayama et al. 2010, Dean et al. 2014). Asymmetric effects of the mutualists on

each other as a result of *C. fasciculata* carbon limitation could further create interactive effects on plant fitness. For example, although rhizobia increased ant abundance, ants decreased allocation to rhizobia. Yet in this system rhizobia are likely providing a greater relative fitness benefit per carbon allocated than ants since rhizobia dramatically increased plant fitness but ants provided no fitness benefit and even reduced fitness.

Although greater ant attraction is typically considered beneficial for the plant, we find that ants are parasitic on *C. fasciculata* in this experiment, despite significantly reducing arthropod density. While surprising and counter-intuitive for a relationship commonly considering mutualistic, the lack of fitness benefits from ants is consistent with previous findings of less benefits with: facultative relationships; plants tended by multiple ant species; and with EFN bearing plants. Facultative relationships often comprise plants that may sometimes benefit from ant presence, but are not dependent on ants for survival and reproduction (Rico-Gray and Oliveira 2007, Webber et al. 2007). Moreover, there is often looser facultative associations for EFN based ant-plant symbioses than those producing domatia and/ or food bodies (Rico-Gray and Oliveira 2007). These loose associations provide greater opportunity for cheating by ant species. Free-loader ants also have been observed in other systems, such as *Acacia drepanolobium*, *Acacia hindsii*, *Cordia nodosa*, and *Duroia hisuta*, among others (Clement et al. 2008, Frederickson and Gordon 2009, Palmer et al. 2010). As previously mentioned, these negative effects may be due to reduced carbon allocation to rhizobia when resources are being allocated to EFN, although ants also failed to benefit plant growth or fitness even in the absence of rhizobia. Some plants species reduce EFN allocation in absence of herbivores (Mondor et al. 2006) or ants (Bixenmann et al. 2011). In our system, in the absence of ants, nectar tended to dry up in the field by the end of August despite continued herbivore pressure, while plants in the

presence of ants continued to produce nectar until harvest in October (Keller et al. pers. obs.). Although our results align with another *C. fasciculata* study that demonstrated ants reducing herbivore load but not increasing plant fitness (Kelly 1986), other studies on a variety of ant taxa have found *C. fasciculata* fitness benefits from ants (Barton 1986, Rutter and Rausher 2004). Moreover, ants may not have been entirely negative in this system; ants increased contamination of non-inoculated plants. Therefore, in habitats where rhizobia are limiting or spatially heterogeneous, ants may provide fitness benefits by increasing the likelihood that a plant contacts compatible rhizobia beyond the root growth zone. This would also be beneficial for the ants since they may benefit from increased resources associated with rhizobia inoculated plants.

Multi-mutualist effects on higher trophic levels

With increasing recognition of the importance of multiple mutualist interactions to ecological and evolutionary responses of hosts their environment (e.g. Chamberlain and Rudgers 2011, Afkhami et al. 2014), future research should continue to explore how variation in multiple mutualistic interactions may affect communities across trophic levels. For example, for host species associating multiple different mutualists, each of which may alter aspects of the abiotic and biotic environment, differential allocation to partners can drive variation in community patterns (Miller and Hay 1996). Here, we find that rhizobia increased arthropod density only when ants were absent. Ants reduced the increased arthropod densities on rhizobia inoculated plants to levels observed on uninoculated plants. The heavy arthropod load and aphid population densities associated with rhizobia inoculation indicate that they are preferentially preying upon inoculated plants. Non-ant arthropods may also be using the excess nectar available on plants with ants excluded, as herbivores were occasionally observed feeding at nectaries. Interestingly,

since we find reduced arthropod pressure in the presence of ants, this indicates that the ecological benefits of ants are not resulting in a fitness benefit in this system.

Conclusions

Associating with multiple types of mutualists that provide very different benefits may provide protection against numerous plant stressors (Afkhani et al. 2014). Perhaps plants harboring multiple mutualists may differentially benefit from each of these symbionts in a mosaic of interaction outcomes across the species range and even temporally within a site (Bronstein 2009). At our site, we find evidence for trade-offs in carbon allocation between rhizobia and ants, where ants reduced nodulation. Interestingly, these effects were assymetric, and the presence of rhizobia promoted higher ant abundances. These conflicts between mutualists affect plant fitness; although rhizobia increased plant fitness, ants negated the fitness benefits provided by rhizobia despite reducing the abundance of potential herbivores. In our study, abiotic resource limitation was likely stronger than herbivory, resulting in greater fitness benefits from rhizobia than ants.

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APPENDIX

Table S5.1. ANCOVA tables of rhizobia and ant treatment effects with aboveground biomass or extrafloral nectary number as covariates on ant abundance and plant fitness (seeds and pods).

	<i>df</i>	<i>F</i>	<i>P-value</i>
Ant abundance			
Rhizobia	1, 29	0.00	0.99
Aboveground Biomass	1, 29	9.42	0.005
Rhizobia $\times$ Abovegr. Biomass	1, 29	3.11	0.089
Rhizobia	1, 29	0.002	0.965
EFN	1, 29	4.39	0.044
Rhizobia $\times$ EFN	1, 29	0.31	0.583
Plant Fitness (Seeds)			
Rhizobia	1, 61	2.85	0.097
Ants	1, 61	7.00	0.010
Aboveground Biomass	1, 61	77.25	<0.0001
Rhizobia $\times$ Ants	1, 61	0.45	0.507
Rhizobia $\times$ Abovegr. Biomass	1, 61	2.80	0.099
Ants $\times$ Abovegr. Biomass	1, 61	3.81	0.055
Rhizobia $\times$ Ants $\times$ Abovegr.	1, 61	1.84	0.180
Plant Fitness (Pods)			
Rhizobia	1, 61	7.85	0.007
Ants	1, 61	11.69	0.001
Aboveground Biomass	1, 61	109.12	<0.0001
Rhizobia $\times$ Ants	1, 61	0.17	0.684
Rhizobia $\times$ Abovegr. Biomass	1, 61	5.42	0.023
Ants $\times$ Abovegr. Biomass	1, 61	6.42	0.134
Rhizobia $\times$ Ants $\times$ Abovegr.	1, 61	1.04	0.312

Table S5.2. Likelihood ratio tests of the effects of rhizobia and ant treatments on arthropod number. Aboveground biomass and extrafloral nectar number as included as covariates.

	χ^2	<i>df</i>	<i>P-value</i>
Arthropod Abundance			
Rhizobia	12.23	1	0.0005
Ants	3.57	1	<i>0.059</i>
Aboveground Biomass	15.88	1	<0.0001
Rhizobia $\times$ Ants	0.12	1	0.725
Rhizobia $\times$ Abovegr. Biomass	6.80	1	0.0091
Ants $\times$ Abovegr. Biomass	0.01	1	0.924
Rhizobia $\times$ Ants $\times$ Abovegr.	0.94	1	0.331
Ants $\times$ EFN	0.10	1	0.748
Rhizobia $\times$ Ants $\times$ EFN	0.49	1	0.485

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