THE ROLE OF RESOURCE MUTUALISM IN PLANT RESPONSE AND ADAPTATION TO ABIOTIC ENVIRONMENTS

By

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ABSTRACT

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Symbiotic interactions between microbes and plants are ubiquitous in nature. These symbioses can facilitate a plant's ability to tolerate biotic and abiotic stress. For example, resource mutualists, such as arbuscular mycorrhizal fungi and nitrogen-fixing bacteria can not only aid nutrient acquisition but also confer tolerance to drought and pH stress. Using an annual legume, *Amphicarpaea bracteata,* and nitrogen-fixing bacteria, *Bradyrhizobium* sp., as a model system, I investigated whether symbiotic microbes mediate plant fitness responses and adaptation to abiotic stressors, including soil moisture, limited light availability, and nitrogen limitations.

First, using a large reciprocal transplant experiment, I demonstrated that soil moisture is likely an important selective agent driving plant adaptation. Additionally, I found that symbiotic rhizobia influence patterns of plant adaption to soil moisture. Given the intimate relationship between plants and symbiotic microbes, such as mycorrhizae, endophytes and rhizobia, such patterns may be prevalent in nature. My results also highlight the importance of examining both biotic and abiotic factors in adaptation studies.

Second, because rhizobia are notoriously difficult to manipulate in the field, and to further identify soil moisture as the selective agent driving plant local adaptation, I conducted a multi-factorial greenhouse experiment manipulating soil moisture, plant genotype and rhizobia genotype (both collected from the same wet or dry sites). While I found weak evidence of plant

adaptation to soil moisture, I found that rhizobia performance was strongly affected by the match between rhizobium origin and plant origin (wet or dry sites), suggesting that plant divergence across wet and dry sites results in traits that differentially benefit rhizobium genotypes isolated from wet versus dry sites.

Finally I tested for plant population variation in plant response to other key selective agents on the legume-rhizobium mutualism (light and nitrogen availability). I found that plants and rhizobia responded differently to changes in resource availability. Symbiosis was most beneficial for rhizobia under high light and low nitrogen conditions, as predicted by resource mutualisms theory. For plants, however, symbiosis was beneficial in low nitrogen treatments regardless of light conditions. These asymmetric effects of both traded resources are, in part, driven by plants' ability to control nodulation under unfavorable conditions.

To my partner, Anna K. Jonsson.

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CHAPTER 1:

INTRODUCTION

Background

Plant populations are likely to face novel environmental stressors as a result of climate change and other anthropogenic disturbances. Plants can potentially overcome these environmental stresses by: 1) dispersing to less stressful habitats (e.g. Howe and Miriti 2004, Cousens et al. 2008); 2) adaptation (e.g. Wright et al. 2006, Franks et al. 2007); or 3) associating with other organisms (e.g. Clay et al. 1985, Johnson 1993, Al-Karaki et al. 2004, Kannadan and Rudgers 2008), such as microbes, that increase their stress tolerance. For example, symbiotic rhizobia occupy root nodules of leguminous plants and provide plants with fixed atmospheric nitrogen; in return, plants provide photosynthetic carbon to rhizobia. If nitrogen becomes more difficult to access in stressful environments, rhizobia may facilitate tolerance and plant adaptation to abiotic stress by providing nitrogen directly to plants. Further, because of their short generation times, genetic diversity and dispersal ability, rhizobia may evolve rapidly to potentially ameliorate the negative fitness consequences of environmental stress for their plant hosts (Rodriguez and Redman 2008, Friesen et al. 2011)

Using an annual native legume, *Amphicarpaea bracteata,* and nitrogen-fixing bacteria, *Bradyrhizobium* sp, as a model system, my dissertation research investigated whether symbiotic microbes mediate plant fitness responses to soil moisture, light limitation (which limits carbon availability), and nitrogen (N) limitation. I explored two main questions: 1) How do resource mutualists influence plant adaptation to soil moisture (Chapters 2-3), and 2) How does the symbiosis between plants and rhizobia respond to changes in the availability of both traded

resources, C and N (Chapter 4)? Addressing the first question helps to expand our understanding of the mechanisms contributing to plant adaptation and how species interactions influence adaptation. Given the intimate relationships between plants and symbiotic microbes, it is likely that symbiotic microbes play a crucial role in plant adaptation. Surprisingly, this is an understudied topic in evolutionary biology, although the ecological importance of symbiotic microbes has been widely appreciated for a decades (e.g. Johnson 1993, Schwartz and Hoeksema 1998, Heath 2010).

While soil moisture is a well-characterized stressor to both plants and soil microbes, there are other potential key abiotic factors that can alter plant-rhizobia mutualism. Thus, my second question aimed to investigate how the symbiosis between plants and rhizobia respond to changes in the availability of both traded resources, C and N. Plant control of resource allocation to rhizobia under unfavorable conditions may contribute to the observed stability of this mutualism between plants and rhizobia for over 60 million years.

Together, my dissertation research expands our understanding of how rhizobia influence plants responses to various abiotic stressors in natural systems at both ecological and evolutionary levels. This information may ultimately help us predict changes in abundance and distribution of legume species in response to novel environmental stressors in natural systems.

Organization of the Dissertation

Chapter 2: To examine whether *A. bracteata* is adapted to soil moisture, I conducted a reciprocal transplant experiment at three sites in Southwest Michigan. Each site included a population inhabiting a wet environment and a population inhabiting a dry environment. The use

of three locales with paired wet and dry sites allowed me to not only test for adaptation, but also identify whether soil moisture is a key agent of differential selection. I found some evidence for adaptation to soil moisture conditions. In wet sites, plants originating from the wet sites had significantly higher fitness than plants originating from dry sites, suggesting adaptation to wet soil moisture conditions. However, in dry sites, plants originating from dry and wet sites performed similarly. I also found that that the proportion of nodulating plants depended on both source and destination soil moisture type. In wet destination sites, plants originating from wet sites were more likely to produce nodules than plants originating from dry sites. In the dry destination sites however, plants originating from wet and dry sites were equally likely to nodulate. These differences in nodulation paralleled the observed fitness responses. In sum, this study suggests that soil moisture is an important selective agent driving plant adaptation and that symbiotic rhizobia likely contribute to adaption to soil moisture.

Chapter 3: My reciprocal transplant experiment (Chapter 2) tested whether plants were adapted to soil moisture in the field, but a manipulative experiment is necessary to definitively examine whether soil moisture is the key selective agent and whether microbial symbionts influence patterns of adaptation. In collaboration with Dr. Jennifer Lau, I conducted two greenhouse experiments to examine 1) how symbiotic rhizobia influence plant growth and fitness responses to a soil moisture gradient; and 2) how symbiotic rhizobia and different genotypes of rhizobia affect plant adaptation to soil moisture.

In the first experiment, we found that rhizobia were beneficial only within a range of moisture conditions (20ml and 40ml water addition); rhizobia did not increase plant growth in the lowest and highest soil moisture treatments. In the second experiment, we manipulated soil moisture conditions, plant populations and rhizobia populations. Contrary to our predictions, we

found limited evidence that plants were adapted to soil moisture or that rhizobia adaptation to soil moisture conditions or host plant populations influences plant fitness. Instead, we found that rhizobia populations are adapted to plants originating from the same soil moisture type.

Chapter 4: The final chapter of my dissertation tests for plant population variation in response to other key selective agents on the legume-rhizobium mutualism. In collaboration with Dr. Jennifer Lau, I examine the hypothesis that symbiotic function is governed by the relative availability of carbon (light) and nitrogen. Using *A. bracteata* and rhizobia as a model system, we conducted a greenhouse experiment manipulating nitrogen and light availability. We found that plants and rhizobia responded differently to changes in resource availability. Symbiosis was most beneficial for rhizobia under high light and low N conditions, as predicted by a descriptive model (Johnson et al. 1997). For plants, however, symbiosis was beneficial in low N treatments regardless of light conditions. These asymmetric effects of both traded resources are, in part, driven by plants' ability to control nodulation under unfavorable conditions. Reduced allocation to rhizobia by plants under less beneficial conditions may contribute to the maintenance of plant-rhizobia symbiosis for over 60 million years.

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CHAPTER 2:

THE ROLE OF RESOURCE MUTUALISM IN PLANT ADAPTATION TO ABIOTIC ENVIRONMENTS

INTRODUCTION

The study of local adaptation has captured the attention of evolutionary biologists for decades (e.g. Darwin 1859, Clausen et al. 1940, Dobzhansky 1950), in part because local adaptation is recognized as a key mechanism maintaining genetic variation (Hedrick et al. 1976, Hedrick 1986, Wade and Kalisz 1990) and may ultimately drive speciation (Schluter 2001, Turelli et al. 2001, Via 2001). However, few studies identify the environmental selective pressures that drive local adaptation (selective agents). Identifying these factors is a crucial step to understand the causes of natural selection (Wade and Kalisz 1990).

One of the major challenges in identifying agents of selection in nature is that organisms typically live in complex communities where they cope with many abiotic stressors and interact with multiple species (Nuismer and Gandon 2008). These biotic and abiotic factors can interact to influence plant fitness. For example, herbivores have been shown to increase the negative effects of drought, light and nutrient stress on plants (Hawkes and Sullivan 2001). Thus, the effects of abiotic stressors on plant fitness and potentially the evolution of plant traits may depend on other species in the community.

Recent work has revealed that microbial symbionts play vital roles in plant communities (Reviewed in van der Heijden et al. 2008, Bever et al. 2010), and there is increasing evidence that microbial symbionts can facilitate host tolerance to abiotic stress (reviewed by Rodriguez and Redman 2008, Friesen et al. 2011). For example, endophytes have been shown to reduce herbivory and drought stress (Clay et al. 1985, Kannadan and Rudgers 2008). Algal symbionts

not only provide nutrients to host corals but also play a critical role in thermal tolerance (Jones et al. 2008, Correa and Baker 2011). Resource mutualists, such as arbuscular mycorrhizal fungi and nitrogen-fixing bacteria can aid nutrient acquisition, especially in low nutrient environments (Johnson 1993, Schwartz and Hoeksema 1998, Leidi and Rodriguez-Navarro 2008, Heath et al. 2010) and can also help mitigate the effects of drought and low pH (Goicoechea et al. 1997, Clark et al. 1999, Al-Karaki et al. 2004). Given the key roles microbes play in mediating host responses to potential selective agents, variation in how hosts interact with microbial symbionts may influence patterns of adaptation.

Symbiotic microbes can influence patterns of adaptation mainly in two ways. First, interactions with microbes may alter the strength and direction of selection acting on their plant hosts, altering patterns of plant adaptation. Second, microbes may adapt to habitat-specific stress. If these microbes are mutualists, then microbial adaptation may increase plant fitness and/or help confer stress tolerance to any host plant in that habitat. In this scenario, symbionts evolve tolerance and can ameliorate stress for their plant hosts, but the plant hosts themselves are not necessarily adapted to a stress (Rodriguez et al. 2008).

Here I examined the hypothesis that plant adaptation is driven in part by interactions with rhizobium symbionts (Rodriguez and Redman 2008, Friesen et al. 2011). Symbiotic rhizobia occupy root nodules of leguminous plants where they convert atmospheric nitrogen (N_2) to ammonia (NH_3), making it available to host plants. In return, plants provide photosynthetic carbon (C) to rhizobia (Denison and Kiers 2004, Bottomley and Myrold 2007).

Soil moisture is a well-characterized agent of selection on many physiological and morphological plant traits (Dudley 1996, Sherrard et al. 2009), and rhizobia can reduce the negative effects of drought on plant fitness (Athar and Johnson 1996, Goicoechea et al. 1997,

Serraj et al. 1999, Aroca and Ruiz-Lozano 2009), although in some systems, rhizobia have minimal effects on plant drought response or even increase the negative effects of drought (reviewed by Serraj et al. 1999). For example, drought reduced *Medicago sativa* biomass by 72% in the absence of rhizobia but by 45% when plants were inoculated with rhizobia (Goicoechea et al. 1997). Further, rhizobia may also increase plant tolerance to saturated soil moisture environments because plants can have difficulty accessing N directly from wet soils as N mineralization decreases (Schuur and Matson 2001, Sleutel et al. 2008). My prior work showed that rhizobia reduced the negative fitness effects of both wet and dry conditions on plant growth (Chapter 3). These studies suggest that rhizobia may mitigate the negative fitness effects of both dry and wet soil moisture conditions on plants. As a result, genetic changes in rhizobium populations (Raverkar et al. 2005, Romdhane et al. 2009, Zilli et al. 2013) or genetic variation in how plants interact with rhizobia may influence plant fitness responses to soil moisture.

In this study, I first tested whether plants originated from dry or wet environments (plant source site) or where plants are transplanted (destination site) influence nodulation. Second, I tested whether plants are adapted to soil moisture conditions or are locally adapted to their home sites. If plant populations transplanted into their "home" soil moisture type have greater fitness than populations from the contrasting soil moisture type, it suggests that soil moisture is a selective agent driving adaptation. If plant populations transplanted back into their home sites have greater fitness than plants originating from “foreign” sites, it suggests that plants are locally adapted (Kawecki and Ebert 2004). Finally, I also investigated whether interactions with rhizobia influence patterns of plant adaptation to soil moisture. If patterns of plant adaptation depend on whether plants associate with rhizobia, it suggests that rhizobia play an important role in plant adaptation.

To address these questions, I conducted a replicated reciprocal transplant (Kawecki and Ebert 2004, Wright et al. 2006) between populations that differ in soil moisture conditions. Including multiple populations from each habitat type allows me to differentiate selection caused by the identified environmental variable (in this case, soil moisture or factors tightly correlated with soil moisture) from selection caused by idiosyncratic environmental variation among populations (Kawecki and Ebert 2004, Wright et al. 2006, Blanquart et al. 2013).

METHODS

Natural history

The annual native legume *Amphicarpaea bracteata* (hog peanut) forms a symbiotic relationship with nitrogen-fixing bacteria *Bradyrhizobium sp* (hereafter referred to as rhizobia). *A. bracteata* has a mixed mating system: it produces both chasmogamous and cleistogamous aboveground flowers (hereafter referred as aerial seeds) and belowground cleistogamous flowers (hereafter referred as subterranean seeds). Subterranean seeds are four to six times larger than aerial seeds, account for more than 90% (and often >99%) seedling recruitment (Parker 1991) and have restricted dispersal (< 2m; Trapp, 1988). Aerial seeds have lower germination rates than subterranean seeds but are tolerant to harsh environments and potentially have higher dispersal than subterranean seeds (Schnee and Waller 1986). The number of aerial flowers is positively correlated with aboveground plant size (Schnee and Waller 1986, Callahan and Waller 2000). To minimize maternal effects, seeds used in this experiment were the offspring of greenhouse-reared plants originating from aerial seeds collected between 2012 and 2013 from the six local sites described below.

Field sites

I studied three locales in southwest Michigan (Brook Lodge, Pierce Cedar Creek and Carter Lake, MI, 26 - 45 km apart) where *A. bracteata* naturally occurs. Each locale contains two sites, one in a "wet" and one in a "dry" habitat (6 sites total, 0.5 - 5km apart) (Supplementary Material Table 2.A1). Wet sites are sedge meadows with on average 45.45±3.90% (May – July 2013) and 48.3±0.95% (July – August 2014) volumetric water content (VWC, measured using HydroSence II, Campbell Scientific Inc., North Logan, Utah). Dry sites are forest understory with 13.43±2.73% (May- July 2013) and 11.8±2.47% (July-August 2014) VWC on average. Wet and dry sites had on average 8.24±1.96 mg N/g soil and 4.61±1.02 mg N/g soil respectively (averaged across 2012 and 2014). VWC significantly differed between wet and dry sites across two years ($t_{33.4}$ = 14.12, $P < 0.001$), while total soil N did not ($t_{7.5}$ = 1.64, P = 0.14, 2012 and 2014 data combined). Previous studies showed three distinct *A. bracteata* lineages (Wilkinson et al. 1996, Parker 1996, Parker et al. 2004). Our *A. bracteata* populations are genetically differentiated from each other and do not appear to be grouped by habitat type or locale, based on restriction site associated DNA (RAD) data (Supplementary Material Figure 2.A1).

Experimental design and treatments:

I conducted a replicated reciprocal transplant experiment, where I transplanted seedlings from each site back into their home site and also into replicated dry and wet sites. I transplanted six seedlings from each of 15 full-sib families from each of the six source sites into each of six destination sites (6 source sites x 6 destination sites x 15 families x 6 replicates; N=3240 seedlings total). However, some families had fewer replicates because of seed limitation, so seeds were supplemented with different families from the same source site to maintain equal

sample sizes across all source sites (See Supplementary Material Table 2.A2 for the full replication table). The experiment ran for a total of 20 weeks.

Seeds were physically scarified by nicking the seed coat between April 30-May 5 2014 and germinated in petri dishes with wet filter paper in the dark for approximately 7 days. Between 12-15 May 2014, germinated seeds were transplanted into randomly selected cells (14 cm^3) in 200 cell plug trays (filled with sterilized potting media (Sunshine Mix LP5®). If the seeds failed to emerge within one week, they were replaced by newly germinated seeds of the same family. Seedlings were grown in a common garden greenhouse condition for *ca*. 20 days. At this point, most of the plants had developed at least one true leaf.

On 22 May 2014, each of the six destination sites was sprayed with herbicide (Roundup® 2% glyphosate in water; Monsanto, St. Louis, MO) to minimize vegetation cover. Although the vegetation cover grew back within a month, this step was necessary to help identify experimental plants early in the experiment. In each destination site, I constructed three 4.5 m x 2.5 m plots, surrounded by deer-exclusion fences (1.5 m height), and transplanted seedlings originating from each of the six source sites into randomly selected locations within each destination site (180 plants per plot, 20cm spacing between plants, Supplemental Material Table2.A2). I recorded survival monthly between May – August 2014; however, plant survival was very high throughout the growing season (Mean ± SE: 93% ±0.02), so I did not include it in the analysis. To estimate plant fitness, I harvested aboveground biomass and counted subterranean seeds on a subset (N=1620) of surviving plants because digging up subterranean seeds of all experimental plants was infeasible within a reasonable timeframe (9 – 29 September 2014). To assess whether each plant formed successful associations with rhizobia, presence of root nodules was also recorded for the subsampled plants. I was unable to record total number of nodules of each plant

because it was logistically infeasible to excavate plants without losing nodules in the field. While I recognize that this is a course measure of nodulation, the results were consistent with results from a similar greenhouse experiment where I counted the number of nodules (Chapter 3).

Statistical analysis:

To test whether plants originating from wet or dry soil moisture sources (source population type) differ in likelihood of nodulation (presence/absence) when planted under wet or dry destination soil moisture types (destination type), I performed a logistic regression using generalized linear mixed model (GLIMMIX procedure) in SAS (SAS Institute Inc. 2011. SAS 9.3). Source population type, destination type, and their interaction were included as fixed factors. Plant family nested within source population, source population nested within source population type, destination site nested within destination type, and interactions between destination site and source population were included as random factors. When interactions were statistically significant, I performed Tukey's honestly significant difference test.

I tested whether plants are adapted to soil moisture conditions, whether interactions with rhizobia influence plant fitness responses to soil moisture. and whether plants are locally adapted to their "home" sites or home site soil moisture types with generalized linear model analysis (GLIMMIX procedure) in SAS (SAS Institute Inc. 2011. SAS 9.3). I used number of subterranean seeds as an estimate of fitness. A negative binomial distribution was used to fit the distribution of the subterranean seed numbers. The model included destination site type (wet or dry), source population type (wet or dry), nodulation (presence or absence of nodules) and their interactions as fixed factors. Destination site nested within destination type, source population nested within source population type, and family nested within source population were included

as random factors. When interaction effects were significant, I performed Tukey's honestly significant difference test.

A significant destination moisture type x source population moisture type interaction, such that plants have higher fitness than plants from the other habitat when grown under similar soil moisture conditions as their "home" soil moisture type, provides evidence that *A. bracteata* is adapted to soil moisture conditions. A significant destination moisture type x source moisture type x nodulation interaction provides evidence that interactions with rhizobia influence patterns of plant adaptation to moisture conditions and potentially that genetic variation among plant populations in how they interact with rhizobia contributes to plant adaptation to moisture conditions. Finally, a significant destination site x source site interaction, such that populations planted in their home destination sites outperform populations originating from other source sites shows evidence for local adaptation to local site conditions above and beyond any adaptation to soil moisture.

RESULTS

Does plant source or destination soil moisture type influence nodulation?

Nodulation (proportion of plants producing nodules) depended on both source population type and destination soil moisture (destination type) (significant destination type x source type interaction in Table 2.1 and Figure 2.1). In wet destination sites, genotypes originating from the wet sites were more likely to nodulate than genotypes originating from dry sites (Tukey HSD test $P < 0.001$). Although the proportion of nodulating plants originating from dry sites was greater in dry than wet destination sites, in dry destination sites, plants originating from wet and dry sites were equally likely to nodulate in dry environments (Figure 2.1, Tukey HSD test $P = 0.95$).

Are plants adapted to soil moisture conditions and/or locally adapted to their "home" site?

Adaptation to Soil Moisture: I detected evidence for adaptation to soil moisture conditions (significant destination type x source type interaction in Table 2.2); plants originating from wet sites produced significantly more subterranean and aerial seeds than plants originating from dry sites in wet destination environments, and plants originating from dry sites produced significantly more (Figure 2.2, Figure 2.A3). Note that number of aerial seeds is not presented here because only 12.2% of surviving plants produced aerial seeds and aerial seeds likely contribute little to population growth. However, the pattern showed a classic local adaptation where dry-originating plants had higher fitness than wet-originating plants in dry sites and wet-originating plants had higher fitness than dry-originating plants in wet sites. (see Supplementary Material Figures 2.A2 and 2.A3, 2.A4C &D). Nodulation influenced plant fitness differently in wet vs. dry destination sites and for plants originating from wet vs. dry source sites (significant nodulation x destination type and nodulation x source type interactions in Table 2.2). Although the destination type x source type x nodulation interaction was not statistically significant (Table 2.2), in wet destination sites, nodulating plants originating from wet sites produced more subterranean seeds than nodulating plants originating from the dry sites (Figure 2.2 Tukey HSD test $P < 0.001$). In these wet environments, non-nodulating plants produced fewer seeds than nodulating plants (non-nodulating 0.61±0.08, nodulating 1.97±0.11; Tukey HSD test $P < 0.001$) and non-nodulating plants from wet sites produced a similar number of subterranean seeds as plants from dry sites (Figure 2.2 Tukey HSD test $P = 0.557$). Together, these results suggest that patterns of adaptation to wet environments are only observed when wet-adapted plants actively associate with rhizobium symbionts. In contrast, in dry destination sites, I found evidence of

adaptation to dry soil conditions only for non-nodulating plants (Figure 2.2B; Tukey HSD test P = 0.05). Nodulated plants originating from wet and dry sites produced a similar number of subterranean seeds (Figure 2.2, Tukey HSD test P = 1) and fewer seeds than non-nodulating plants from dry source populations (Figure 2.2). Thus, in dry environments nodulation does not yield increased plant fitness, but in wet environments nodulation increases plant fitness, particularly for plants originating from wet sites (Figure 2.1 &2.2).

Local Adaptation to home site: Although plant fitness varied among destination sites and source sites (significant destination x source interaction Table 2.2), plants that were transplanted back into their "home" site did not have significantly higher fitness than plants originating from "foreign" sites (Figure 2.3). Similarly, although the proportion of nodulating plants differed among destination sites and I detected a marginally significant destination site x source site interaction, plants were no more likely to nodulate in their home sites (Table 2.1, Figure 2.3).

DISCUSSION

Environmental Variation and Fitness Benefits of Mutualism

I found that nodulation depended on the interaction between plant source population and destination environmental conditions. In wet destination sites, plant genotypes originating from wet sites were more likely to produce nodules than genotypes originating from dry sites. However, in dry destination sites, plants originating from wet and dry sites were equally likely to nodulate. All of the wet sites in this experiment were located adjacent to a wetland or stream and the soil moisture content was very high (> 45% VWC). Under such conditions, decomposition and N mineralization decrease due to oxygen limitation to soil microbes, often making soil N less easily accessible to plants (Stanford and Epstein 1974, Schuur and Matson 2001, Sleutel et

al. 2008). In such environments, plants may be more likely to nodulate because interactions with rhizobia may be needed to ameliorate low N availability. Interestingly, only plants originating from wet environments increased nodulation in wet destination sites and benefitted from interacting from the naturally occurring rhizobia found in those sites (see below).

Are plants locally adapted to their home site and/or adapted to soil moisture conditions?

While many studies have documented local adaptation (e.g. Clausen et al. 1941, Antonovics et al. 1971, Ågren and Schemske 2012, Chen and Schemske 2015), the key selective agents driving local adaptation often remain unidentified. This is in part because in a community, both biotic and abiotic factors influence plant fitness simultaneously and disentangling multiple factors to identify putative selective agent(s) is very challenging.

The strong source population type x destination type interactions detected here suggest that either a) soil moisture is indeed a key selective agent contributing to patterns of adaptation in this system or b) other unmeasured factors correlated with soil moisture are selective agents. Factors such as light availability, temperature, herbivory, and competition often correlate with soil moisture (Linhart and Grant 1996). Regardless of the specific environmental variable responsible, these gross differences between dry and wet environments appear to be the key drivers of plant adaptation, given that I found no evidence for local adaptation to specific local sites (Figure 2.3). Similarly, a study of *Collinsia sparsiflora* adaptation to serpentine soil showed that although plants were adapted to serpentine soil, they were not adapted to specific home site conditions (Wright et al. 2006). In these examples, extremely different environmental conditions drive population differentiation, with little differentiation between sites within a given habitat type (wet or dry soil moisture here; serpentine or non-serpentine in Wright et al 2006).

Local adaptation is expected when natural selection differs across sites. Here, selection likely differs across wet or dry sites, but likely is very similar between sites within the same soil moisture type. Such findings may be common--a recent meta-analysis of plant local adaptation studies revealed that only 45.3 % of comparisons showed evidence of local adaptation, suggesting that local adaptation is less frequent than commonly assumed (Leimu and Fischer 2008). Lack of local adaptation to specific home site conditions (above and beyond soil moisture) could be attributed to several factors including small population size (Leimu and Fischer 2008), presence of locally adapted natural enemies such as herbivores and pathogens (Thompson et al. 2002, Schweitzer et al. 2014), temporal environmental variation (Stearns 1992), and minimal differences in selection pressures between sites.

Do interactions with rhizobia influence patterns of plant adaptation to soil moisture?

Here I found that association with mutualistic rhizobia and the fitness effects of associating with rhizobia depended on both the plant population source type (wet or dry) and the destination soil moisture type and appeared to influence plant fitness responses to soil moisture. In particular plant genotypes from wet sources were more likely to nodulate and had higher fitness in wet soil moisture conditions. In contrast, in dry sites, plant genotypes from dry and wet sources were equally likely to nodulate and forming successful associations with rhizobia actually reduced fitness in these environments. These results are consistent with previous studies showing that microbial symbionts may influence patterns of plant adaptation to abiotic stressors (Heath et al. 2010, Friesen et al. 2011, Lau and Lennon 2011, 2012, Porter et al. 2011), in some cases, by increasing nitrogen availability to plants through nitrogen fixation (Chapter 4 Figure 4.3)

Interestingly, in wet destination sites, plants originating from dry sites were significantly less likely to nodulate than plants from wet sites (Figure 2.1). One hypothesis explaining differential nodulation is that matching of evolutionary history of both plants and rhizobia increases fitness of both parties (Heath and Tiffin 2007). Given that complex chemical interactions take place between plants and rhizobia during the formation of symbiosis (Perret et al. 2000, Bottomley and Myrold 2007), plants originating from dry sites may not form symbioses with rhizobia originating from wet sites as effectively as source-matching plants and rhizobia.

In contrast to wet soil moisture conditions, forming successful associations with rhizobia did not increase plant fitness in dry destination sites, and dry-originating plants did not have greater subterranean seed numbers than wet-originating plants when nodulated. However, non-nodulated dry-originating plants outperformed non-nodulated wet-originating plants. Given that nodulating plants tended to produce fewer subterranean seeds in dry sites than non-nodulating plants (Supplementary Material Figure 2.A4 A&B), rhizobia may be costly in dry environments and may hinder rather than facilitate adaptation to dry sites.

Increased costs of rhizobia in dry sites are surprising (Figure 2.2), given that dry soil restricts microbial decomposition and N mineralization (Al-Ithawi et al. 1980, Fuentes et al. 2003), and plants have difficulty accessing N in soluble form (Al-Ithawi et al. 1980, Bennington and McGraw 1995). The results are also contrary to theoretical expectations and empirical studies, showing that microbial symbionts can help maintain plant fitness in stressful conditions and potentially even facilitate plant adaptation to environmental stress (Johnson 1993, Goicoechea et al. 1997, Schwartz and Hoeksema 1998, Clark et al. 1999, Al-Karaki et al. 2004, Thrall et al. 2007, 2008, Leidi and Rodriguez-Navarro 2008, Rodriguez and Redman 2008, Heath et al. 2010, Porter et al. 2011).

Because we could not manipulate the presence of rhizobia and the source of rhizobia (wet or dry) in the field, it is impossible to determine whether genetic differences between rhizobia populations found in wet or dry destination sites explain our results or whether our findings are simply the result of fitness outcomes of legume-rhizobium mutualisms shifting depending on environmental conditions (context dependency, Bronstein 1994). Both may be possible. For example, mycorrhizae isolated from arid environments increased host plant drought tolerance more than those isolated from mesic environments (Stahl and Smith 1984, Bethlenfalvay et al. 1989).

Likewise, stressful environments may alter the carbon and nitrogen supply-demand balance. As a result, from the plants' perspective, associating with rhizobia may become less beneficial under certain environmental conditions (Heath 2010). Under drought, plants tend to close stomata in order to avoid desiccation (Hamidou et al. 2007). Stomata closure decreases carbon dioxide input and reduces photosynthetic efficiency (Hamidou et al. 2007). As a result, plants may not be able to keep up with rhizobia's high C demand (Harris et al. 1985, Kaschuk et al. 2009). In extreme cases, rhizobia can become parasitic, taking up photosynthate without providing fixed nitrogen (Sachs and Simms 2006, Kiers and Denison 2008). Consistent with this hypothesis, in dry destination sites, plants that did not nodulate had higher fitness regardless of their origin (Figure 2.1 & 2.2).

Future directions

Correlation does not imply causation, and other unmeasured variables that covary with soil moisture could be the primary selective agent driving the patterns of adaptation observed here (Wade and Kalisz 1990, Kawecki and Ebert 2004). An experiment manipulating the

putative selective agent either in the field or in the greenhouse is necessary to establish a causal relationship between the environmental factor and adaptation (Wade and Kalisz 1990). Manipulating genotypes of both plants and rhizobia under different moisture conditions will allow us to confirm whether soil moisture is a primary selective agent driving adaptation in this systems, to explicitly test whether rhizobia, as opposed to other soil microbes, influence plant adaptation to soil moisture conditions, and to differentiate between the two main mechanisms by which microbes can influence adaptation (genetic changes in rhizobia vs. context dependency).

Conclusion

In sum, this study suggests that soil moisture is likely an important selective agent driving adaptation and that how plants interact with symbiotic rhizobia may play an important role in plant adaptation. Given the intimate relationship between plants and symbiotic microbes, such as mycorrhizae, endophytes and rhizobia, such patterns may be prevalent in nature. My results also highlight the importance of examining both biotic and abiotic factors in local adaptation studies.

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APPENDICES

Appendix A

Tables and Figures

Table 2.1. Results from logistic regression testing the effects of destination soil type (wet or dry) and source soil type (wet or dry) on probability of nodulating. Family nested within source, destination nested within destination type and source nested within source type were included as random factors. *indicates marginal significance.

Source	*DF*	*F or* χ^2	*P*
Destination Type	1,4	00.02	0.88
Source Type	1,4	**10.67**	**0.03**
Dest.Type x Source Type	1,24	**21.56**	**<0.001**
Family (Source)	1	0.58	0.44
Destination (Dest.Type)	1	**233.39**	**<0.001**
Source (Source Type)	1	1.81	0.18
Destination (Dest.Type) * Source (Source Type)	1	3.64	**0.06***

Table 2.2. Results from a generalized linear model (negative binomial distribution) testing the effects of destination moisture type (wet or dry), source moisture type (wet or dry) and nodulation (nodulating or non-nodulating) on number of subterranean seeds. Plant family was nested within source site, destination site was nested within destination type, and source site was nested within source type.

Source	*DF*	*F or* χ^2	*P*
Destination Type	1,4	0.10	0.77
Source Type	1,4	3.28	0.14
Nodulation (N)	1,1426	**76.06**	**<0.001**
D.Type x S.Type	1,24	**20.14**	**<0.001**
D.Type x N	1,1426	**16.56**	**<0.001**
S.Type x N	1,1426	**5.94**	**0.015**
D,Type x S. Type x N	1,1426	0.79	0.374
Family (Source)	1	**210.38**	**<0.001**
Destination (D.Type)	1	**164.56**	**<0.001**
Source (S.Type)	1	0.52	0.47
Destination (D.Type)* Source (S.Type)	1	**63.86**	**<0.001**

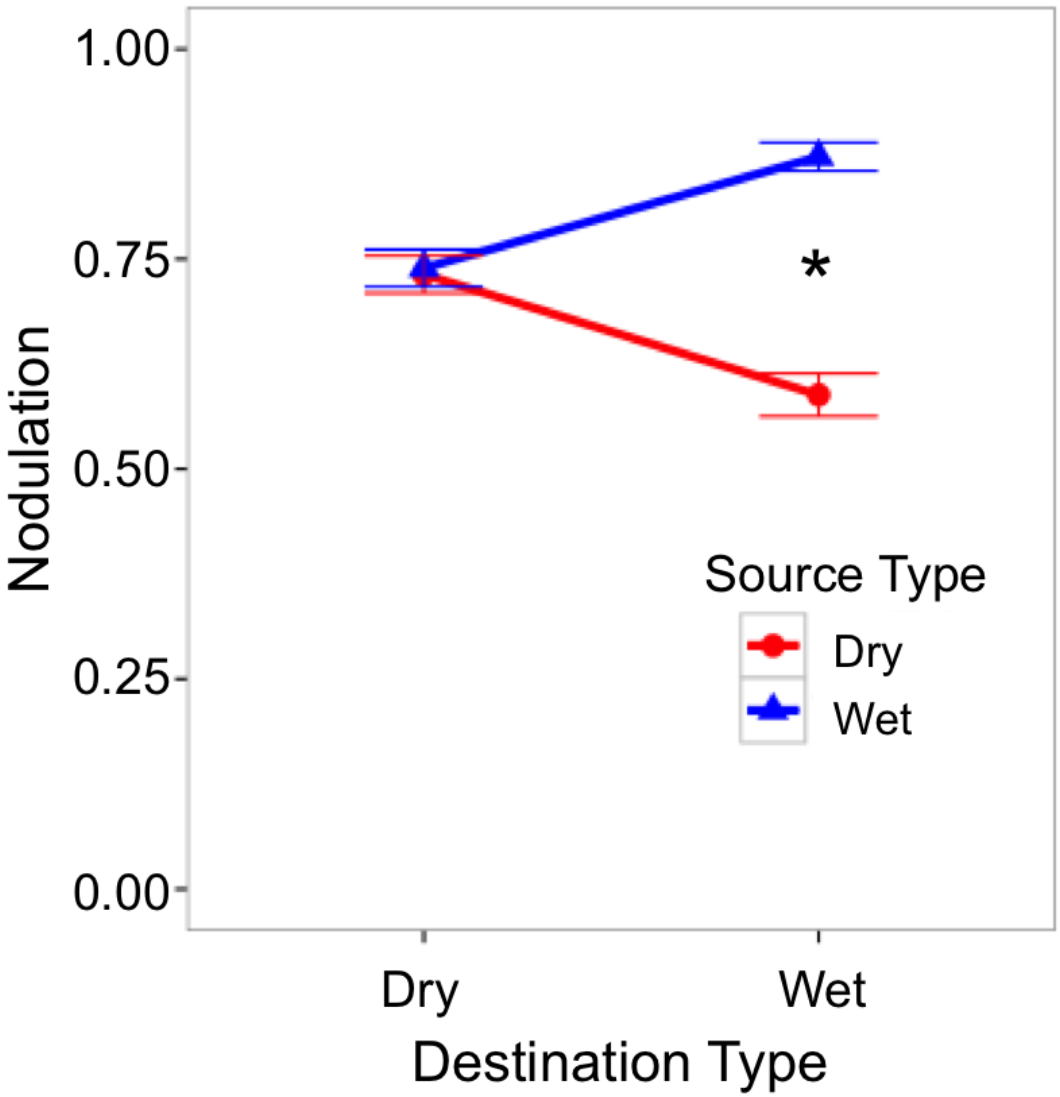

Figure 2.1. Proportion of plants forming associations with rhizobia (nodulating). Blue symbols indicate plants originating from wet source soil types; red symbols indicate plants originating from dry source soil types. Asterisks indicate significant differences (Tukey HSD test <0.05) between plants originating from wet vs. dry source types growing under the same destination type.

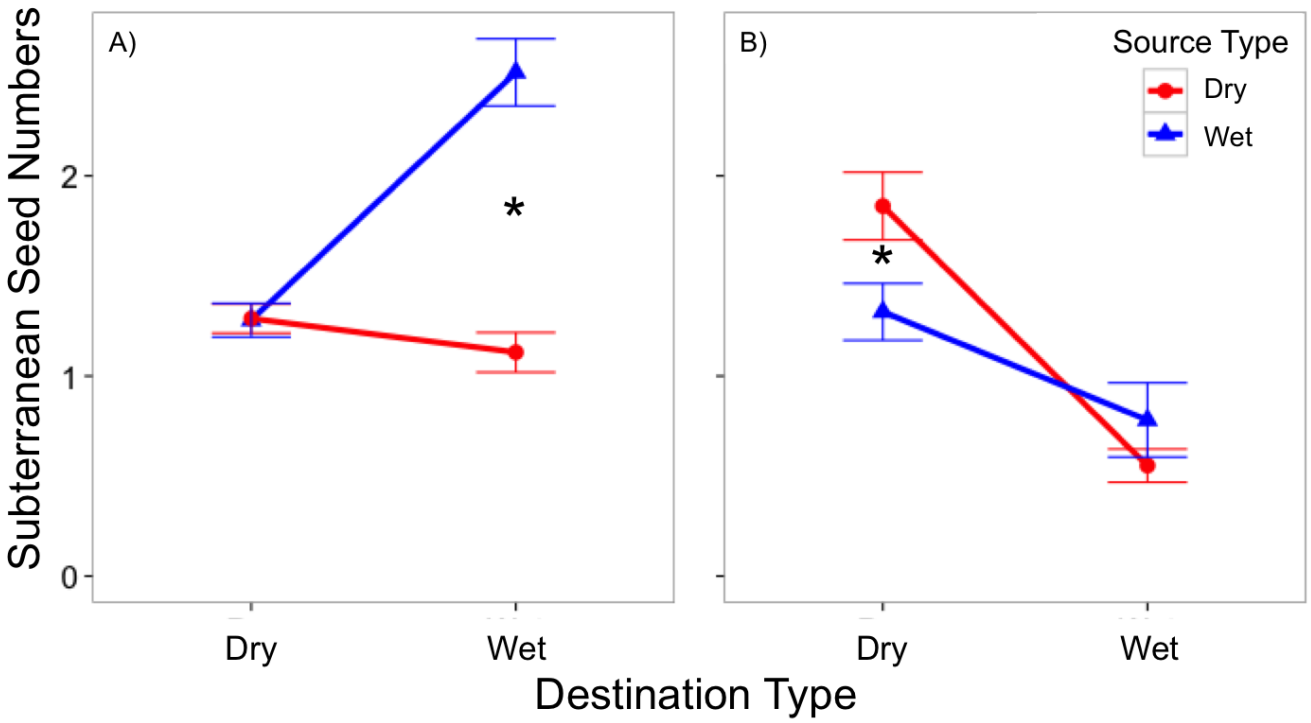

Figure 2.2. Number of subterranean seeds of nodulated (A) and non-nodulated (B) plants originating from wet or dry source types growing under wet or dry destination soil types. Asterisks indicate significant differences (Tukey HSD test ** <0.05) between plants originating from wet vs. dry source soil types.

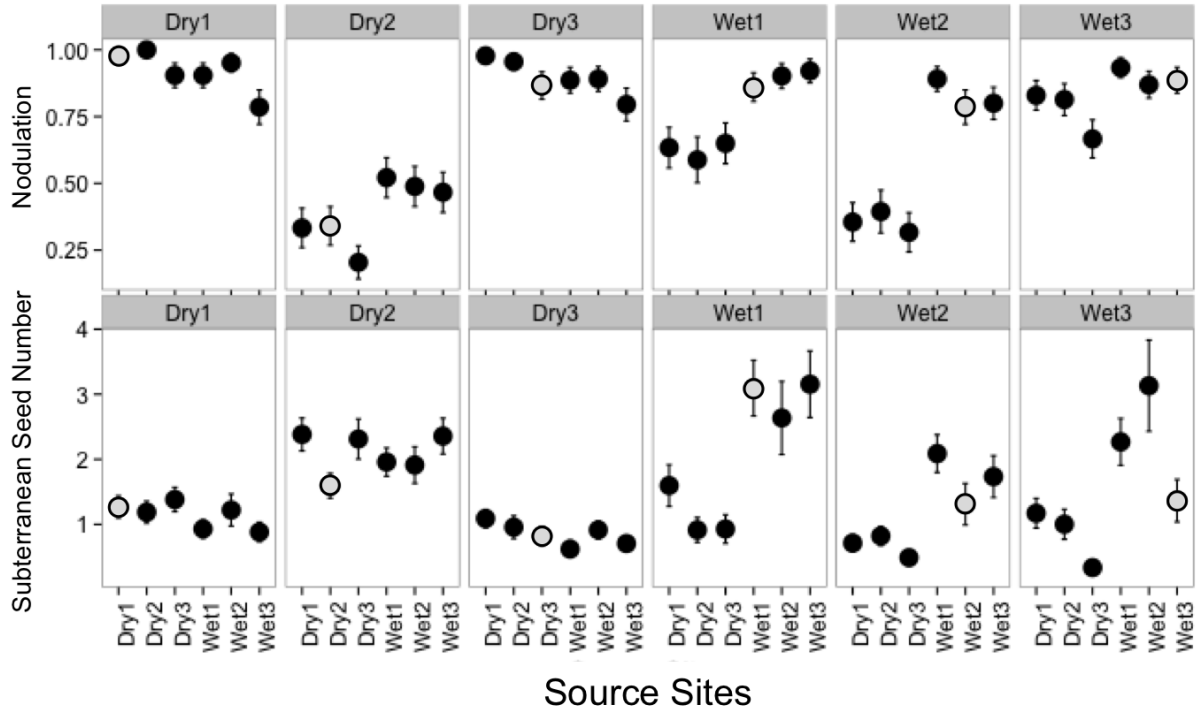

Figure 2.3. Plants originating from six source populations planted in six different destination sites. Each panel indicates a different destination site. Each point within a panel indicates a different source site. Grey symbols are plant populations planted into their "home" sites, and black symbols indicate plant populations planted into "foreign" sites. Plants growing in their "home" site do not have higher likelihood of nodulation (top panels) or subterranean seed numbers (bottom panels) than plants originating from "foreign" sites. Error bars indicate 1 standard error of the means.

Appendix B

Supplementary Materials

Supplemental Materials

Table 2.A1. Six field sites used in replicated reciprocal transplant experiment. Site acronym, average volumetric water content (VWC) and average photosynthetically active radiation (PAR,) between July and August 2014, total soil nitrogen content (N/gSoil) in June 2014 and GPS coordinates are summarized below. In July and August 2014, three PAR and VWC measurements and five soil samples (10cm soil cores) were taken along a transect (1 m intervals) for each *A. bracteata* population. PAR was measured using AccuPAR LP-80 Ceptometer (Decagon Devices, Inc, Pullman, USA) under clear sky conditions within 2 h of solar noon. Total soil N analysis was conducted using the field-collected soil in early July 2014. We performed a KCl extraction using homogenized soil (5 soil samples per site) and estimated soil ammonium and nitrate availability with an Alpkem/ OI Analytic Flow Solution IV analyzer (Model 3550) (see Eilts et al. 2011).

Site Name	**Acronym**	**VWC (%)**	**PAR (μmol m-2 sec-1)**	**Total Soil N (N/gSoil)**	**GPS**	
Brook Lodge A	Wet-1	48.87±0.35	1397 ± 189.1	10.13	N42°21.357	W085°22.822
Brook Lodge B	Dry–1	6.73±0.96	75±33.7	5.35	N42°21.498	W085°22.611
Pierce Cedar Creek H	Wet-2	46.67±1.45	288±50.2	8.65	N42°32.646	W085°17.444
Pierce Cedar Creek C	Dry-2	21.97±2.11	130±51.2	8.42	N42°32.063	W085°17.619

Table 2.A1. (Cont'd)

Carter Lake A	Wet–3	49.48±0.72	956±27.7	14.43
Carter Lake B	Dry–3	6.59±1.40	60±22.7	5.03

Table 2.A2. Summary of sample replication of plants originating from six source sites planted into each of six destination sites (N =3240).

		Destination						
Source	**Plant Family**	**Wet1**	**Dry1**	**Wet2**	**Dry2**	**Wet3**	**Dry3**	**Total**
Wet1	7	6	6	6	6	6	6	36
	11	6	6	6	6	6	6	36
	14	6	6	6	6	6	6	36
	15	6	6	6	6	6	6	36
	18	6	6	6	6	6	6	36
	21	6	6	6	6	6	6	36
	23	6	6	6	6	6	6	36
	F1	6	6	6	6	6	6	36
	F10	6	6	6	6	6	6	36
	F12	6	6	6	6	6	6	36
	F2	6	6	6	6	6	6	36
	F3	6	6	6	6	6	6	36
	F4	6	6	6	6	6	6	36
	F6	6	6	6	6	6	6	36
	F9	6	6	6	6	6	6	36
Wet1 Total		90	90	90	90	90	90	540
Dry1	3	7	6	6	7	6	6	38
	4	3	3	3	1	5	5	20
	5	7	6	7	6	6	7	39
	6	3	3	5	2	5	6	24
	8	6	6	6	6	6	6	36
	9	6	6	6	7	6	6	37
	11	6	5	6	5	6	6	34
	13	7	8	6	7	7	6	41
	15	6	6	6	6	6	6	36
	17	8	8	6	7	7	6	42
	18	13	18	10	16	9	6	72
	19	8	10	8	7	8	7	48
	20	5	5	6	6	6	6	34
	21	8	7	7	7	6	7	42

Table 2.A2. (cont'd)

	F1	6	5	6	5	6	6	34
Wet2	1	6	6	6	6	6	6	36
	2	6	6	6	6	6	7	37
	5	6	6	6	6	6	8	38
	7	6	6	6	6	6	6	36
	12	6	6	6	6	6	6	36
	13	7	6	6	6	6	6	37
	14	6	6	6	6	6	5	35
	16	6	6	6	6	6	6	36
	17	6	6	6	6	6	6	36
	18	6	6	6	6	6	6	36
	20	5	5	6	5	6	3	30
	21	6	6	6	6	6	6	36
	23	6	6	6	6	6	6	36
	24	6	7	6	7	6	7	39
	F1	6	6	6	6	6	6	36
Wet2 Total		90	90	90	90	90	90	540
Dry2	1	1	1	6	1			9
	2	6	3	7	9	9	9	43
	5	4	1	1	1	1		8
	6	3	4	9	9	8	6	39
	9	6	8	3	4	4	5	30
	12	3	4	4	2	3	3	19
	13	6	5	4	4	2	3	24
	15	6	5	6	8	5	7	37
	16	12	7	10	8	11	10	58
	17	7	6	6	6	6	5	36
	18	2	6	4	6	6	6	30
	19	8	9	8	5	6	8	44
	20	8	6	5	6	8	9	42
	21	2	2	1	3	2	1	11
	23	3	4	2	5	4	7	25
	24		4	5	4	5	5	23
	26	4	3	4	5	5	4	25
Dry2 Total		81	78	85	86	85	88	503
Wet3	1	6	6	6	6	6	6	36
	2	6	6	6	6	6	6	36

Table 2.A2. (cont'd)

	5	6	6	6	6	6	6	36
	6	6	6	6	6	6	6	36
	7	6	6	6	6	6	6	36
	9	6	6	6	6	6	6	36
	10	6	6	6	6	6	6	36
	14	6	6	6	6	6	6	36
	19	6	6	6	6	6	6	36
	21	6	6	6	6	6	6	36
	23	6	6	6	6	6	6	36
	25	6	6	6	6	6	6	36
	26	6	6	6	6	6	6	36
	27	6	6	6	6	6	6	36
	29	6	6	6	6	6	6	36
Wet3 Total		90	90	90	90	90	90	540
Dry3	1	5	3	5	5	3	2	23
	2	5	14	5	9	3	8	44
	5	16	7	7	7	8	6	51
	9	5	4	3	3	5	6	26
	11	6	5	6	6	6	5	34
	14	3	3	2	2	3	5	18
	15	6	12	24	20	21	12	95
	16	6	5	6	4	6	5	32
	17	5	6	5	6	5	5	32
	18	7	8	4	5	7	8	39
	19	6	7	4	5	5	6	33
	21	5	5	4	4	3	5	26
	24	5	3	5	4	4	5	26
	26	4	2	4	4	6	6	26
	28	6	6	6	6	5	6	35
Dry3 Total		90	90	90	90	90	90	540
Grand Total		**540**	**540**	**540**	**540**	**540**	**540**	**3240**

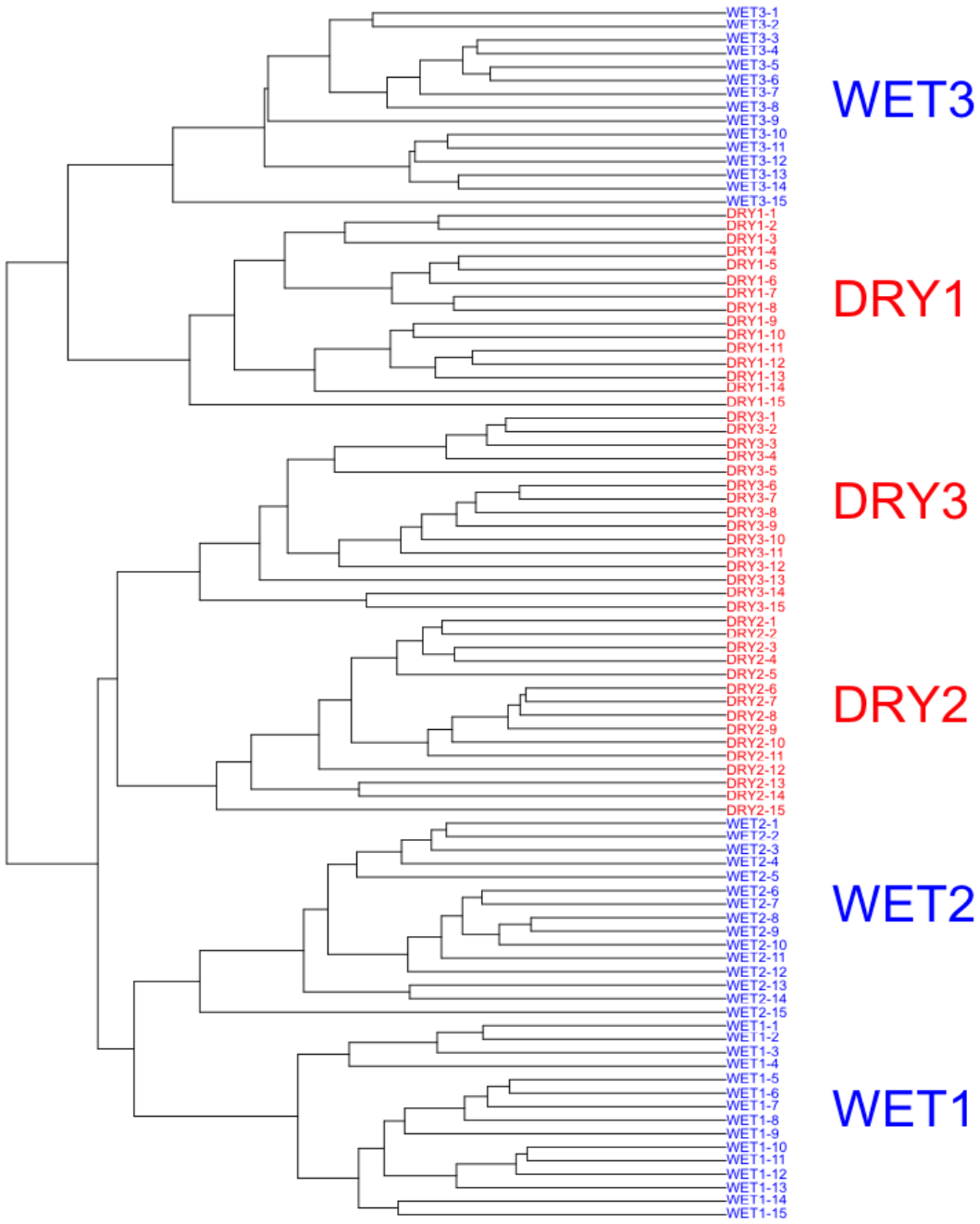

Figure 2.A1. Phylogeny of 90 *Amphicarpae bracteata* samples originating fro six sites (15 replicates per site).Three of the six sites were located in wet soil moisture habitat (blue) and the other three sites are located in dry soil moisture habitat (red).

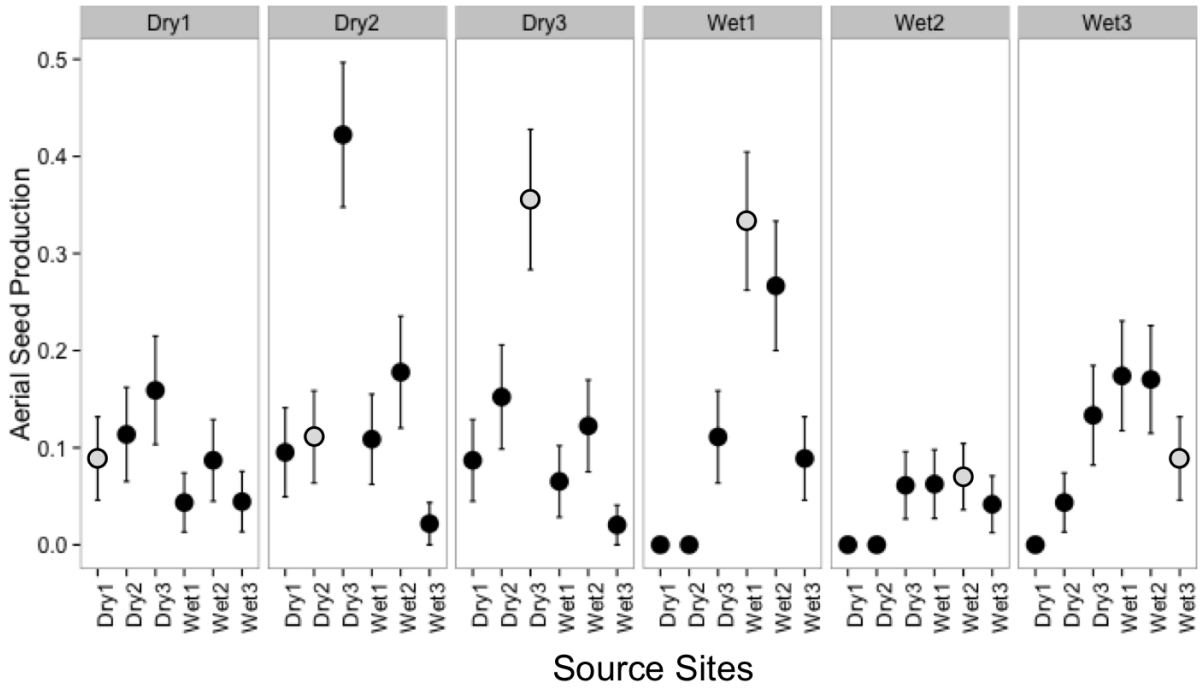

Figure. 2.A2. Proportion of aerial seed producing plants originating from each of the six source populations planted into each destination site. Each panel indicates a different destination site. Each point within a panel indicates a different source site. Grey symbols are plant populations planted into their "home" sites, whereas black symbols indicate plant populations planted into "foreign" sites. Plants growing in their "home" site are no more likely to produce aerial seeds than the plants originating from "foreign" sites, except for Dry3-originating plants. Error bars indicate 1 standard error of the means.

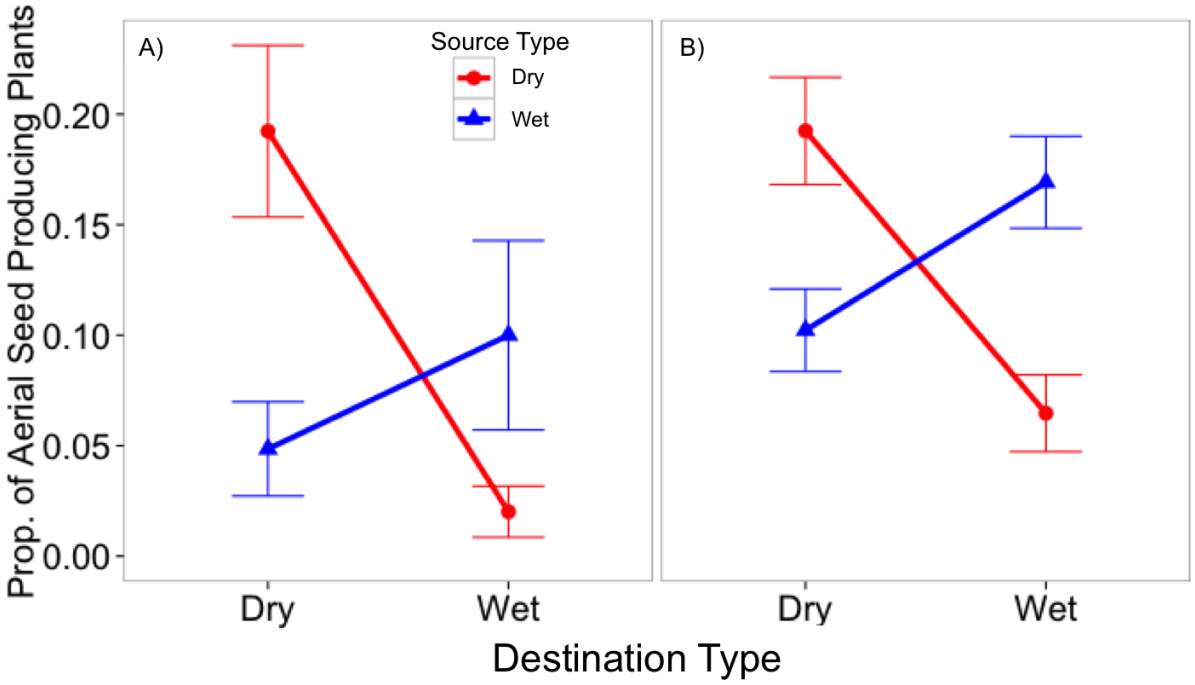

Figure 2.A3. Proportion of nodulated (a) and non-nodulated (b) plants originating from wet or dry source populations growing under wet or dry destination soil types producing aerial seed.

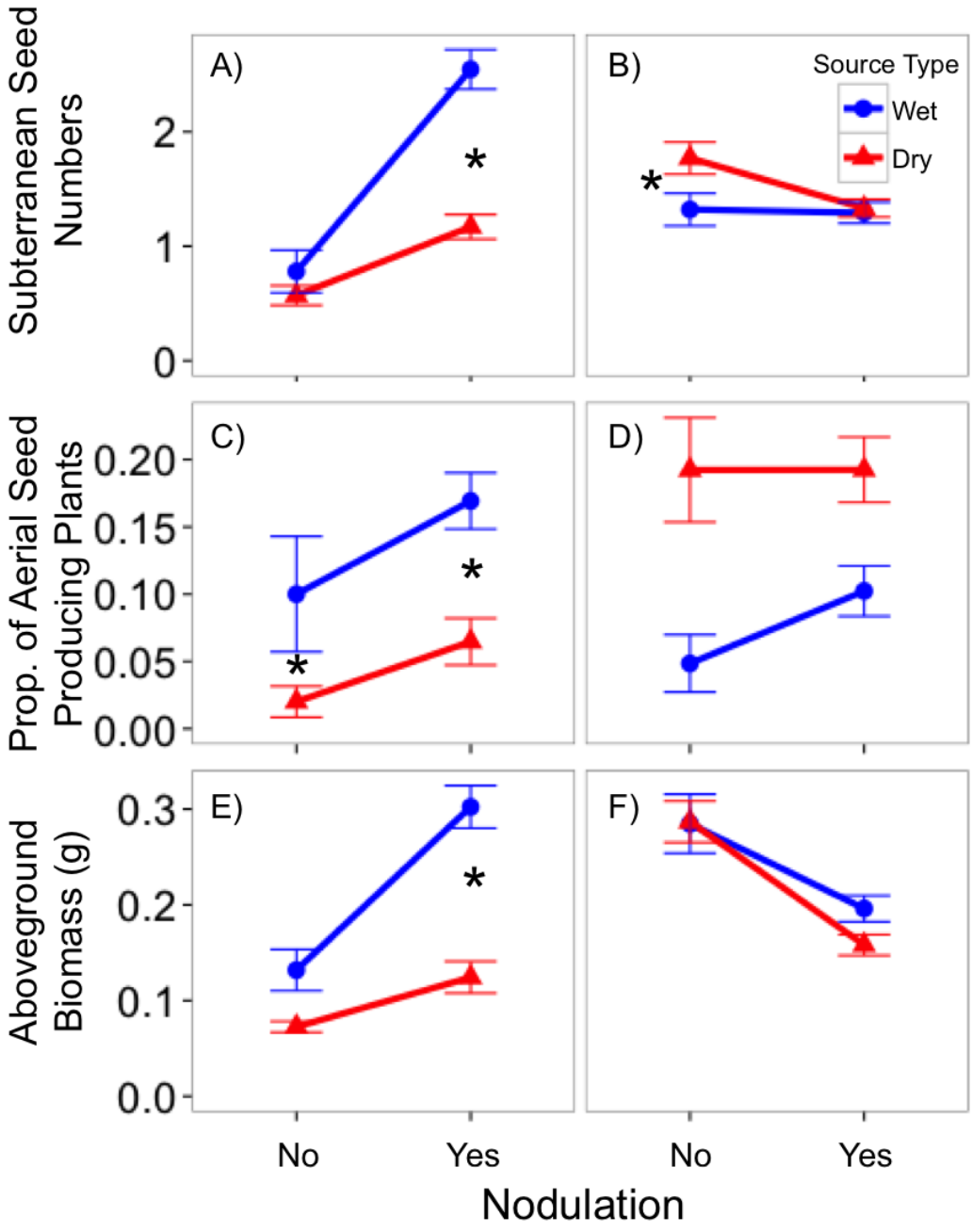

Figure 2.A4. Number of subterranean seeds, proportion of aerial seed producing plants and aboveground biomass (g) when nodulated and not-nodulated in wet destination (a,c,e) and dry destination sites (b,d,f). Asterisks indicate significant differences (Tukey HSD test <0.05) between plants originating from wet vs. dry source growing under the same destination soil type.

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CHAPTER 3

ECOLOGICAL AND EVOLUTIONARY EFFECTS OF SOIL MOISTURE ON A LEGUME-RHIZOBIUM SYMBIOSIS

INRODUCTION

Symbiotic interactions between microbes and plants are ubiquitous in nature. Approximately 80% of vascular plant species depend on mycorrhizal symbiosis for nutrients (Schussler et al. 2001), 20-30% of grass species associate with endophytes conferring stress tolerance (Leuchtmann 1993, Rodriguez et al. 2009), and many legume species associate with symbiotic rhizobia to obtain biologically fixed nitrogen (Sprent and James 2007).

Microbial symbionts can facilitate a plant's ability to tolerate biotic and abiotic stress (reviewed in Rodriguez and Rodman 2008). For example, foliar endophytes can reduce herbivory and increase drought tolerance (Clay et al. 1985, Kannadan and Rudgers 2008) and can increase a plant species' range by expanding the realized ecological niche (Friesen et al. 2011, Afkhami et al. 2014). Resource mutualists, such as arbuscular mycorrhizal fungi and nitrogen-fixing bacteria, can not only aid nutrient acquisition (Johnson 1993; Schwartz & Hoeksema 1998;Leidi & Rodriguez-Navarro 2008; Heath et al. 2010) but also confer tolerance to drought and pH stress (Goicoechea et al. 1997; Clark et al. 1999; Al-Karaki et al. 2004).

While there are many examples of the presence of microbial symbionts confering tolerance to environmental stress, there are relatively fewer studies examining how microbial evolution under different conditions influences their effects on host stress tolerance. Owing to their short generation times, high genetic variation, large population size, and high dispersal ability, microbes can evolve rapidly in response to environmental stress. In some cases, plant tolerance

to environmental stress may driven by plant adaptation, mediated by symbiont adaptation, or both. For example, recent studies sugget that plant adaptation to abiotic stress is driven in part by local adaptation of microbial symbionts (Rodriguez and Redman 2008, Friesen et al. 2011). The ability of *Pinus sylvestris* seedlings to tolerate copper stress, for example, was confered by adaptation of ectomycorrhial fungus, *Suillus luteus*, to copper (Adriaensen et al. 2005). Thus rapid evolution of microbial symbionts may facilitate adaptive plant responses to environmental stress.

There are two primary mechanisms by which microbial adaptation can mediate environmental stress for a host plant. First, the habitat-adapted symbiotic hypothesis (*sensu* Rodriguez et al. 2008) posits that symbionts adapting to habitat-specific stress can confer stress tolerance to any host plant in that habitat. In this scenario, symbionts evolve tolerance and can ameliorate stress for their plant hosts, but the plant hosts themselves are not necessarily adapted to a stress. For example, *Acropora millepora*, a common hard coral species, can acquire increased thermal tolerance by associating with zooxanthellae species adapted to high temperatures (Császár et al. 2010). Despite the low overall heritability of coral host traits and a long generation time (>20 yrs for the majority of corals), coral hosts appear to be maintaining their fitness against increased temperature because of the presence of habitat-adapted microbial symbionts. Another example of habitat-adapted symbionts is an adaptation of symbiotic *Attamyces* fungi of leaf cutter ants to cold temperature (Mueller et al. 2011). Cold-tolerant fungi strains increase garden productivity of leaf cutter ants and consequently increase colony fitness, leading to a range expansion to temperate habitats.

Second, as predicted by the co-adaptation hypothesis (Williams 1996, Thompson 2005), symbionts can be adapted to host plants originating from the same local site (host-adapted

symbionts). If symbiosis fitness benefits translate directly into increased host fitness, then host-adapted symbionts may mediate environmental stress better than non-host-adapted symbionts. For example, Wilkinson et al. (1996) found that the growth of the native legume *Amphicarpaea bracteata* was higher not only when associating with rhizobia from the same site, but also when associating with rhizobia isolated from genetically similar plants from different sites. However, there is surprisingly little evidence for the host-adapted symbiosis hypothesis in plant-microbe mutualisms, although positive host-symbiont fitness correlations are common in plant-rhizobia symbioses (Friesen 2012, but see Heath 2010).

To differentiate between the effects of habitat- vs. host-adapted symbionts on host fitness, first, one needs to identify a putative selective agent likely to influence hosts. One approach to identify selection pressures contributing to local adaptation is to conduct replicated reciprocal transplant experiments between replicate populations that differ in a key environmental variable (Kawecki & Ebert 2004; Wright et al. 2006). Then, one must factorially manipulate the genotypes of symbiotic microbes and host plants, as well as the putative selection pressure, to identify the role symbiotic microbes play in shaping plant responses to environmental stress.

Here we used a plant-rhizobia symbiosis as a model system to examine the role of resource mutualists in plant adaptation to extreme soil moisture conditions (wet and dry). The plant-rhizobia interaction is generally considered to be a mutualism, but there is increasing evidence showing that this interaction is context dependent, with outcomes varying from mutualistic to commensalistic depending on abiotic conditions and host genotypes (Thrall et al. 2007, Chamberlain et al. 2014, Suwa and Lau *In review*). We focus on soil moisture because it is a well-characterized selective agent on many physiological and morphological traits of plants (Dudley 1996, Sherrard et al. 2009) and is also known to influence legume-rhizobium

interactions (Chaves et al. 2003, Cornwell and Grubb 2003, Kannadan and Rudgers 2008). A recent reciprocal transplant experiment using *A.bracteata* revealed that plants are adapted to wet moisture conditions but adaptation depended on whether they formed associations with rhizobia (Chapter 2): These findings suggest that rhizobia affect the relative fitness of plants originating from wet vs. dry environments when transplanted into wet vs. dry soil moisture environments; however, this study did not manipulate soil moisture conditions or rhizobia due to logistical challenges in the field.

Expanding on the previous chapter (Chapter 2), we conducted two complementary greenhouse experiments to test ecological (presence of rhizobia) and evolutionary (genetic identity of rhizobia) effects of resource symbionts on plant responses to soil moisture. We asked: 1) How do symbiotic rhizobia influence plant growth and fitness responses to a soil moisture gradient; and 2) How do symbiotic rhizobia influence plant adaptation to soil moisture? We hypothesized that rhizobia confer plant tolerance to extreme soil moisture conditions both ecologically by reducing nutrient stress often experienced in extreme soil moisture conditions and evolutionarily through either rhizobia adaptation to soil moisture (habitat-adapted symbiosis) or local host plants (host-adapted symbiosis).

METHODS

Natural History

We studied the annual native legume, *Amphicarpaea bracteata* (hog peanut) and its nitrogen-fixing symbiotic bacteria, *Bradyrhizobium* sp (hereafter referred to as rhizobia). *A. bracteata* has a mixed mating strategy, possessing aerial (cleistogamous or chasmogamous) and subterranean (cleistogamous) flowers (Schnee and Waller 1986). Subterranean seeds are four to

six times larger than aerial seeds and account for most seedling recruitment (Parker 1991). Previous field observational studies in southwest Michigan revealed that *A. bracteata* occur in habitats ranging between very high and very low soil moisture and that populations differ in size, trichome density and SLA across the two soil moisture types (Suwa unpublished data). All seeds used in this experiment are the aerial cleistogamous seeds of greenhouse-reared plants originating from subterranean seeds collected between 2012 and 2013 from the ten sites described in Experiments 1 and 2 below (Supplementary Material Table 3.A1). Seeds were propagated in the greenhouse for one generation to minimize environmental maternal effects.

Experiment 1: Ecological effects of soil moisture on a plant-rhizobia interaction

Experimental Design and Treatments

To test whether soil moisture affects root nodulation and whether rhizobia affect plant responses to soil moisture, we conducted a greenhouse experiment, manipulating soil moisture and rhizobia presence on five plant populations (4 moisture levels x 2 rhizobia treatments x 5 plant populations).

Prior to applying experimental treatments, we filled pots (754 cm^3) with a 3:3:3:1 mix of potting media (Sunshine Mix LP5®), peat moss (Greensmix Sphagnum Peat Moss®, Waupaca Northwoods LLC), sand (Quikrete Tubesand® No. 1159, Quikrete International Inc.), and perlite (Horticultural Perlite®, Midwest Perlite Inc.). To minimize soil contamination, we homogenized and autoclaved the soil mixture twice at 121°C, 31 psi setting. Because the soil mixture was low in nutrient content, we supplemented the plants with a small amount of fertilizer: 20ml of 0.317g /L non-nitrogen soluble trace element mix (Peters® S.T.E.M.™, Erreris®) on 23 July and 10

August 2013.

Plant Populations: In 2011, we collected *A. bracteata* subterranean seeds from five sites in southern Michigan (Supplementary Material Table 3.A1). These subterranean seeds were reared in the greenhouse and served as maternal plants for the seeds used in the following experiments. We collected aerial seeds from greenhouse maternal plants and surface sterilized them with 30% commercial bleach (5.25% NaOCI) for one minute and rinsed with DI water for 10 minutes. We used aerial seeds in the following experiments because subterranean seeds do not have thick enough seed coats to protect the seeds from the sterilization process that was necessary to ensure eradication of rhizobia. We scarified each seed by nicking the seed coat and germinated them in a petri dish in the dark for seven days. Due to unequal germination success, we had variable replication for each treatment combination (1-17 replicates per soil moisture x rhizobia x plant population combination; N =323). We planted all the germinated seedlings on 5 July 2013 and watered evenly for the first ten days after planting.

Soil Moisture treatment: Beginning 15 July 2013, we added 10ml, 20ml, 40ml, or 80ml of water every other day throughout the experiment. These water addition treatments corresponded to gravimetric water contents of 9.7±3.9 %, 36.41±4.2%, 78.50±9.3% and 122.85±12.0% (GWC), respectively, based on volumetric water content (VWC) measurements of a separate set of 12 pots (3 replicates per moisture treatment) throughout the growing season using HydroSence II. (Campbell Scientific Inc., North Logan, Utah) GWC was estimated by measuring GWC once and correlated with average VWC of three replicated pots for each moisture treatment. This range of soil moisture conditions roughly corresponds to the range of soil moistures observed in

the field (9.8-103% GWC Supplementary Material Table 3.A1).

Rhizobia Genotypes: Eleven days after the soil moisture gradient was established, we inoculated each plant with rhizobia or a sterile media control. The rhizobia inocula contained 10 rhizobium isolates, 2 isolates from each of the five plant populations. Rhizobium isolates were isolated from two naturally occurring *A. bracteata* plants from each of the five populations (Supplementary Material Table 3.A1) in mid July 2012. Thus each plant population had potential to interact with co-evolved rhizobia strains. To obtain rhizobium isolates, we surface sterilized root nodules with commercial bleach (5.25 % NaOCI) for 1 min, followed by triple rinsing them with sterile water. We plated nodules on modified arabinose gluconate media (MAG media; van Berkum 1990) multiple times to isolate single colonies of rhizobia following standard techniques (Somasegaran and Hoben 1994), We archived rhizobia isolates at -80°C and later prepared inoculant by incubating each isolate in MAG liquid media in a shaking incubator at 28 □C (180 rpm) for five days. All of the liquid cultures were diluted to an optical density of 0.5 using a spectrophotometer to standardize the number of rhizobia cells per isolate and all the cultures were combined. We used this mixture to inoculate the plants with 4ml of inoculant (approximately 1 x 10^{10} cells). The plants in the control treatment were inoculated with an equal amount of autoclaved MAG media. We then covered the soil surface with *ca*. 100 ml of autoclaved Turface MVP® (PROFILE Products LLC, Buffalo Grove, IL) to minimize rhizobia cross contamination.

Data Collection

Eleven weeks after seedlings were planted, we measured leaf chlorophyll content (an

indicator of plant nitrogen status) with a SPAD-502 plus chlorophyll meter (Spectrum Technologies, Aurora, IL, USA), harvested above- and belowground biomass and collected and counted rhizobium nodules. Nodules and plant biomass were dried at 65ºC for at least 3 days before weighing.

Data Analysis

To test how rhizobia influence plant growth and fitness responses to a soil moisture gradient, we included the soil moisture treatment (4 moisture levels), plant population (5 levels), and rhizobium inoculation treatment (2 levels) as fixed factors, and bench (4 levels) as a random factor. We square root transformed aboveground biomass to improve normality. For nodule numbers, we used a negative binomial distribution. When interaction effects were significant, pairwise comparisons between treatments were conducted using Tukey's honestly significant difference (HSD) test. All analyses were conducted in Proc Mixed in SAS, except for nodule number which was analyzed used Proc GLIMMIX (SAS v.9.3; SAS Institute, Inc., Cary, North Carolina, U.S.A.).

Experiment 2: Evolutionary effects of soil moisture on a plant-rhizobia interaction

Experimental Design and Treatments

To test whether genetic variation in rhizobia influence plant adaptation to soil moisture, we conducted a multi-factorial greenhouse experiment manipulating soil moisture, plant genotypes, and rhizobia genotypes {2 moisture levels x 6 plant populations x 7 rhizobium treatments (6 rhizobium populations + 1 no rhizobium control)} at W. K. Kellogg Biological

Station at Michigan State University (2 December 2013- 20 March 2014). Each treatment combination was replicated 10 times, except one plant population ("Dry 2") had reduced replication due to low germination (Total N = 772).

We prepared the potting soil mixture in the same way as the first experiment. Because the soil mixture was low in nutrient content, we supplemented the plants with a small amount of fertilizer: 20ml of 0.317g /L non-nitrogen soluble trace element mix (Peters® S.T.E.M.™, Erreris®) on 20 January 2014 and 20ml of 158ppm N 24-8-6 fertilizer (Scotts MiracleGro® Product, Inc.) on 29 January 2014.

Plant Populations: In 2011, we collected *A. bracteata* subterranean seeds from paired dry and wet sites in three locales (6 sites total, gravimetric water content of wet and dry populations 87.84. ±14.96% and 22.63±5.16% respectively in July 2014). Dry and wet sites within each locale are 0.2 – 1.1 km apart from each other, and locales were 14.3 – 36 km apart (Table 3.A2). These subterranean seeds were reared in the greenhouse and served as maternal plants for the F1 cleistogamous aerial seeds that were used in the following experiment. We sterilized and scarified the aerial seeds using the same methods descried in the first experiment (see above). Then, we planted all the seeds between 14 -18 December 2013. On 24 December 2013, we replaced seedlings that had not yet emerged with new seedlings from the same population.

Moisture treatment: On 28 December 2013, we began applications of two soil moisture treatments (low and high) to simulate the range of soil moisture conditions experienced by *A. bracteata* in the field. For low and high moisture treatments, 15ml and 40ml of water, respectively, were applied every other day throughout the experiment. Soil moisture was

regularly measured on a separate set of 12 pots throughout the growing season using HydroSence II (Campbell Scientific Inc., North Logan, Utah). We measured GWC once and correlated with VWC to estimate GWC of each soil moisture treatment. On average, plants experienced 120.16±5.53 % and 20.74±1.13% GWC when growing in high and low soil moisture treatments, respectively, roughly representinf the full range of soil moisture conditions observed in the field (Supplementary Material 1).

*Rhizobia Genotypes:*Ten days after initiation of the moisture treatment, we inoculated each plant with one of seven rhizobia treatments: a sterile media control and six mixtures. Each mixture contained three rhizobium isolates isolated from one of the six populations in the field using the methods described in Experiment 1. We used a mixed inoculation because mixed inocula better simulate the diversity of rhizobia typically encountered under natural field conditions than single strain inoculations. Control plants were inoculated with an equal amount of sterile MAG media. We repeated the same procedure one week later to ensure inoculation. We then covered the soil surface with autoclaved Turface MVP® to minimize rhizobia cross contamination.

Data Collection

Nine weeks later, we harvested the plants and estimated rhizobium fitness traits (total nodule numbers and nodule mass) and plant fitness components (aboveground biomass, number of aerial and subterranean seeds, and subterranean seed mass). Nodules and plant biomass were dried at 65ºC for at least 3 d before weighing. Additionally, we measured plant physiological traits and traits related to resource allocation including belowground biomass, total carbon (C) and nitrogen (N) content (%) in leaf tissues, water use efficiency (WUE) through δ13C and

nitrogen fixation through δ15N (Rodriguez-Echeverria et al. 2009). For nutrient analyses, we ground and weighed the dried leaf tissues into tin cups and sent for ^{13}C and ^{15}N natural abundance and total C and N content analysis (Stable Isotope Facility, UC Davis, USA) using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Statistical Analysis

To examine whether adaptation of rhizobia to habitat type or local host plants influence plant adaptation to soil moisture, we examined plant C content, N content, δ13C, δ15N, proportion of aerial seed producing plants, number of subterranean seeds, and total subterranean seed mass. We analyzed proportion of plants with aerial seeds, as opposed to number of aerial seed produced because only 13.2 % of surviving plants produced aerial seeds. Eighty-nine percent of the experimental plants survived so we did not include plant survival in the analysis. To estimate rhizobia fitness components, we counted nodule numbers and weighed total nodule mass (g). We used a binomial distributions for the proportion of aerial seed producing plants. A negative binomial distribution was used for count variables (number of subterranean seeds, total number of nodules) and a Gaussian distribution for continuous variables (aboveground biomass, total subterranean seed mass and mean nodule mass). Some of the continuous variables (aboveground biomass and mean nodule mass) were log-transformed to improve normality.

To test whether symbiotic rhizobia influence plant adaptation to soil moisture through adaptation to habitat or to local host population, we analyzed the data in two ways by performing generalized mixed models using Proc Mixed and GLIMMIX in SAS (SAS v.9.3; SAS Institute, Inc., Cary, North Carolina, U.S.A.). First, we included moisture treatment (2 moisture levels),

plant moisture type (wet or dry), and rhizobia moisture type (wet and dry) as fixed factors. Bench, plant population (nested within plant moisture type), rhizobia population (nested within rhizobia moisture typed), and the plant population x rhizobia population interaction were included as random factors. Plants inoculated with MAG media (control) were not included in these analyses. No nodules were formed on control plants, suggesting that contamination was minimal. Interaction terms in the model used to test specific predictions regarding habitat-adapted and host-adapted rhizobia are summarized in Table 3.A3.

Additionally, we tested how the presence of rhizobia, regardless of genetic identity, influenced plant adaptation to soil moisture. In this model, we included moisture treatment (2 moisture levels), plant moisture type (wet or dry), and inoculation (inoculated/non-inoculated) as fixed factors. Bench and plant population (nested within plant moisture type) were included as random factors. This analysis parallels our previous study which investigated plant adaptation to wet and dry habitats in the field (Chapter 2*)*.

RESULTS

Experiment I: How do rhizobium mutualists influence plant response to soil moisture?

Soil moisture effects on nodulation: Soil moisture significantly affected the number and mass of root nodules (Figure 3.1; total nodule numbers $F_{3,136} = 38.1$ $P < 0.001$; total nodule mass $F_{3,135} = 51.63$, $P < 0.001$; mean nodule mass $F_{3,135} = 4.02$ $P = 0.009$). Averaged across all populations, plants produced the most nodules and greatest total nodule mass under 40ml water addition, and produced the fewest nodules and total nodule mass in the 10ml water addition treatment (Figure 3.1). However, the effects of soil moisture on total nodule mass also varied among plant

populations (significant moisture x plant population interaction $F_{12,135} = 2.35$, $P = 0.009$, Supplementary Material Figure 3.A1). This variation is mostly driven by one plant population (CCI), and total nodule mass of this population was only significantly reduced under very low (10ml) water addition treatments.

Rhizobia effects on plant response to soil moisture: Rhizobia altered plant response to soil moisture treatments for aboveground biomass and chlorophyll content (significant moisture x rhizobia inoculation, Table 3.1, Figure 3.2). Non-inoculated plants produced the greatest aboveground biomass under high soil moisture conditions (80ml water addition treatments), while inoculated plants produced the greatest aboveground biomass under 40ml water addition (Table 3.1, Figure 3.2A). Rhizobia only significantly increased aboveground biomass under intermediate soil moisture conditions (20 and 40mL). Rhizobia significantly increased chlorophyll content across all water addition treatments, but the magnitude of this effect was greatest under 20ml and 40ml water addition treatments. The effects of soil moisture and rhizobia were independent for root:shoot ratio (Table 3.1): increased soil moisture and rhizobia addition decreased root:shoot ratio by 60.7 % (0ml vs. 80ml water addition) and 25.3%, respectively.

Experiment II: How do symbiotic rhizobia influence plant adaptation to soil moisture?

Plant adaptation: We found weak evidence of plant adaptation to high soil moisture conditions (Figure 3.3, Table 3.2). While wet-originating plants produced significantly more aboveground biomass and were more likely to produce aerial seeds than dry-originating plants under the high moisture treatment, dry- and wet-originating plants did not differ in subterranean seed numbers

under either low or high moisture conditions (Figure 3.3B). Plants consistently produced more subterranean seeds and aboveground biomass under high than low soil moisture conditions regardless of their origin (Figure 3.3A&B). Results from analyses oftotal seed numbers (sum of aerial and subterranean seeds)were qualitatively very similar to the ones using subterranean seed numbers only (data not shown).

Additionally, root:shoot ratio responses to soil moisture differed between plant types (significant moisture x plant type interaction; $F_{1.544} = 16.07$, $P < 0.001$, Figure 3.3C). In low soil moisture conditions, dry-originating plants had significantly lower root:shoot ratios than wet-originating plants while in wet soil moisture conditions, dry- and wet-originating plants did not differ in root:shoot ratio (Figure 3.3C).

Although rhizobia populations did not differ in their effects on plant growth and fitness components and did not differentially influence plant adaptation to soil moisture (no significant rhizobia type or rhizobia type x plant type x soil moisture interactions, Table 3.2), the presence of rhizobia did: when grown in the presence of rhizobia, wet-originating plants produced significantly more aboveground biomass than dry-originating plants in high soil moisture treatments (Figure 3.4B). When plants were grown in the absence of rhizobia, plants originating from dry vs. wet environments did not differ in any fitness component in either high or low conditions. (Figure 3.4A).

While we found weak evidence of plant adaptation to soil moisture, we detected a significant plant type x rhizobia type interaction on total N content and N fixation (Table 3.4, Figure 3.5): Dry-originating plants inoculated with dry-originating rhizobia tended to have lower δ15N (i.e. higher N-fixation) and 22% higher total leaf N content than those inoculated with wet-originating rhizobia. Similarly, wet-originating plants inoculated with wet-originating rhizobia

tended to have a lower δ15N values and 20% higher total leaf N content than plants inoculated with dry-originating rhizobia (Table 3.4, Figure 3.5).

Additionally, we detected a significant soil moisture x plant type interaction for both N content and δ15N (Table 3.4). In low soil moisture treatments, dry and wet-originating plants had similar total N contents (dry –originating plants 2.12 ± 0.071; wet-originating plants 2.29±0.10), although wet-originating plants had lower δ15N than dry-originating plants, indicating greater N-fixation rates (dry-originating plants -0.19 ±0.21; wet-originating plants -1.02±0.21). In high soil moisture treatments, wet-originating plants had significantly greater total N content than dry-originating plants (dry-originating plants 1.46 ±0.068 wet-originating plants 2.01 ±0.081) and had lower δ15N, indicating higher N-fixation rates (-1.40 ±0.19). Dry-originating plants had positive δ15N values, indicating no N-fixation (0.73 ± 0.22). Total C and WUE, measured as δ13C, were only affected by moisture treatment: Plants grown in high soil moisture conditions had significantly lower WUE and lower total leaf C (Table 3.4).

Adaptation of rhizobia to habitat type or local host plants: Dry-originating rhizobia appear to be adapted to plants originating from dry environments. (Figure 3.6B&D, Table 3.5 significant plant type x rhizobia type interaction). Total nodule numbers and total nodule mass were greater for dry- compared to wet-originating rhizobia associating with dry-originating plants. However, dry- and wet-originating rhizobia produced similar nodule numbers and total nodule mass when associated with wet-originating plants (Figure 3.6 B& D).

Contrary to our prediction, we found no evidence of rhizobia adaptation to soil moisture conditions (Figure 3.6A & C, no Moisture x Rhizobia Type interaction Table 3.5), but rhizobia produced greater numbers and total mass of nodules under high soil moisture conditions,

regardless of their soil moisture type (Figure 3.6A & C). Similarly, we found no evidence of rhizobia adaption to local host plant populations above and beyond effects driven by wet vs. dry sites (Supplementary Material Figure 3.A2& 3.A3), and only one of six rhizobia populations had highest fitness when associating with plants from its home site (rhizobia originating from Wet2 site; Supplementary Material Figure 3.A2).

DISCUSSION

The benefits rhizobia provided to plant hosts depended on soil moisture conditions. Rhizobia were most beneficial when nodule production was the highest under intermediate soil moisture conditions and least beneficial when nodule production was lowest under low soil moisture conditions. We detected some evidence that plants were adapted to soil moisture conditions, particularly in high soil moisture conditions when grown in the presence of rhizobia. However, rhizobium fitness benefits to plant hosts were not determined by rhizobia adaptation to soil moisture conditions (Figure 3.6 A&C) or adaptation to local plant hosts (Figures 3.A2 & 3.A3), even though dry-originating rhizobia produced more and larger nodules and fixed more nitrogen when associating with plants originating from dry environments.

How do rhizobium mutualists influence plant responses to soil moisture?

Our first experiment revealed that there is an upper and lower soil moisture threshold where symbiotic rhizobia are beneficial to plants. Under very low and very high soil moisture treatments (10mL and 80mL), rhizobia did not significantly benefit plant growth (Fig 3.2A). Severe drought (10mL) appears to induce nodule senescence in our study (Supplementary Material Figure 3.A4), as reported by other studies (Gogorcena et al. 1995, Escuredo et al. 1996).

Although physiological and biochemical mechanisms inhibiting rhizobia activity need further investigation, the N-feedback hypothesis suggests that extreme drought restricts volumetric phloem flow into the nodule (Hartwig et al. 1994), slowing down the flow of the fixed N products through xylem and leading to accumulation of N products in nodules (Serraj et al. 1998). Accumulation of N products triggers a negative feedback, inhibiting nodule activity (Serraj et al. 1998). Although we do not have N fixation data for Experiment 1, total nodule mass and chlorophyll content (an indicator of plant nitrogen status) was lowest in the driest treatment in our study (Fig 3.1.B and 3.2C), perhaps indicating reduced nitrogen fixation activity. Under very high soil moisture (80ml water addition) treatments, rhizobia provided little growth benefit to their plant hosts, even though plants nodulated to the same extent as plants in lower water addition treatments (20ml water addition) where beneficial effects on plants were detected (Figure 3.1 & 3.2). Previous studies on crop legumes reported a similar reduction in rhizobia benefit to plants under flooded conditions (Minchin and Pate 1975, Minchin and Summerfield 1976, Bacanamwo and Purcell 1999). This has been attributed to a reduction in oxygen supply to the nodules under flooded conditions (Gallacher and Sprent 1978, Dakora and Atkins 1991, Pugh et al. 1995). Although nitrogenase is denatured by oxygen concentrations above 5mmol m^{-3}, rhizobia requires oxygen for aerobic respiration (Hunt and Layzell 1993). Furthermore, recent studies on *Glycine max* suggest that nitric oxide (NO) is produced by nitrite reductase in soybean nodules during flooding events, resulting in negative effects on nitrogenase activity and N-fixation (Sánchez et al. 2011)

Evolutionary effects of soil moisture on plant-rhizobia interactions

Our first experiment showed that soil moisture influences the fitness outcome of the legume-rhizobium mutualism. However, our second experiment showed only minimal evidence of plant adaptation to soil moisture and no evidence that locally adapted rhizobia increase plant fitness in high and low soil moisture conditions. Plants originating from wet environments produced more aboveground biomass than plants originating from dry environments when grown in high soil moisture conditions, but only significantly so in the presence of rhizobia (Figure 3.4) – a finding that suggests that variation among plant populations in how they associate with rhizobium symbionts may contribute to adaptation to dry vs. wet environmental conditions. This result parallels those observed in a previous field study on this system that showed that adaptation to wet sites were strongest when plants successfully formed associations with naturally occurring rhizobia (Chapter 2). However, the evolutionary history of rhizobia had no detectable effects on plant fitness responses to soil moisture despite influencing plant nutrient content and N-fixation rates (Table 3.2 no significant moisture x rhizobia interaction, Table 3.4 and Figure 3.5). Instead, we found that rhizobia from dry sites are adapted to host plants originating from dry sites: When associating with dry-originating plants, rhizobia originating from dry environments produced 3.3 times more nodules numbers, two times more total nodule mass, and fixed more nitrogen than rhizobia originating from wet sites (Figures 3.5, 3.6B & D). Because nodule numbers and size are correlated with rhizobia fitness (Kiers et al. 2003, Simms et al. 2006, Heath and Tiffin 2007, but see Ratcliff and Denison 2009), these results indicate that dry-originating rhizobia are adapted to dry-originating plant hosts (Figure 3.6 B&D). Wet- and dry-originating rhizobia performed similarly on wet-originating host plants, however. Our results suggest differentiation between dry- and wet-adapted plant populations in plant traits affecting

the formation and growth of nodules and differentiation between dry- and wet-adapted rhizobia in the benefits they receive from their plant hosts.. Surprisingly, variation in nodulation and nitrogen fixation did not result in variation in plant fitness. In other words, plants inoculated with wet- or dry-originating rhizobia had equal fitness across both moisture treatments (Table 3.2).

Above and beyond the interactions with host type (wet or dry), we detected little evidence for rhizobia adaptation to their local host populations and no evidence that plant populations are adapted to local rhizobia. Our results are consistent with previous studies reporting lack of tight coadaptation between host plants and symbiotic microbial partners (Wilkinson et al. 1996, Burdon and Thrall 2009, Heath 2010, Barrett et al. 2012, Hoeksema et al. 2012). While natural selection may favor increased mutualism benefit among sympatric than allopatric pairs (Barrett et al. 2012), there are several factors that limit co-adaptation of local plant and symbiotic rhizobia. They include spatial variation in biotic and abiotic environments (Thrall et al. 2007, Thompson 2005), differences in generation time between interacting species (Hoeksema and Forde 2008), and variation in dispersal ability (Gomulkiewicz et al. 2000, Nuismer et al. 2000) and partner specificity (Wilkinson and Parker 1996, Barrett et al. 2012).

Conclusion

Experimental designs manipulating putative selective agents likely to drive local adaptation and influence plant-microbe interactions, combined with reciprocal transplant experiments, are key to elucidate complex interactions between plants and microbes under different abiotic conditions (Nuismer and Gandon 2008). We found that rhizobia significantly increase plant fitness, but only within a certain range of moisture conditions (20 and 40ml water addition). Despite the large fitness effects of rhizobia on host plants and the variability in

legume-rhizobium interactions across a soil moisture gradient, we detected little evidence that plant adaptation is determined by rhizobia adaptation to soil moisture conditions or host plant populations. Instead, we found that rhizobia populations are adapted to plants originating from the same soil moisture type. In this case, host and symbiont fitness responses were asymmetric and did not translate into correlated fitness responses to common environmental conditions

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APPENDICES

Appendix A

Tables and Figures

Table 3.1. Results from ANOVA testing the effects of soil moisture, rhizobia inoculation, plant population and their interactions on aboveground biomass (g), root:shoot ratio, and chlorophyll content for Experiment 1. Bench was included as a random effect.

	Aboveground Biomass			Root:Shoot Ratio			Chlorophyll Content		
	df	F or χ^2	P	df	F or χ^2	P	df	F or χ^2	P
Moisture (M)	3,281	**41.61**	**<0.001**	3, 278	**123.71**	**<0.001**	3, 280	**6.67**	**<0.001**
Population (P)	4, 281	**2.94**	**0.021**	4, 278	**2.46**	**0.046**	4, 265	**2.6**	**0.036**
Rhizobia (R)	1, 281	**24.26**	**<0.001**	1, 278	**57.28**	**<0.001**	1, 280	**279.14**	**<0.001**
M x P	12, 281	0.88	0.568	12, 278	1.03	0.426	12, 280	0.86	0.584
M x R	3, 281	**5.69**	**<0.001**	3, 278	1.43	0.235	3, 280	**17.9**	**<0.001**
R x P	4, 281	0.66	0.619	4, 277	2.6	**0.037**	4, 280	1.45	0.219
M x P x R	1, 281	1.46	0.137	1, 278	0.7	0.750	1, 280	0.86	0.585
Random Effect									
Bench	1	0	1	1	**9.7**	**0.002**	1	0.4	0.527

Table 3.2. Results from ANOVA testing the effects of soil moisture, plant type (wet or dry), rhizobia type (wet or dry) and their interaction on aboveground biomass (g) and subterranean seed numbers for Experiment 2. Bench, plant population, rhizobia population and the plant population x rhizobia population interaction were treated as random effects.

	Aboveground Biomass			Subterranean Seed Numbers		
	df	F or χ^2	P	df	F or χ^2	P
Moisture (M)	1,544	**259.75**	**<0.001**	1,544	**37.95**	**<0.001**
Plant Type (PT)	1,544	2.14	0.144	1,544	0.19	0.666
Rhizobia Type (RT)	1,544	0	0.980	1,544	0.21	0.649
M x PT	1,544	**13.47**	**<0.001**	1,544	0.03	0.874
M x RT	1,544	0	0.988	1,544	2.5	0.115
PT x RT	1,544	0.22	0.632	1,544	0.62	0.431
M x PT x RT	1,544	0.18	0.675	1,544	0	0.996
Random Effects						
Bench	1	**57.6**	**<0.001**	1	1.08	0.149
Plant Population	1	**5.3**	**0.021**	1	**8.12**	**0.004**
Rhizobia Population	1	0	1	1	0	1
Plant Pop x Rhizobia Pop	1	0	1	1	0	1

Table 3.3. Results from ANOVA testing the effects of soil moisture, plant type (wet or dry), rhizobia presence (inoculated or not inoculated) and their interactions on aboveground biomass (g) and subterranean seed numbers for Experiment 2. Bench and plant population were treated as random effects for aboveground biomass.

	Aboveground Biomass			Subterranean Seed Numbers		
	Df	F or χ^2	P	df	F or χ^2	P
Moisture (M)	1,660	124.79	**<0.001**	1,664	6.75	**0.010**
Plant Type (PT)	1,660	0.09	0.761	1,664	0.39	0.53
Inoculation (I)	1,660	10.36	**0.0014**	1,664	0.38	0.54
M x PT	1,660	4.64	**0.032**	1,664	1.66	0.20
M x I	1,660	4.83	**0.028**	1,664	3.26	0.072*
PT x I	1,660	2.25	0.144	1,664	0.21	0.65
M x PT x I	1,660	0.72	0.40	1,664	1.14	0.24
Random Effects						
Bench	1	103.3	**<0.001**	1	1.23	0.27
Plant Population	1	12.4	**<0.001**	1	11.16	**<0.001**

Table 3.4. Results from ANOVA testing the effects of soil moisture, plant type (high or low), rhizobia type (wet or dry) and their interactions on total carbon content, δ13C (as an estimate of water use efficiency), total nitrogen content and δ15N (as an estimate of biological N fixation) in a leaf tissue for Experiment 2. Bench, plant population, rhizobia population and the plant population x rhizobia population interaction were treated as random effects.

	Total Carbon			δ13C		
	df	F or χ^2	P	df	F or χ^2	P
Moisture (M)	1,199	**241.74**	**<0.001**	1, 199	**74.49**	**<0.001**
Plant Type (PT)	1,4	1.86	0.245	1,4	0.39	0.566
RhizobiaType (RT)	1,4	0.55	0.501	1,4	2.68	0.178
M x PT	1,198	1.04	0.309	1,199	2.35	0.127
M x RT	1,198	2.08	0.159	1,198	0.56	0.455
PT x RT	1,198	0.28	0.600	1,198	0.44	0.509
M x PT x RT	1,198	0.99	0.321	1,198	0.35	0.556
Random Effects						
Bench	1	0	1	1	**4.5**	**0.034**
Plant Population	1	**27.9**	**<0.001**	1	**46.6**	**<0.001**
Rhizobia Population	1	**18.9**	**<0.001**		0	1
Plant Pop x Rhizobia Pop	1	1.8	0.180		0	1

Table 3.4 (Cont'd)

	Total Nitrogen			δ15N		
	df	F or χ^2	P	df	F or χ^2	P
Moisture (M)	1,176	**49.8**	**<0.001**	1,176	3.3	0.07*
Plant Type (PT)	1,4	2.5	0.189	1,4	3.77	0.12
Rhizobia Type (RT)	1,4	0.01	0.930	1,4	0	0.98
M x PT	1,176	**8.16**	**0.005**	1,176	**18.62**	**<0.001**
M x RT	1,176	0.37	0.543	1,176	0.05	0.819
PT x RT	1,24	**21.63**	**<0.001**	1,24	**7.86**	**0.010**
M x PT x RT	1,176	1.13	0.290	1,176	0.19	0.666
Random Effects						
Bench	1	0	1	1	0	1
Plant Population	1	**27.9**	**<0.001**	1	**55.3**	**<0.001**
Rhizobia Population	1	**18.9**	**<0.001**	1	**12.3**	**<0.001**
Plant Pop x Rhizobia Pop	1	0.2	0.18	1	14.4	**<0.001**

Table 3.5. ANOVA table to test the effects of soil moisture, plant type (wet or dry), rhizobia type (wet or dry) and their interactions on total nodule numbers, mean nodule mass, and total nodule mass for Experiment 2. Bench, plant population, rhizobia population and the plant population x rhizobia population interaction were treated as random effects.

	Total Nodule Numbers			Mean Nodule Mass			Total Nodule Mass		
	df	F or χ^2	P	df	F or χ^2	P	df	F or χ^2	P
Moisture (M)	1,547	**213.94**	**<0.001**	1,501	**212.2**	**<0.001**	1,537	**396.95**	**<0.001**
Plant Type (PT)	1,547	**14.91**	**<0.001**	1,501	1.13	0.29	1,537	**92.95**	**<0.001**
Rhizobia Type (RT)	1,547	**5.94**	**0.015**	1,501	**27.74**	**<0.001**	1,537	1.65	0.1998
M x PT	1,547	1.50	0.22	1,501	**9.67**	**0.002**	1,537	**79.28**	**<0.001**
M x RT	1,547	1.05	0.31	1,501	3.58	0.06	1,537	2.6	0.1071
PT x RT	1,547	**19.80**	**<0.001**	1,501	**48.25**	**<0.001**	1,537	**8.23**	**0.0043**
M x PT x RT	1,547	0.08	0.78	1,501	0.39	0.53	1,537	2.51	0.1137
Random Effects									
Bench	1	**29.25**	**<0.001**	1	**18.4**	**<0.001**	1	**35.70**	**<0.001**
Plant Population	1	**113.48**	**<0.001**	1	**38.6**	**<0.001**	1	**4.10**	**0.043**

Table 3.5. (cont'd)

Rhizobia Population	1	**12.52**	**<0.001**	1	0.20	0.65	1	**7.10**	**0.008**
Plant Pop x Rhizobia Pop	1	3.21	0.073	1	**6.20**	**0.013**	1	1.30	0.25

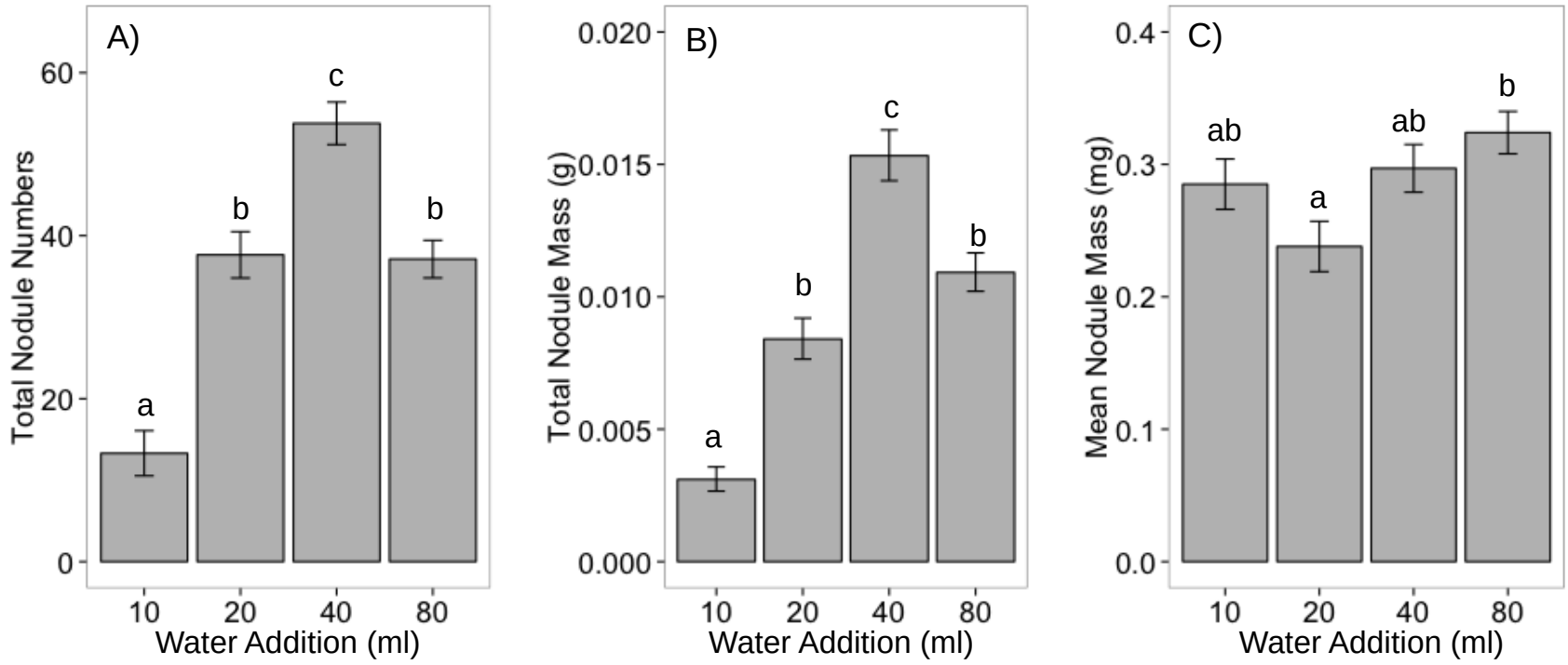

Figure 3.1. Least square means ± 1SE of total nodule numbers, total nodule mass (g) and mean nodule mass (mg) of inoculated plants growing under four different soil moisture conditions in Experiment 1. Bars with different letters differ significantly from each other ($P < 0.05$, Tukey's honestly significant difference test (HSD).

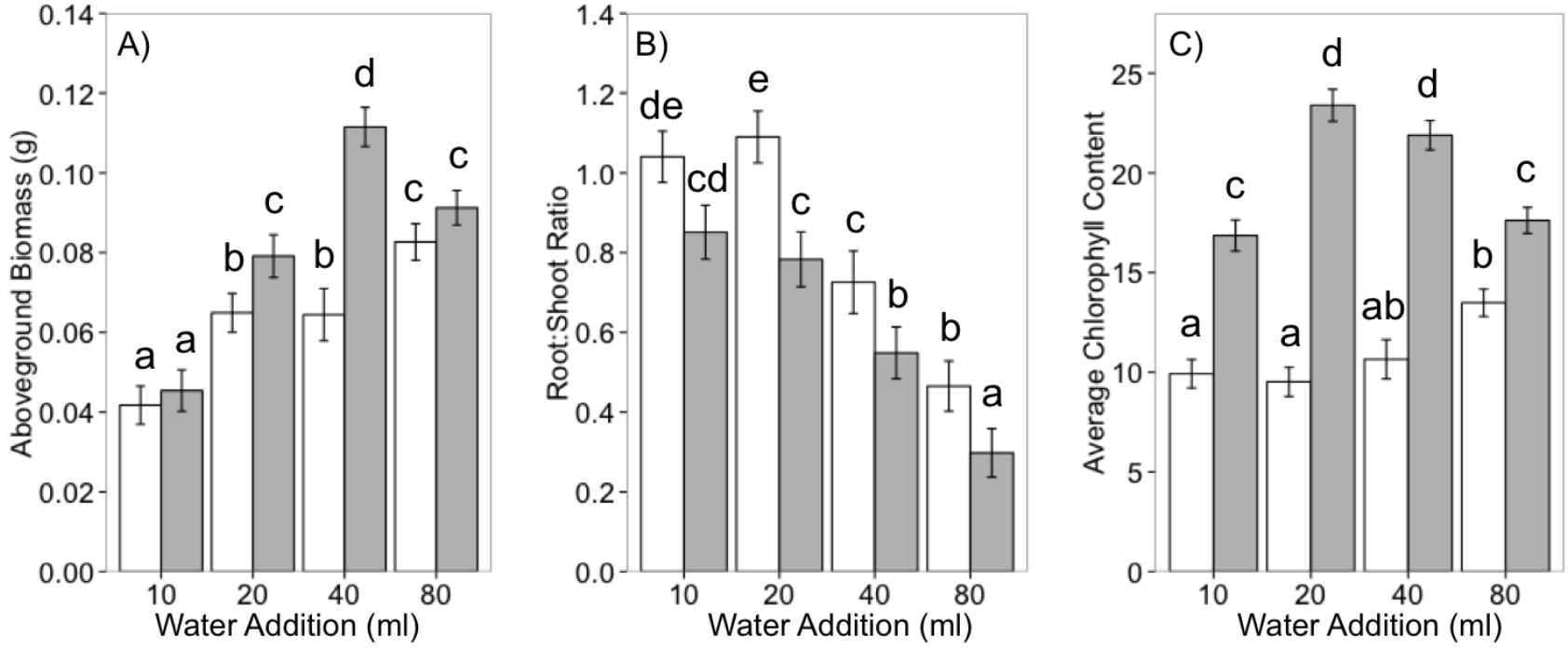

Figure 3.2 Least square means ± 1SE of aboveground biomass (g), root:shoot ratio, and chlorophyll content of plants growing under four different soil moisture conditions in Experiment 1. Plants were either not inoculated (white bars) or inoculated (grey bars). Bars with different letters differ significantly from each other ($P < 0.05$, Tukey's honestly significant difference test (HSD).

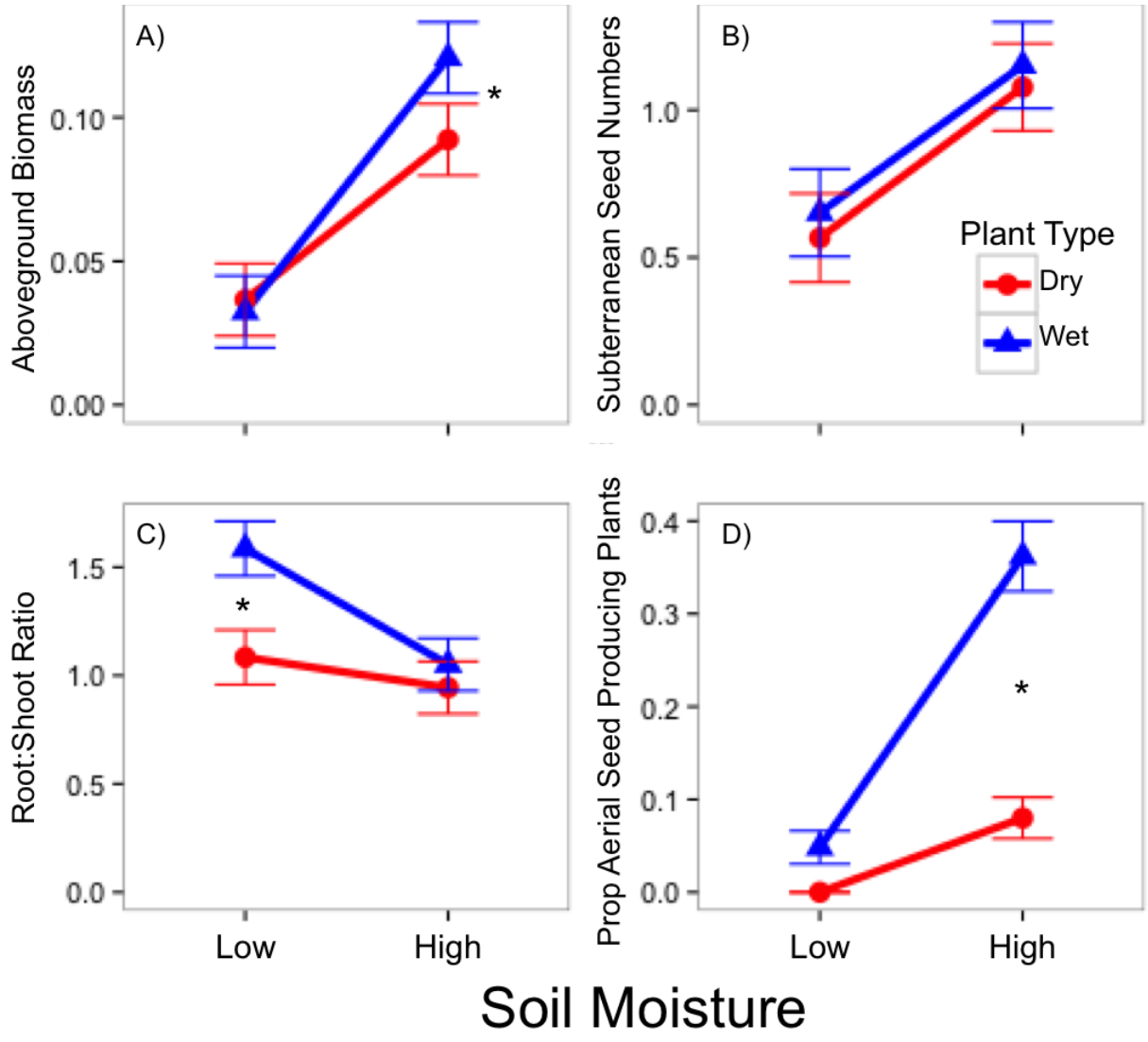

Figure 3.3 Least square means ± 1SE of aboveground biomass (g) (A), number of subterranean seeds (B), root:shoot ratio (C), and proportion of plants producing aerial seed (D) of plants originating from wet (blue line) or dry (red line) moisture types grown under high and low soil moisture conditions in Experiment 2. * indicates statistically significant difference between dry- vs. wet-originating plants within a soil moisture treatment ($P < 0.05$, Tukey's honestly significant difference test (HSD).

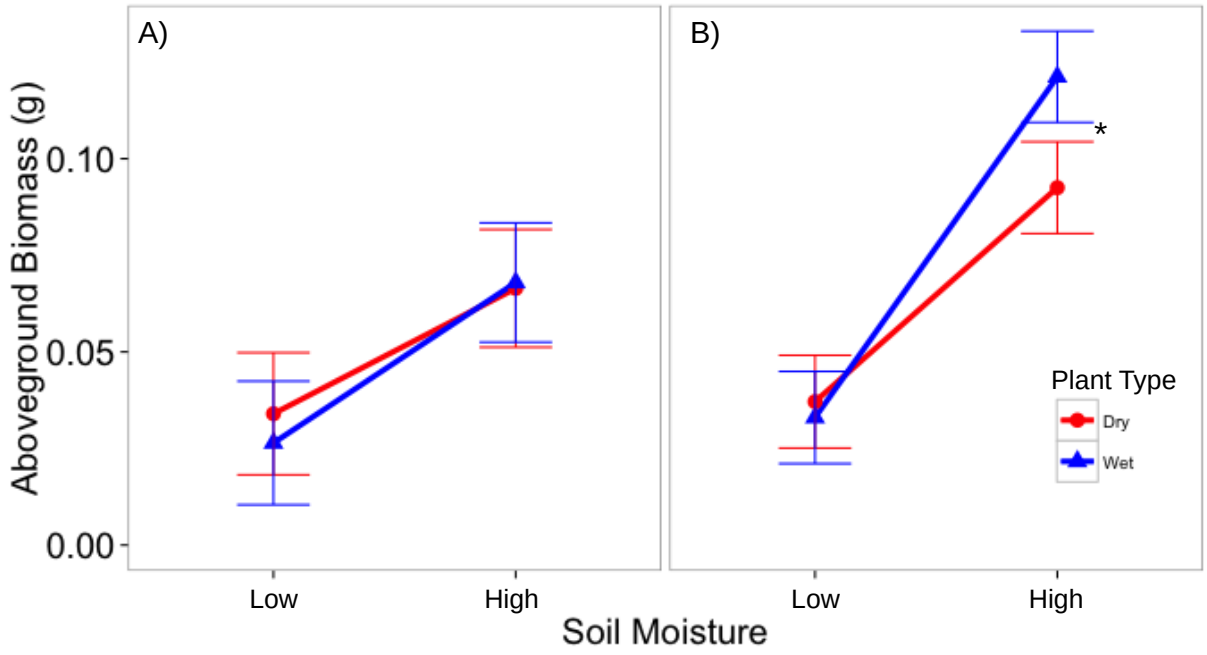

Figure 3.4. Least square means ± 1SE aboveground biomass (g) of non-inoculated (A) and inoculated (B) plants. Plants originating from wet (blue line) or dry (red line) moisture sites were grown under high and low soil moisture conditions in Experiment 2. * indicates statistically significant difference between dry- vs. wet-originating plants within a soil moisture treatment ($P < 0.05$, Tukey's honestly significant difference test (HSD).

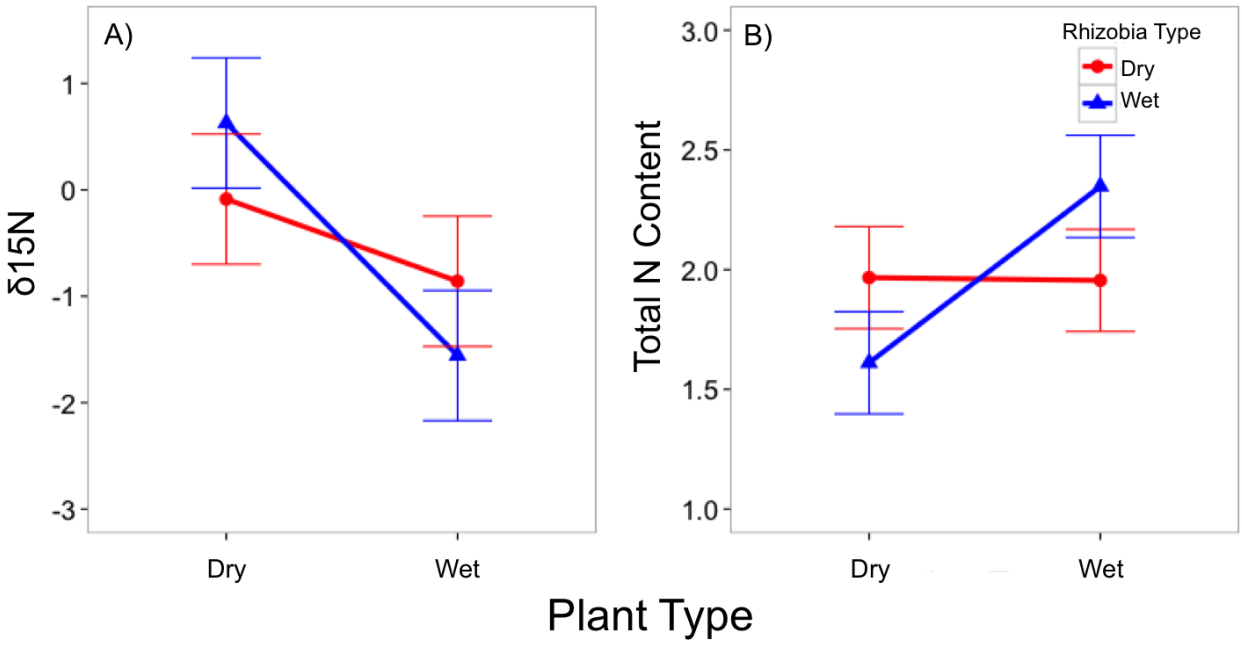

Figure 3.5. Least square means ± 1SE of δ15N (A) and total nitrogen content (%) (B) of plants originating from either wet or dry moisture type inoculated with rhizobia originating from either wet (blue line) or dry (red line) moisture type in Experiment 2. Zero to negative values of $\delta^{15}N$ typically indicates nitrogen fixation (Rodriguez-Echeverria et al., 2009).

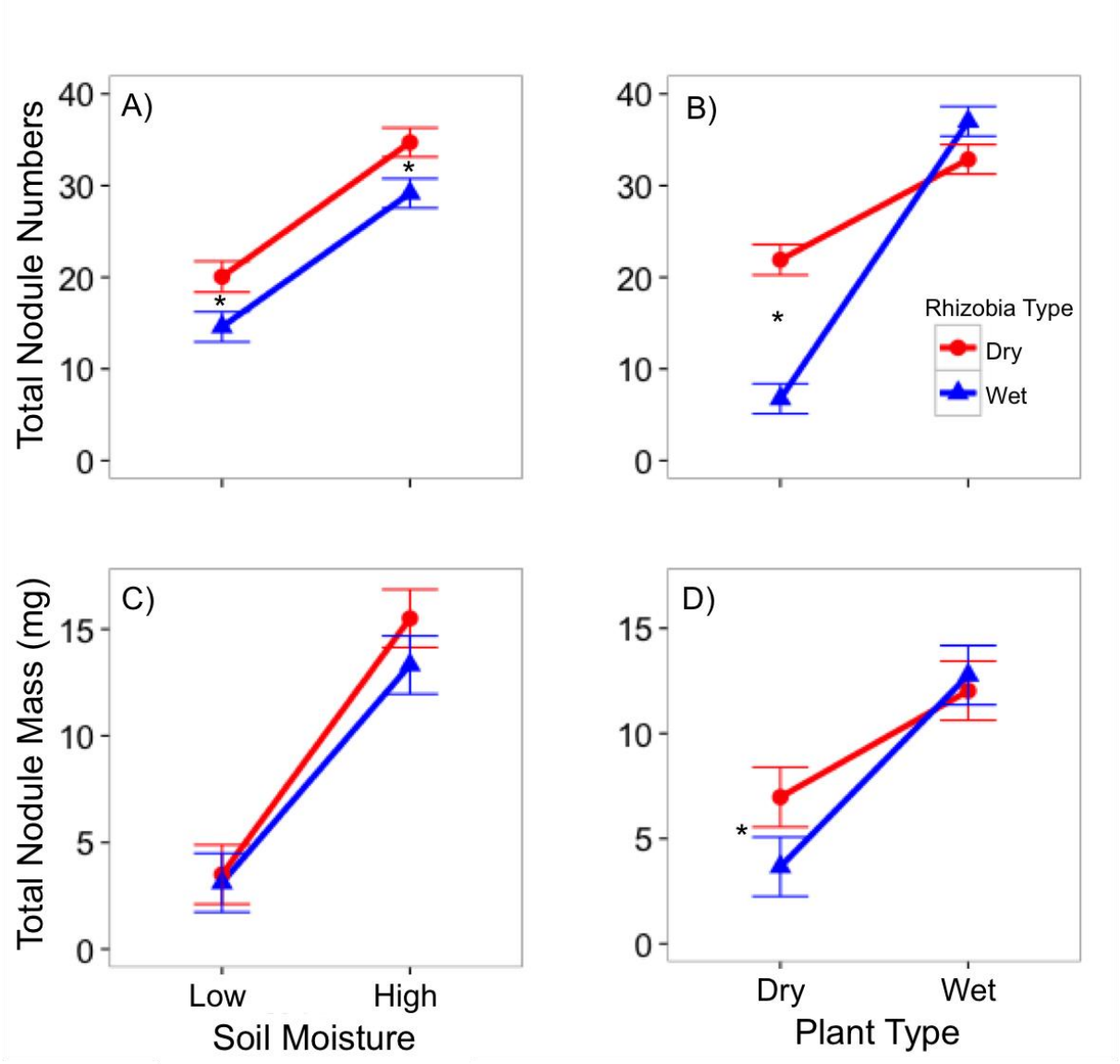

Figure 3.6. Least Square means of total nodule numbers (A & B) and total nodule mass (g) (C &D) of rhizobia originating from either wet (blue line) or dry moisture environments (red line) grown under low or high soil moisture condition (A & C) or associated with plants originating from either dry or wet environments (B & D) in Experiment 2. * indicates statistically significant difference between dry- vs. wet-originating plants within a soil moisture treatment ($P < 0.05$, Tukey's honestly significant difference test (HSD).

Appendix B

Supplementary Materials

Supplementary Materials

Table 3.A1. Field sites used in ecological (Experiment 1) and evolutionary (experiment 2) experiments. Average gravimetric water content (GWC), average photosynthetically active radiation (PAR,) and total soil nitrogen content (N/g Soil) data were collected in May-June 2012 and July 2014. In both 2012 and 2014, three PAR and five soil samples (10cm soil cores) were taken along a transect (1 m intervals) for each *A. bracteata* population. PAR was measured using AccuPAR LP-80 Ceptometer (Decagon Devices, Inc, Pullman, USA). Measurements were taken in clear sky conditions within 2 h of solar noon. GWC was calculated as [wet soil (g) – dry soil (g)]/dry soil (g) * 100. Total soil N analysis was conducted using the field-collected soil in late May-early June 2012 and early July 2014. We performed a KCl extraction using homogenized soil (five soil samples per site) and estimated soil ammonium and nitrate availability with an Alpkem/ OI Analytic Flow Solution IV analyzer (Model 3550) (see Eilts et al. 2011)

Exp	**Year**	**Site Name**	**Acronym**	**GWC (%)**	**PAR (μmol m-2 sec-1)**	**Total Soil N (N/gSoil)**	**GPS**
1& 2	2012	Pierce Cedar Creek C	CCC/ Dry-2	24.10	130±51.2	11.66	N42°32.063 W085°17.619
1	2012	Pierce Cedar Creek B	CCB	30.09	10.3±-0.33	10.87	N42°32.568 W085°17.950

Table 3.A1 (cont'd)

1	2012	Pierce Cedar Creek I	CCI	9.80	42.7±6.36	5.39	N42°32.063 W085°17.444
1	2012	Nature Center A	NCA	20.28	18±1.53	9.48	N42°21.696 W085°34.764
1	2012	Nature Center B	NCB	50.69	29.7±11.7	19.82	N42°21.654 W085°34.764
2	2014	Brook Lodge A	Wet-1	66.41	1397 ± 189.1	10.13	N42°21.357 W085°22.822
2	2014	Brook Lodge B	Dry–1	18.12	75±33.7	5.35	N42°21.498 W085°22.611
2	2014	Pierce Cedar Creek H	Wet-2	103.06	288±50.2	8.65	N42°32.646 W085°17.444
2	2014	Pierce Cedar Creek C	CCC/Dry-2	30.76	130±51.2	8.42	N42°32.063 W085°17.619
2	2014	Carter Lake A	Wet–3	94.06	956±27.7	14.43	N42°40.361 W085°18.036
2	2014	Carter Lake B	Dry–3	19.00	60±22.7	5.03	N42°40.458 W085°18.026

Table 3.A2. Distance between each of the six sites (km) used in this study. Distance was calculated based on latitude and longitude coordinates.

Sites	BLA	BLB	CCH	CCC	CLA	CLB
BLA		0.4	22.2	21.1	35.8	36
BLB			21.8	20.7	35.5	35.7
CCH				1.1	14.3	14.5
CCC					15.4	15.6
CLA						0.2
CLB						

Table 3.A3. Specific predictions and the statistical methods used to test whether habitat-adapted vs. host-adapted rhizobia facilitate plant adaptation to soil moisture (Experiment 2).

Predictions	**Statistical Test**	**Statistical interpretation**
Plants are adapted to “home” soil moisture type	ANOVA: Significant moisture treatment x plant moisture type on plant fitness, followed by Tukey HSD test.	Plant populations transplanted into their "home" soil moisture type have greater fitness than populations from the contrasting soil moisture type
Rhizobia are adapted to “home” soil moisture (i.e. habitat-adaptation)	ANOVA: Significant moisture treatment x rhizobia moisture type interaction on rhizobia fitness, followed by Tukey HSD test	Rhizobia genotypes transplanted into their "home" soil moisture type have greater fitness than the populations from the contrasting soil moisture type

Table 3.A3. (cont'd)

Habitat-adapted rhizobia facilitate plant adaptation to soil moisture	ANOVA: Significant moisture treatment x rhizobia moisture type interaction on plant fitness, followed by Tukey HSD test	Plant populations have greater fitness when the rhizobia type and moisture treatment match
Rhizobia are adapted to local host plants (i.e. host-adaptation").	ANOVA: Significant plant population x rhizobia population on rhizobia fitness, followed by Tukey HSD test	Matching of plant and rhizobia populations leads to greater *rhizobia fitness* than mismatching
Plants are adapted to local rhizobia	ANOVA: Significant plant population x rhizobia population on plant fitness.	Matching of plant and rhizobia populations leads to greater *plant fitness* than mismatching

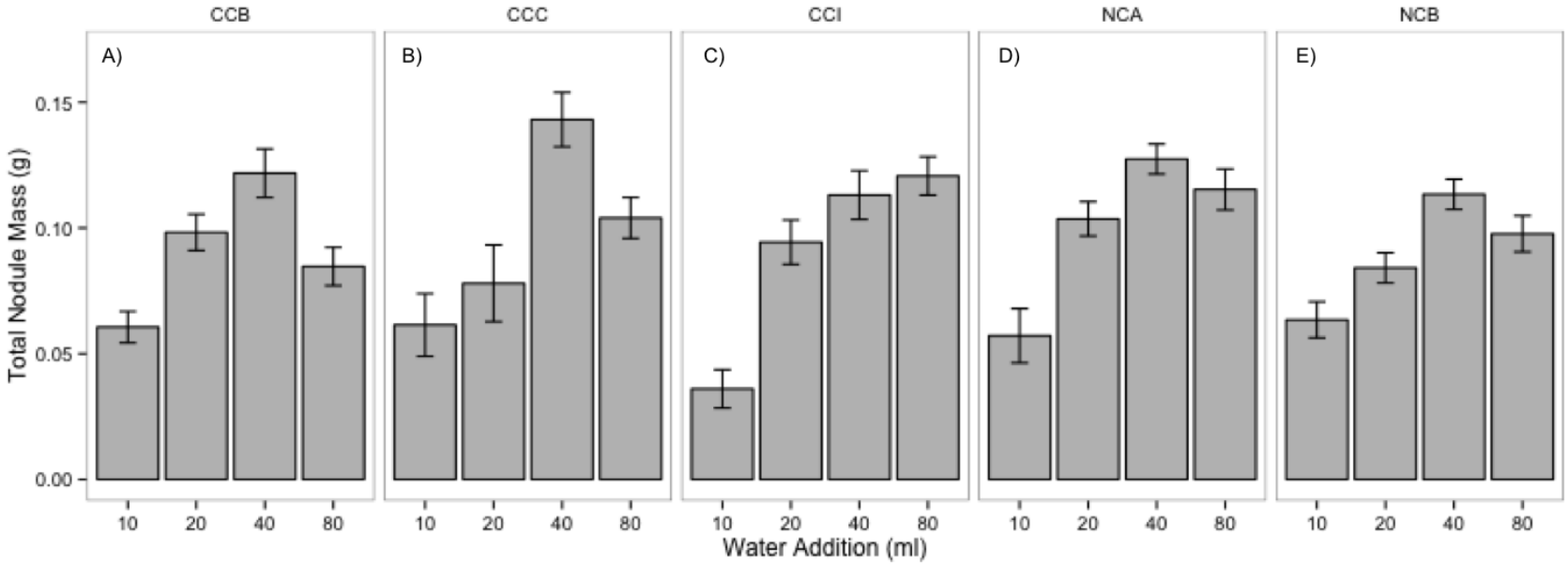

Figure 3.A1. Total nodule mass (g) of plants from five different sites (panels) growing under four levels of water addition treatments in Experiment 1 (mean + SE).

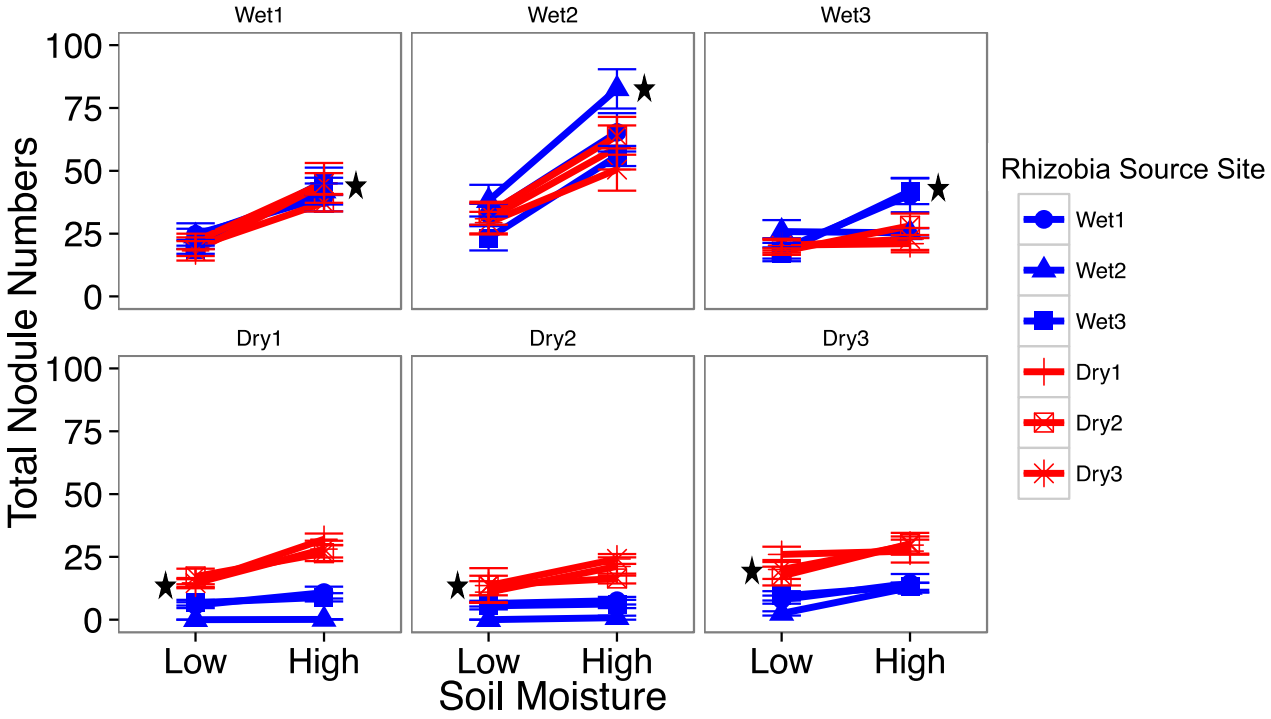

Figure 3.A2. Nodule numbers of plants growing under either high or low soil moisture condition and inoculated by rhizobia originating from each of site. Each panel indicates one of the six host plant populations and different lines indicate rhizobia inoculation from different sites. Blue lines indicate rhizobia originating from wet soil moisture type and red lines indicate rhizobia originating from dry soil moisture type. Black stars show the combination when plant population, rhizobia population and sol moisture condition match.

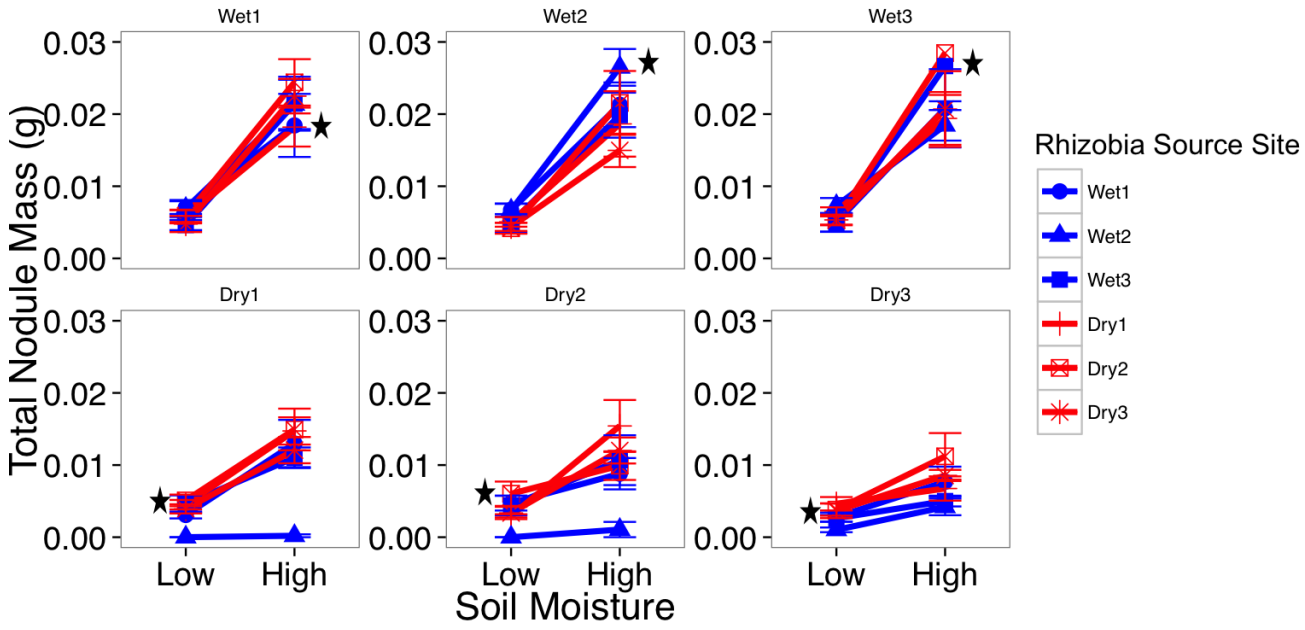

Figure 3.A3. Total nodule mass (g) of plants growing under either high or low soil moisture condition and inoculated by rhizobia originating from each of site. Each panel indicates one of the six host plant populations and different lines indicate rhizobia inoculation from different sites. Blue lines indicate rhizobia originating from wet soil moisture type and red lines indicate rhizobia originating from dry soil moisture type. Black stars show the combination when plant population, rhizobia population and soil moisture condition match.

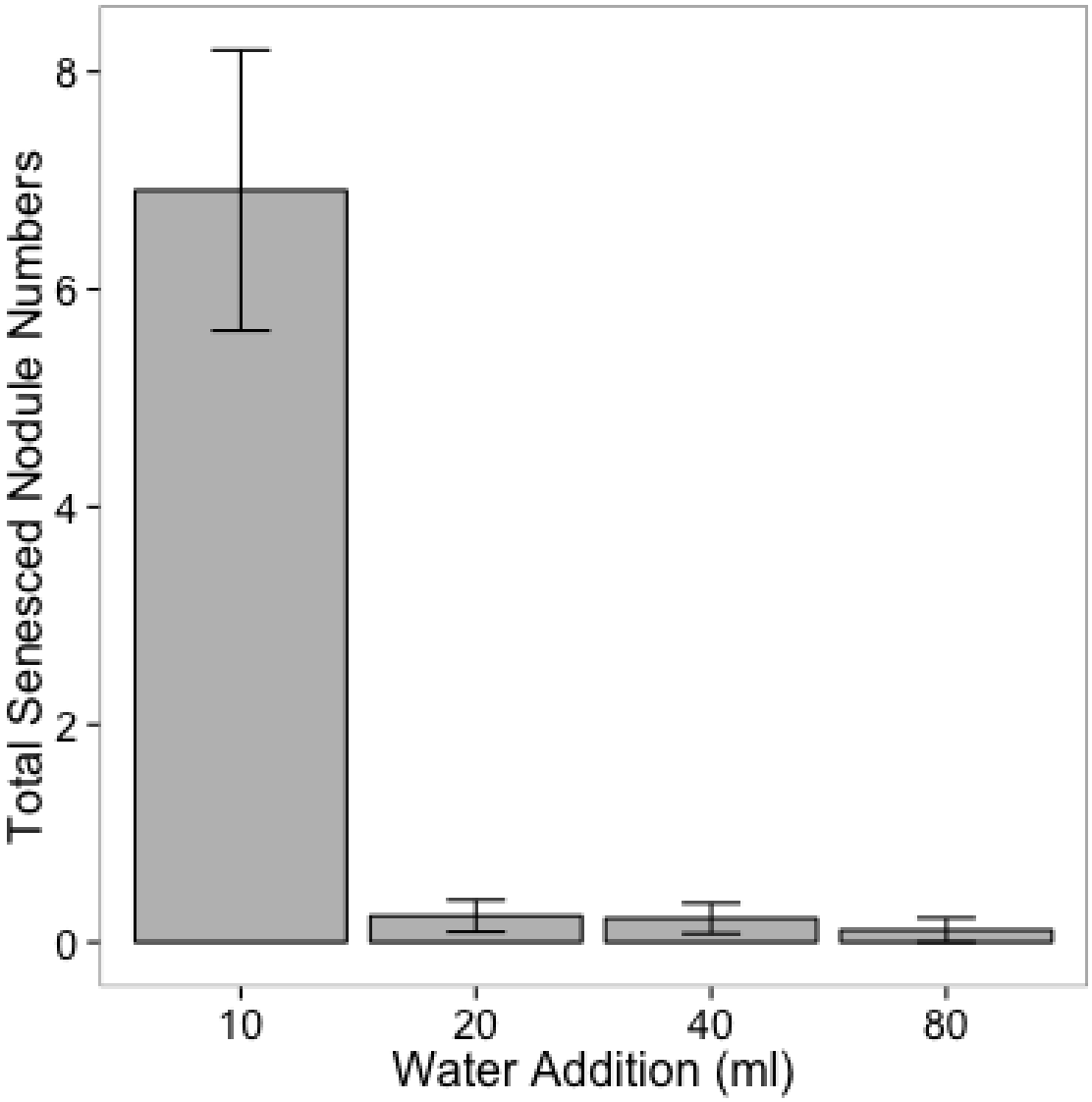

Figure 3.A4. Least square means total senesced nodule numbers of inoculated plants growing under four different soil moisture conditions in Experiment 1.

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CHAPTER 4:

LIMITING TRADED RESOURCES CAUSE CONTEXT DEPENDENCY IN MUTUALISM

INTRODUCTION

Mutualism theory predicts that individuals benefit by specializing in production of a resource (or service) and trading it for other limiting resources (Yu 2001, Johnstone and Bshary 2002, Foster and Wenseleers 2006, Grman et al. 2012). As a result, organisms engaged in mutualism can access more resources than they could otherwise acquire on their own (Schwartz and Hoeksema 1998, Hoeksema and Schwartz 2003). Plants are often limited by nutrients such as nitrogen (N) and phosphorus (P), whereas belowground symbiotic microbes, such as mycorrhizae and rhizobia, are often limited by carbon (C) (Eissenstat et al. 1993, Johnson 1993, Johnson et al. 1997). By exchanging these limiting resources, both plants and microbes can increase their fitness (Bruno et al. 2003, Afkhami et al. 2014). In order for the interactions to be mutualistic, the benefits to each interactor must exceed their costs (Johnson et al. 1997, Schwartz and Hoeksema 1998).

However, mutualism is often context-dependent, and the net benefit of the symbiotic interaction may depend on environmental conditions (Bronstein 1994, Neuhauser and Fargione 2004, Heath and Tiffin 2007, Hoeksema et al. 2010). Both mathematical and descriptive models (Johnson et al. 1997, Schwartz and Hoeksema 1998, Gomulkiewicz et al. 2007) predict that when the availability of limiting resources changes the cost-benefit ratio of engaging in trade, the interaction will shift along a continuum from mutualism to parasitism. For example, symbiotic rhizobia occupy root nodules of leguminous plants where they convert atmospheric nitrogen to ammonium (NH^+_4), making it available to host plants in

exchange for photosynthetic carbon (C). However, the outcome of plant-rhizobia interactions depends on N availability, which in turns affects the cost-benefit ratio of exchanging N and C (Heath and Tiffin 2007, Lau et al. 2012). The cost can outweigh the benefit when N availability in the soil is high because it is less expensive for legumes to obtain N directly from the soil than to allocate C to rhizobia (Herridge and Pate 1977, Kaschuk et al. 2009). In some cases, however, host plants can control the formation and growth of nodules to minimize costs associated with symbiosis (Denison 2000, Kiers et al. 2003, Regus et al. 2014), and reducing rhizobium fitness. Thus resource availability and plant plasticity can shift the cost/benefit ratio for both interacting partners.

To test the context dependency of resource mutualism, one needs to manipulate the availability of all traded resources (Johnson 2010) and measure the fitness consequences to both interacting species. To our knowledge, there are only two experiments that have empirically manipulated both traded resource in the legume-rhizobium symbiosis (Lau et al. 2012, Regus et al. 2015). Lau and coauthors (2012) found that rhizobia significantly increased *Glycine max* (soybean) biomass under high light conditions but not under low light conditions and that a fertilizer treatment did not significantly alter rhizobia effects on plants. This study used commercial soybean seeds and rhizobia strains with unknown evolutionary history, making the generalization of the results to natural coevolved systems difficult. Regus et al (2015) examined the effects of seasonal light input, nitrogen and rhizobia genotypes on *Lotus strigosus*. Seasonal light input was manipulated by conducting two experiments, one in the Fall and one in the Winter. They detected net-reduction or elimination of rhizobia benefit to plants under high nitrogen treatments. However, their

experimental design had limited statistical power to test the light effects on plant-rhizobia since they did not explicitly manipulate or replicate light availability.

Here we investigate how the symbiosis between the native annual legume species *Amphicarpaea bracteata* and its symbiotic partner *Bradyrhizobium sp* (hereafter rhizobia) respond to changes in the availability of the traded resources, C and N. Based on Johnson's et al (1997) descriptive model, we predict that high light and low nitrogen is the "most favorable" environment for both partners because each of them can provide the other's limiting resource. Because nitrogen fixation is an energetically costly process, consuming up to 14% of photosythetically-fixed C by the plant (Kaschuk et al., 2009), the carbon costs to the plant can only be supported when C is in surplus (under high light availability). In contrast, when N is abundant in the soil, we predict rhizobia benefits to plants will be reduced or eliminated. In this scenario, plants can obtain N directly from the soil while rhizobia may continue to take up C from the plants. In an extreme case, rhizobia could become parasitic to plants by continuing to take up C when the plant does not need rhizobially-fixed N (Denison 2000, Kiers et al. 2003, Lau et al. 2012). Alternatively, plants may limit association with symbiotic rhizobia either through reduction in nodule formation and/or reduced resource allocation to root nodules (West et al. 2002, Simms et al. 2006, Sachs et al. 2010b, Heath et al. 2010). Plant control over nodule formation depends on many factors including rhizobia genotypes (Endre et al. 2002, Radutoiu et al. 2003, Sachs et al. 2010b, Regus et al. 2014), traded resource availability (Streeter and Wong 1988, Parsons et al. 1993, Heath and Tiffin 2007, Lau et al. 2012) and other environmental contexts (e.g. Porter and Simms 2014, Suwa In Prep).

We experimentally manipulated both light and N availability and measured the fitness consequences for both partners to ask how light and nitrogen availability affect

plant growth and fitness, rhizobia fitness, and the outcome of the legume-rhizobium symbiosis. Because mutualism outcomes can be genotype dependent (Parker 1995, Johnson et al. 1997, Heath and Tiffin 2007) and because plant genotypes may differ in control over carbon allocation to rhizobia (Heath 2010, Regus et al. 2014) we include four plant populations in our study that were collected from a range of light and nitrogen environments.

METHODS

Study system

A. bracteata has a mixed mating strategy, possessing both chasmogamous and cleistogamous (both aerial and subterranean) flowers (Schnee and Waller 1986). In late June to July, they begin growing axillary shoots that produce subterranean seeds. Seeds used in this experiment are the offspring from greenhouse reared maternal plants from subterranean cleistogamous seeds collected from four populations in southwestern Michigan in 2011: Brook Lodge, Pierce Cedar Creek Institute, Fort Custer Training Center and Lux Arbor Reserve (Supplementary Material Table A1). These populations span a range of light and soil nitrogen conditions (Supplementary Material Table A1). Rhizobia strains were also isolated from plant nodules in the same four populations (detail below).

Experimental treatments

To examine the effects of resource availability on *Amphicarpaea bracteata* -rhizobia interactions, we manipulated light and nitrogen conditions and the presence of rhizobia in a greenhouse experiment at W.K. Kellogg Biological Station. The general design is a split-

plot design with light treatments applied at the whole plot level and nitrogen and rhizobia inoculation treatments applied to randomly selected plants within each light treatment. In total, we had 345 plants with 4-18 replicates per plant population per treatment combination. These plants represented 4-11 full-sib families per population. Sample sizes are uneven because of unequal germination of different plant populations.

To surface sterilize seeds and remove potential rhizobia contamination, we immersed seeds in commercial bleach (5.25% NaCIO) for 1 minute followed by 10 minutes of rinsing with deionized water. We physically scarified the sterilized seeds by nicking the seed coat and germinated them in petri dishes with wet filter paper in the dark for approximately 10 days. Thirteen days after imbibition, seedlings were transplanted into square pots (754 cm^3) filled with a 3:3:3:1 mix of potting media (Sunshine Mix LP5®), peat moss (Greensmix Sphagnum Peat Moss ®, Waupaca Northwoods LLC), sand (Quikrete Tubesand® No. 1159, Quikrer Interntional Inc.), and perlite (Horticultural Perlite ®, Midwest Perlite Inc.) and placed under the shade cages (see below) on 27 June, 2013. Plants were bottom watered, to prevent contamination from splashing, every 3-5 days to the point of saturation.

Light Treatment: We manipulated light availability by placing shade cages over the plants in the reduced light treatments. We used 80% shade cloth (Gempler's, Madison, WI), which reduced light transmittance by 75.3% (72.33± 8.08 μmol m^{-2} sec^{-1}). For the ambient light treatment, we placed cages with no shade cloth over the plants (292.50±20.25 μmol m^{-2} sec^{-1}). We prepared 12 cages in total, 6 shade cages and 6 control cages. Photosynthetically

active radiation (PAR) measurements of each light treatment were taken in clear sky conditions within 2 hours of solar noon.

Nitrogen Treatment: We manipulated N availability by applying ammonium nitrate (NH_4O_3) solution to half of the plants. 13 days after the seedling transplant, 4 ml of 20,000 ppm of ammonium nitrate in deionized water was applied (20g NH_4NO_3/ L). Four mL of ammonium nitrate (30,000 ppm) was then applied every two weeks for the remainder of the experiment. An equivalent amount of water was applied to control plants during each N-application as a control. In total, we added 112 mg N to each pot (100cm^2). This amount is similar to the most extreme rates of N deposition in the long-term studies in North America (95 kg N/ha/year; Bobbink et al. 2010). To supplement micronutrients, all experimental plants received 50ml of soluble trace element mix with no nitrogen (Peters Professional® S.T.E.M.) at low application rate (1tsp/9 gallons) on 23 July 2013. In addition, to test the effectiveness of our soil N treatment, following plant harvest we homogenized the potting soil, performed a KCl extraction, and estimated soil ammonium and nitrate availability with an Alpkem/ OI Analytic Flow Solution IV analyzer (Model 3550) (see Eilts et al. 2011). We confirmed that N addition significantly increased total soil inorganic N (Low N: 0.75 ± 0.067 N/g soil, High N: 4.04 ± 0.561 N/g soil, $F_{1,36} = 35.28$, $P < 0.001$); these values are within the range of soil N availability observed in our study populations (Supplementary Material Table A1).

Rhizobia Treatment: One day after the first nitrogen treatment application, we inoculated half of the plants in each resource treatment with a mixture of eight rhizobia strains (two isolates per population, isolated from different nodules of different plants). Rhizobium strains from root nodules were collected and isolated from two *A. bracteata* plants from each of the four

populations (Supplementary Material Table A1) in mid July 2012. We first removed nodules from the root, surface sterilized them with commercial bleach (5.25 % NaOCI) for 1 min, and then triple rinsed them with sterile water. Following standardized techniques (Somasegaran and Hoben 1994), we plated nodules on modified arabinose gluconate media (MAG media; van Berkum, 1990) multiple times to isolate single colonies of rhizobia. We then combined 8 of these strains to create the mixed inocula used here. We used a mixed inocula to insure that all plants were inoculated with at least two strains originating from the same site as the seeds. Mixed inocula better mimic the diversity of rhizobia typically encountered under natural field conditions than single strain inoculations and allow for partner choice. Inoculant was prepared by incubating each strain in 30ml MAG liquid media in a shaking incubator at 28 °C (180 rpm) for five days. All of the liquid cultures were diluted to an optical density of 1.0 using a spectrophotometer to standardize the number of rhizobia cells per strain. Then, 50 ml of each culture were combined, and 400ml of MAG media was added to dilute the inoculant by 50%. We used this mixture to inoculate half of the plants in each resource treatment with 4ml of inoculant (approximately 5×10^{10} cells). The remaining plants were inoculated with an equal amount of MAG media as a control. One week later, we repeated the same procedure to ensure inoculation. Then we covered the soil surface with *ca*. 100 ml of autoclaved Turface MVP® (PROFILE Products LLC, Buffalo Grove, IL) to minimize rhizobia cross contamination.

Data collection

To evaluate nitrogen fixation and nitrogen and carbon content of leaf tissues, we randomly selected the youngest fully developed leaf from one individual within each treatment combination (2 light x 2 nitrogen x 2 rhizobia x 4 plant population = 32) and

ground the dried leaf tissues in a 1.2ml tube with a stainless steel bead using TissueLyser II (Qiagen, Germantown, MD, USA) for four minutes. Samples were weighed in tin cups and sent to the Stable Isotope Facility (UC Davis, USA) to analyze the amount of C and N using a PDZ Europa ANCA-GSL elemental. Nitrogen fixation was estimated using $\delta^{15}N$. Zero to negative values of $\delta^{15}N$ typically indicates nitrogen fixation (Rodriguez-Echeverria et al. 2009).

To estimate plant fitness, we harvested the experiment between 13-20 Sept 2013, and estimated total above- and belowground biomass after drying plant material for >3 days at 65°C. We also counted seeds but none of the plants produced aerial seeds and only 16% of plants produced subterranean seeds. It is likely that greenhouse conditions delayed the phenology of seed production, and as a result, seed production may reflect differences in phenology rather than differences in fitness. Thus, seed data are not discussed further.

To estimate rhizobia fitness, we collected and counted the total number of nodules and estimated nodule biomass after drying nodules for >3 days at 65°C. Thirty-six plants died prior to harvest and were removed from the analysis (no statistical variation in survival among treatments: Light $\chi^2_1 = 0.23$, $P = 0.63$; Nitrogen $\chi^2_1 = 0.16$, $P = 0.69$; Rhizobia $\chi^2_1 = 1.12$ $P = 0.29$, Genotype $\chi^2_3 = 5.75$, $P = 0.13$). Also, three plants in the uninoculated control treatment (1.6%) produced some nodules and were removed from the analysis due to contamination.

Statistical analysis

To test the effects of light, nitrogen and rhizobia on plant nodulation and growth, we used a mixed model ANOVA in R (lme4 Version 3.0.2). Aboveground biomass was natural log transformed and mean nodule mass was square root transformed to improve normality. Nodule numbers were analyzed using a Poisson distribution. Our full model included light, nitrogen,

rhizobia, plant population and all interactions as fixed factors. Greenhouse bench nested within light was included as a random factor, except for the analysis of C content, N content and $\delta^{15}N$ because one randomly-chosen subsample for each treatment combination was analyzed. For all analyses, when we found significant interactions, we performed a Tukey's honestly significant difference test (HSD) to evaluate differences among treatments.

RESULTS

Light and nitrogen effects on rhizobium growth and fitness

Reduced light significantly decreased nodule number by 43.2% but only under low nitrogen conditions (significant L x N interaction, Table 1, Figure 1); nodule numbers were uniformly low under high nitrogen. However, reduced light did not affect mean nodule mass (Table 1, Figure 1). Nitrogen addition significantly reduced nodule numbers and mean nodule mass under both ambient and reduced light treatments (Table 1; Figure 1). In fact, 69.1% of inoculated plants grown under high N did not produce nodules at all, as compared to 4.6% for plants in low nitrogen. For the plants that produced at least one nodule, N addition reduced nodule number by 86.7 % and mean nodule mass by 52.0% (Figure 1). Note that these results were consistent even after standardizing the nodule numbers and mass by plant aboveground biomass (data not shown).

A significant light x nitrogen x plant population interaction effect on total nodule numbers indicates that some plant populations are better than others at reducing nodule production when costs of mutualism outweigh benefits (Table 1, Supplementary Material Figure A1). This pattern is driven by differences in plants ability to reduce nodule numbers under low light in low N environments. Although all populations tended to produce fewer nodules in low

light when grown in low nitrogen conditions, plants originating from Brook Lodge and Pierce Cedar Creek reduced nodule production by 63.7% and 51.9% respectively. In contrast, plants from Fort Custer and Lux Arbor reduced nodulation to a lesser degree (39.5% and 34.2% respectively). Additionally,

under ambient light and high N, plants originating from Brook Lodge and Fort Custer produced virtually no nodules. In contrast, plants from Lux Arbor and Pierce Cedar Creek were not able to control nodulation completely (Lux Arbor: 2.0±0.94, Pierce Cedar Creek: 4.91±1.84).

Light and nitrogen effects on plant growth and fitness

Nitrogen addition strongly altered rhizobia effects on plants (Tables 2&3 and Figures 2&3). Under low N, rhizobia increased plant aboveground biomass by 68%, increased C content by 6%, increased N content by 106% , and reduced root:shoot ratios by 38.8% (Table 2&3 and Figures 2&3). 1δ5N was greater under N addition than control treatment, suggesting that N fixation was significantly reduced in high N treatments (Figure 3). Under high N, however, rhizobia did not affect aboveground biomass, C content, N content, N-fixation or root:shoot ratio, suggesting that rhizobia provided little fitness benefit under these conditions (Table 2&3 and Figure 2&3). Interestingly rhizobia tended to reduce C content under high N, indicating that associating with rhizobia is costly, although the pattern was not statistically significant (Tukey HSD test $P = 0.23$, Supplementary Material Figure A2). A significant light x N x rhizobia interaction on root:shoot ratio indicates that rhizobia altered plants' resource allocation depending on the light and N conditions. For example, under low N, rhizobia reduced root:shoot ratios, but the magnitude of this effect was greater under ambient than reduced light (Table 2 Figure 2C). Under high N, rhizobia had no effect on root:shoot ratio in ambient or reduced light

treatments (Figure 2D).

In addition, we detected a negative correlation between total nodule number and $\delta^{15}N$ for plants grown under low N (Supplementary Material Figure A3, $R^2 = 0.54$, $F_{1,14} = 112.79$, $P < 0.001$), suggesting that nitrogen fixation increased as nodule number increased. Further, nitrogen fixation (and total nodule numbers) was greatest in the ambient light/low N treatment and lowest in high N treatments, regardless of light treatment (Figure 3, Supplementary Material Figure A3), consistent with our hypothesis. Finally, although there was variation in root:shoot ratio among plant populations, all populations responded similarly to light, N and rhizobia treatments (no interactions with population in Tables 2 & 3).

DISCUSSION

Plants have engaged in symbiotic relationships with microbes including, mycorrhizae, endophytes, and rhizobia for ~60 -400 million years (Herendeen et al. 1999, Lavin et al. 2005, Krings et al. 2007, Mondo et al. 2012). Yet, these mutualisms are believed to be inherently unstable (May 1981). Both empirical (Johnson 1993, Johnson et al. 1997, Hoeksema et al. 2010, Lau et al. 2012, Regus et al. 2015) and theoretical (Hoeksema and Bruna 2000, Johnstone and Bshary 2002, Neuhauser and Fargione 2004, Thrall et al. 2007) studies have shown that plant-microbe symbioses can be dynamic, ranging from mutualism to parasitism. In our study system, we predicted that both plants and rhizobia would benefit the most from symbiosis under high light and low N conditions and the least under reduced light and high N conditions. Interestingly, we found that plant and rhizobia responses were asymmetric. For rhizobia, fitness benefit through symbiosis depended on both light and nitrogen availability. For plants, fitness benefit through symbiosis depended mostly on nitrogen.

Effects on rhizobium growth and fitness

For rhizobia, symbiosis was most beneficial under low N and ambient light conditions, where plants require N fixed by rhizobia and have the surplus C to reward the rhizobia. Symbiosis was least beneficial under high N conditions (Figure 1) where plants presumably reduce allocation to rhizobia even when C may be readily available. Not only did rhizobia have the highest fitness under low N and ambient light conditions, but they also fixed more N in this environment (Figure 3), leading to an increase in plant leaf tissue N content (Table 3, Supplementary Material Figure A2). Under high N, biological N fixation declined to zero, consistent with previous studies showing that nitrogen fixation decreases as soil N increases (Streeter and Wong 1988, Van Kessel and Hartley 2000, Leidi and Rodriguez-Navarro 2008, Gelfand and Robertson 2015).

While nodulation always increases rhizobia fitness (Denison and Kiers 2004, Simms et al. 2006, Heath and Tiffin 2007, Sachs et al. 2010a), plants can regulate nodulation to minimize net-costs under different environmental contexts (Lau et al. 2012, Regus et al. 2015). There are at least two mechanisms by which plants regulate associations with rhizobia. First, plants can reduce formation of nodules (partner choice) by discriminating against ineffective rhizobia under unfavorable conditions (Streeter and Wong 1988, Bollman and Vessey 2006, Simms et al. 2006). Second, they can control nodule size by reducing C allocation to nodules (Denison 2000, West et al. 2002, Akcay and Simms 2011) or reducing oxygen flux to ineffective nodules (i.e. host sanction) (Kiers et al. 2003). In our study system, *A. bracteata* appears to both control nodulation by reducing nodule formation and through reduced allocation after nodule formation in high N treatments

(Figure 1). Plants also reduced nodule numbers in response to N addition more under reduced than ambient light, suggesting that plants reduce allocation to rhizobia when surplus carbon is less available (Table 1, significant L x N interactions).

Interestingly, some plant populations were more effective than others at controlling nodulation. Such variation in plants ability to control nodulation among populations may be related to variation in environmental conditions of home sites. For example, the two populations that reduced nodule numbers most in low light treatments (Brook Lodge and Pierce Cedar Creek) originated from sites that were either high light or high soil moisture, environments where surplus carbon may be commonly available (Supplementary Material Table A1).

Effects on plant growth and fitness

In contrast to the strong rhizobia response to light and N, plant responses to rhizobia largely depended on N. Rhizobia increased aboveground biomass, C and N content significantly under low N, while rhizobia had no effects on any plant traits under high N. Light availability did not alter rhizobia benefits to plants, except for root:shoot ratio. On average, rhizobia effects on plants ranged from mutualism to commensalism (but never parasitism), depending on N environment.

A lack of light x rhizobia interaction on plant traits is surprising, given that light is essential for carbon fixation and rhizobia can consume a significant portion of photosynthetially-fixed C (Kaschuk et al., 2009). Previous studies reported a strong effect of light on plant response to rhizobia (Lau et al. 2012). We have two hypotheses explaining our contradictory result. First, although *A. bracteata* can occupy both shady and sunny

environments ranging from 10.3 and 1691 μmol m^{-2} sec^{-1} (August 2012, Suwa unpublished data), all of the *A. bracteata* populations we used in this experiment originated from sites with low light (mean PAR in August 2012:136.8±76.40μmol m^{-2} sec^{-1}). Thus they are likely sciophytic plants, having greater fitness under shady than ambient light conditions. Our results suggest that these plant populations do not require as much light as other legume species, like *Glycine max* which typically grows in open habitats and showed a strong response to light (Bacanamwo and Harper 1997, Lau et al. 2012). It would be interesting to compare *A. bracteata* populations originating from contrasting light habitats. Second, the ambient light treatment in the greenhouse may not have simulated field ambient light conditions. Although the reduced light treatment had 75.3% lower PAR than the ambient light treatment, ambient light in the greenhouse is about 3.5 times lower than ambient light in the field (1029.3 μmol m^{-2} sec^{-1}).

Asymetric responses of rhizobia and plants to light and nitrogen

We found that rhizobia are more responsive to changes in both light and nitrogen conditions than plants. Part of the reason for asymmetrical response may be that plants are effective at controlling symbiosis when the cost outweighs the benefits. Under high N, plants formed significantly fewer nodules, thereby minimizing the cost of associating with C-sinking rhizobia.

However, it is also well documented that plants cannot always regulate the symbiosis (Denison et al. 2003, Bever et al. 2009). In fact, under certain environmental context, plants can be parasitized by symbionts (Lau et al. 2012, Johnson et al. 1997, but see Regus et al. 2015). In our study system, we found that rhizobia can be costly to plants.

Under high N, plants had lower carbon content when inoculated than not inoculated, and in low N treatments, plants had higher C content when inoculated than not inoculated (Supplementary Material Figure A2). However these effects did not translate into plant biomass.

Conclusion

We experimentally evaluated how availability of traded resources influences the fitness outcomes of plant-rhizobia symbiosis. We found that plants and rhizobia respond differently to changes in resources availability. Consistent with our prediction, symbiosis was most beneficial for rhizobia under ambient light and low N conditions. In contrast, for plants, symbiosis was beneficial only under low N, but did not differ between light treatments. This could be in part because plants are effective at limiting allocation to rhizobia when the cost outweighs the benefit of mutualism, although effectiveness of nodulation control varied among plant populations. Plant control of resource allocation to rhizobia under unfavorable conditions may contribute to the observed stability of this mutualism, over 60 million years of symbiosis between plants and rhizobia.

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APPENDICES

Appendix A

Tables and Figures

Table 4.1. Results from a generalized mixed model testing the effects of light (L), nitrogen (N) and plant population (P) on total nodule numbers and mean nodule mass (g). Bench nested within light was included as a random factor. Total nodule number was analyzed using a Poisson distribution and mean nodule mass was square root transformed to meet normality assumptions. Statistically significant effects are shown in bold *(P* < 0.005).

	Total Nodule Numbers			Mean Nodule Mass		
	df	χ_2	*P*	*df*	χ_2	*P*
Light	1	2.81	0.094	1	1.65	0.199
Nitrogen	1	19.41	**<0.001**	1	6.90	**0.009**
Population	3	1.28	0.735	3	2.23	0.525
L x N	1	7.80	**0.005**	1	0.80	0.371
L x P	3	1.46	0.692	3	2.78	0.427
N x P	3	12.72	**0.005**	3	4.66	0.199
L x N x P	3	8.76	**0.033**	3	3.40	0.334
Bench (L)	1	1.67	0.430	1	3.30	0.070

Table 4.2. Results from a generalized mixed model testing the effects of light (L), nitrogen (N), rhizobia inoculation (R) and plant population (P) on aboveground biomass and root:shoot ratio. Bench nested within light was included as a random factor. Aboveground biomass was log transformed. Model selection was performed to remove three way and/or two-way interactions that were not significant. Statistically significant effects are shown in bold *(P* < 0.05).

	Aboveground Biomass			*Root:Shoot Ratio*		
	df	χ_2	*P*	*df*	χ_2	*P*
Light	1	0.01	0.942	1	8.74	**0.003**
Nitrogen	1	54.47	**<0.001**	1	23.63	**<0.001**
Rhizobia	1	6.53	**0.011**	1	13.31	**<0.001**
Population	3	4.50	0.212	3	10.22	**0.017**
L x N	1	1.03	0.311	1	2.23	0.136
L x R	1	1.07	0.301	1	0.44	0.507
L x P	3	6.25	0.100	3	2.08	0.556
N x R	1	15.76	**<0.001**	1	18.91	**<0.001**
N x P	3	3.57	0.311	3	2.39	0.496
R x P	3	2.27	0.519	3	5.76	0.124
L x N x R				1	10.91	**0.001**
L x N x P				3	0.40	0.939
N x R x P				3	3.60	0.308
R x L x P				3	2.14	0.544

Table 4.2. (cont'd)

R x L x P				3	2.14	0.544
Bench (L)	1	3.46	0.06	1	108.00	<**0.001**

Table 4.3. Results from linear mixed model testing the effects of light (L), nitrogen (N), rhizobium inoculation (R) and plant population (P) on carbon content (%), nitrogen content (%) and δN15 of leaf tissues. Statistically significant effects are shown in bold *(P* < 0.005). * marginally significant results.

	% Carbon			% Nitrogen			δN15		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Light	1,13	0.18	0.681	1,13	0.77	0.397	1,13	5.40	0.103
Nitrogen	1,13	9.94	**0.007**	1,13	18.82	**<0.001**	1,13	76.47	**0.003**
Rhizobia	1,13	9.74	**0.008**	1,13	8.33	**0.012**	1,13	9.43	**0.055***
Population	1,13	1.80	0.198	1,13	0.41	0.748	1,13	4.28	0.132
L x N	1,13	0.96	0.345	1,13	0.29	0.600	1,13	3.12	0.176
L x R	1,13	0.52	0.482	1,13	0.56	0.469	1,13	0.08	0.802
L x P	1,13	1.62	0.233	1,13	0.30	0.823	1,13	0.82	0.564
N x R	1,13	25.50	**<0.001**	1,13	8.22	**0.013**	1,13	8.31	**0.063***
N x P	1,13	1.07	0.394	1,13	1.02	0.417	1,13	1.89	0.308
R x P	1,13	0.55	0.658	1,13	0.96	0.441	1,13	3.09	0.189
L x N x R							1,13	9.93	**0.051***
L x N x P							1,13	2.01	0.290
N x R x P							1,13	5.34	0.101
R x L x P							1,13	0.79	0.573

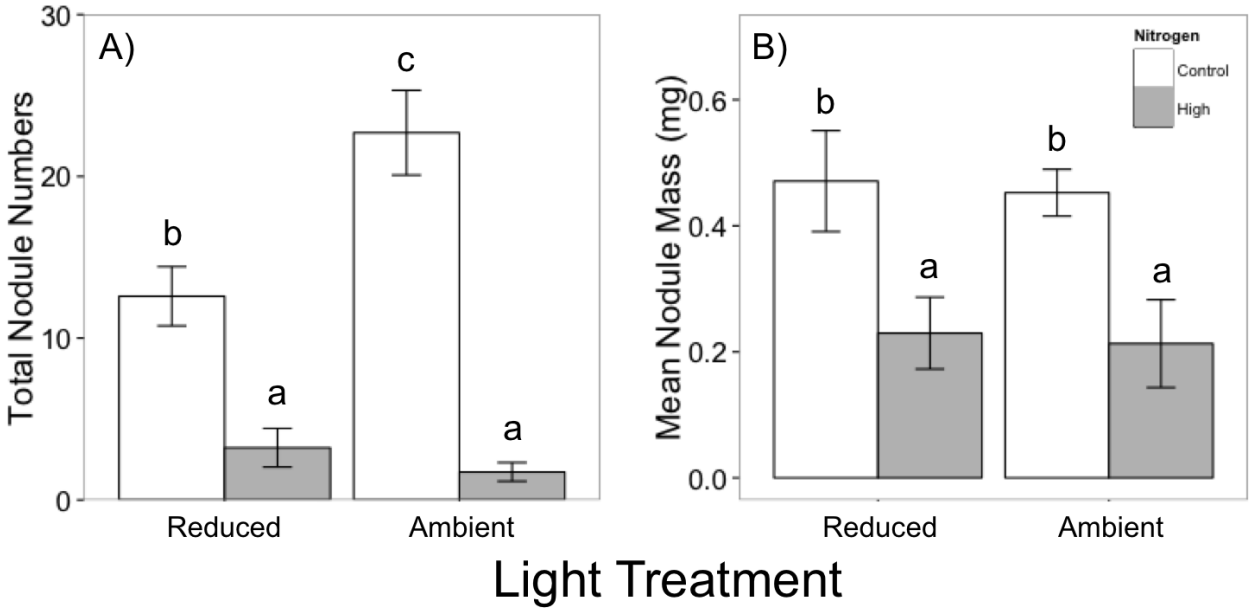

Figure 4.1. Total nodule number (A) and total nodule mass (B) (mean ± SE) of rhizobium-inoculated plants under different light and nitrogen treatment combinations. White and blue bars indicate low and high nitrogen treatment respectively. Bars with different letters differ significantly from each other ($P < 0.05$, Tukey's honestly significant difference test (HSD).

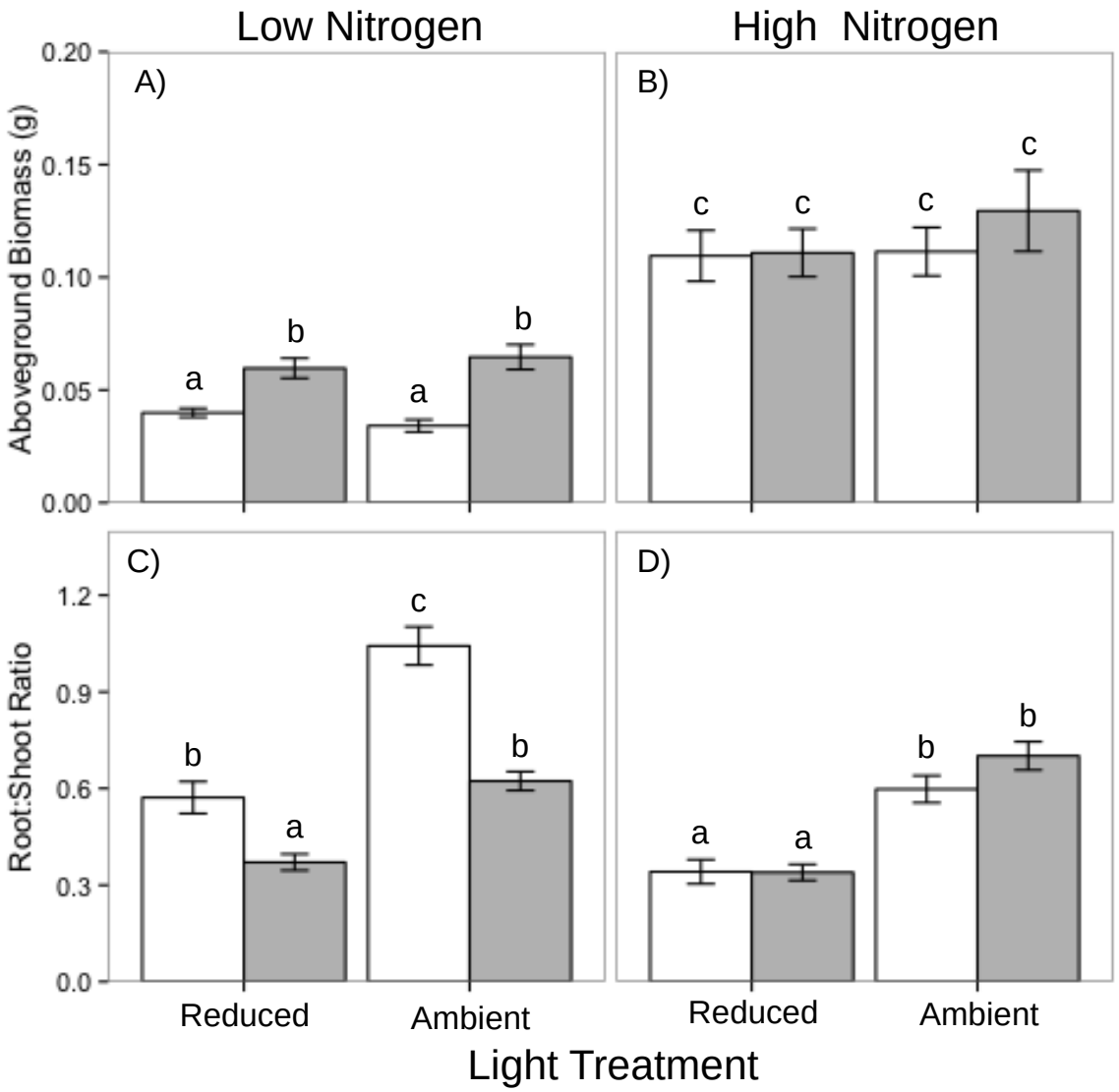

Figure 4.2. Aboveground biomass and root:shoot ratio (A and C) and reduced (B and D) light and low or high nitrogen treatment (mean + SE). White and grey bars indicate plants that were inoculated with media (control) and rhizobia, respectively. Bars with different letters differ significantly ($P < 0.05$, Tukey's honestly significant difference test (HSD).

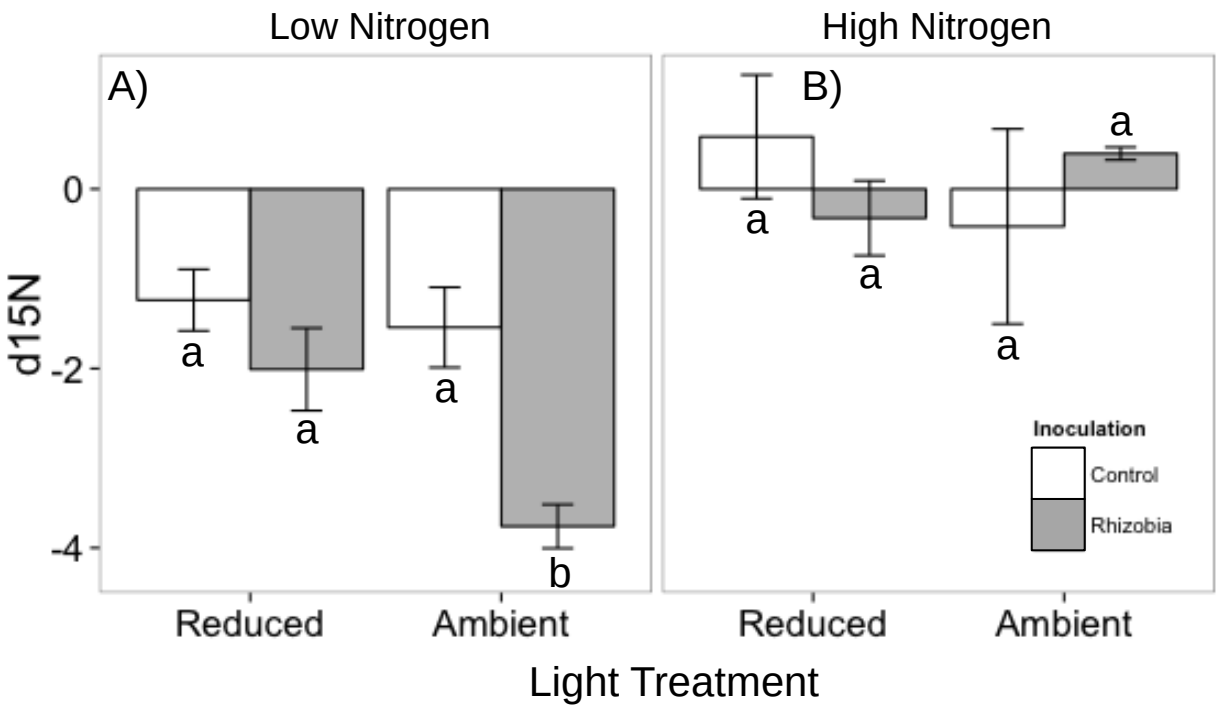

Figure 4.3. δN15 of plant tissues under different light, nitrogen and `rhizobia treatment combinations. More negative δ15N values indicate higher rates of biological nitrogen fixation. Bars with different letters differ significantly ($P < 0.05$, Tukey's honestly significant difference test (HSD).

Appendix B

Supplementary Materials

Table 4.A1. Four field sites used in the greenhouse experiment. Field measurements of: average volumetric water content (VWC, %) in August 2012, average photosynthetically active radiation (PAR, μmol m-2 sec-1) in August 2011 and 2012, total soil nitrogen content (N/gSoil) in May 2012, and GPS coordinates. VWC was obtained using HydroSence II (Campbell Scientific Inc., North Logan, Utah). In August 2012, three measurements of each variable were taken along a transect (1 m intervals) for each *A. bracteata* population. PAR was measured using AccuPAR LP-80 Ceptometer (Decagon Devices, Inc, Pullman, USA)three times along the same transect in as for VWC in 2012. Measurements were taken in clear sky conditions within 2 h of solar noon. Total soil N analysis was conducted using the field-collected soil 2012. We performed a KCl extraction using homogenized soil (5 soil samples per site) and estimated soil ammonium and nitrate availability with an Alpkem/ OI Analytic Flow Solution IV analyzer (Model 3550) (see Eilts et al. 2011).

Site Name	**VMC (%)**	**PAR ($\mu mol\ m^{-2}\ sec^{-1}$)**	**Total Soil N (N/gSoil)**	**GPS**
Brook Lodge	3.40 ± 0.26	804 ± 274	3.13	N42°21.892 W085°22.402
Fort Custer	5.13 ± 0.32	73 ± 38.9	1.53	N42°17.851 W085°19.359
Lux Arbor	4.10 ± 0.75	26 ± 2.52	8.92	N42°28.902 W085°27.841
Pierce Cedar Creek	16.00 ± 1.08	26 ± 14.2	4.90	N42°40.458 W085°18.026

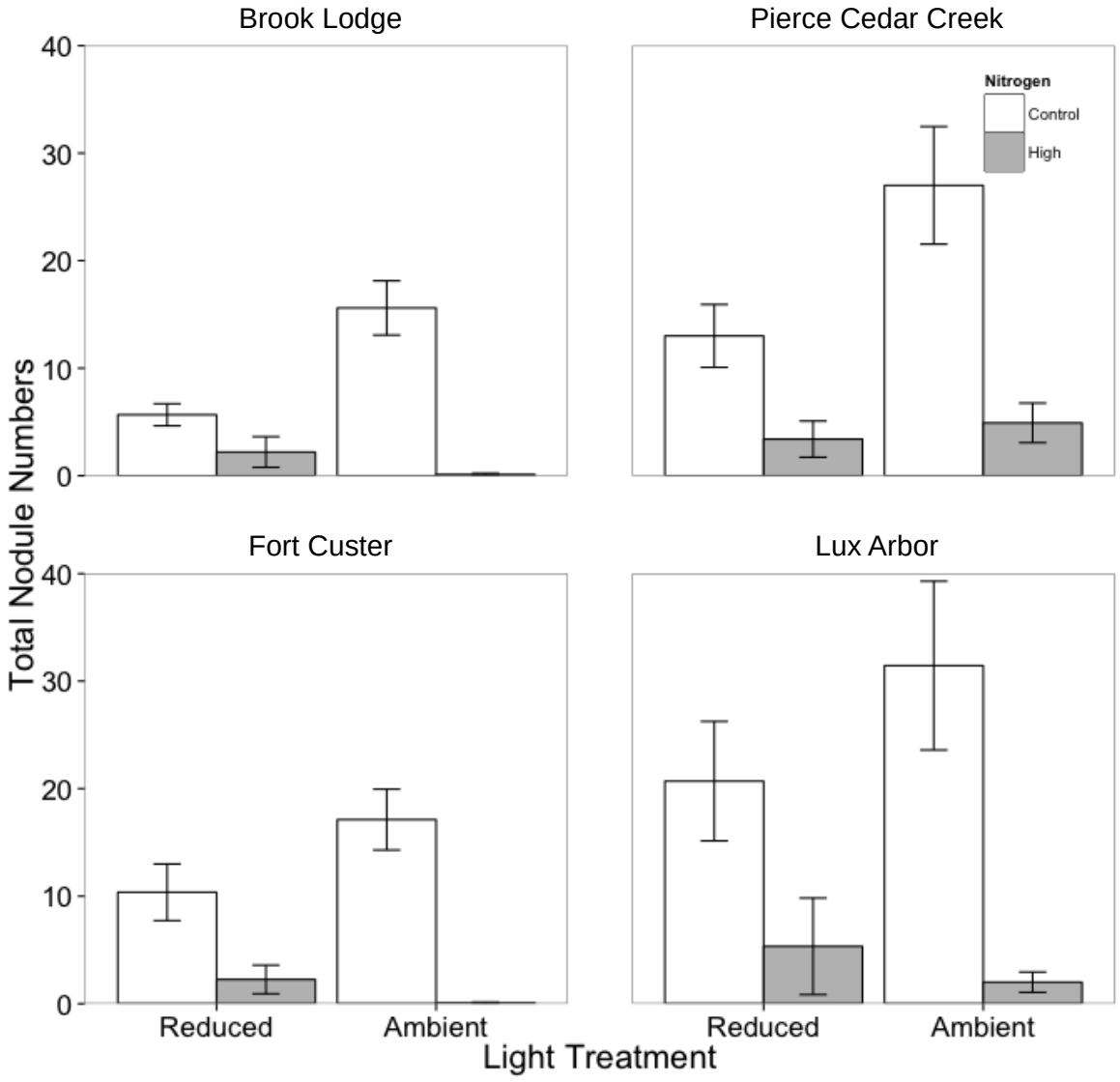

Figure 4.A1. Nodule numbers of plants from each of the four populations growing under different light, nitrogen treatments. Plant populations originated from A) Brook Lodge, B) Pierce Cedar Creek, C) Fort Custer, and D) Lux Arbor. Error bars are standard error of the means. Bars with different letters differ significantly ($P < 0.05$, Tukey's honestly significant difference test (HSD).

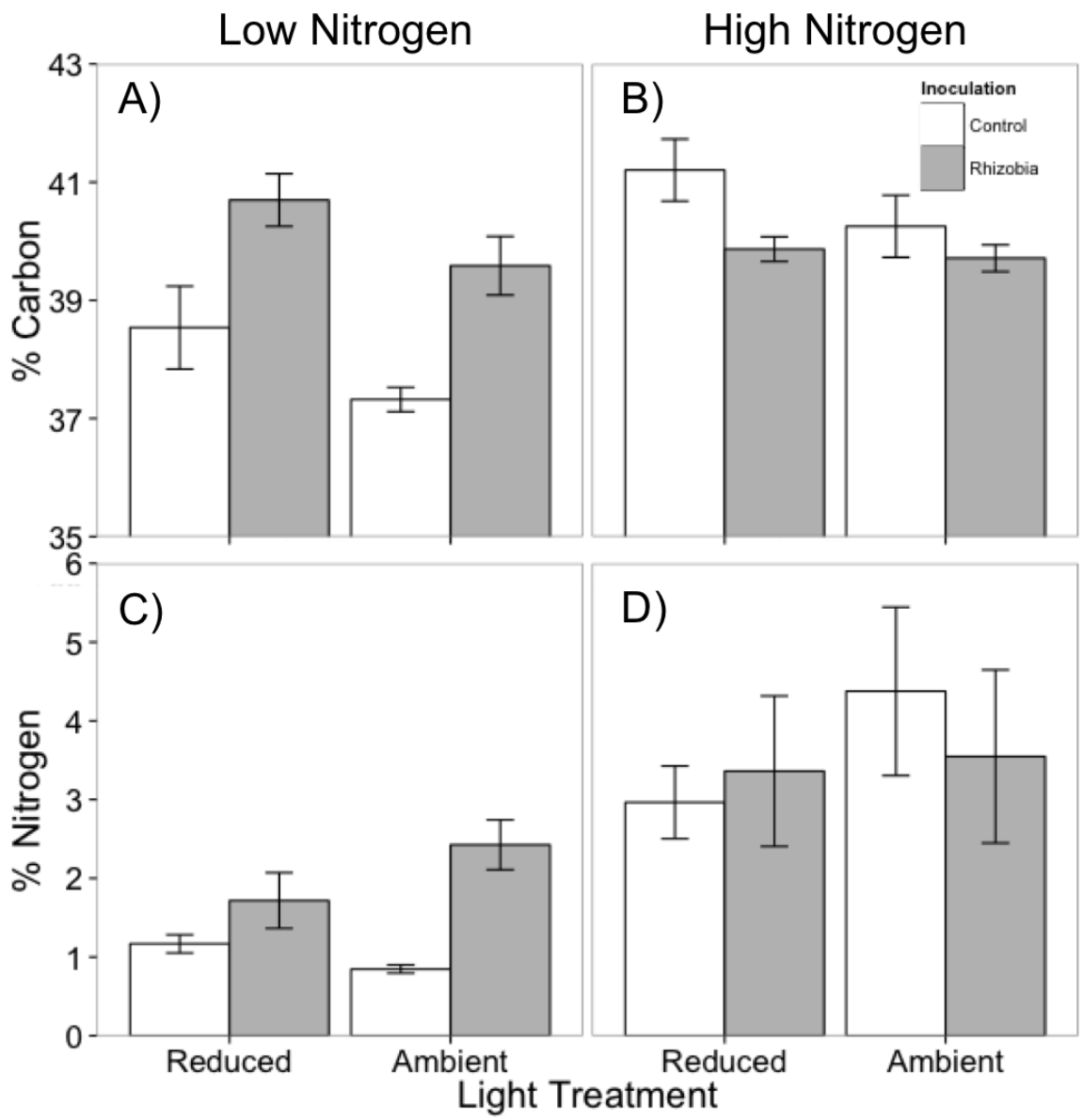

Figure 4.A2. Carbon content (%) and nitrogen content (%) of subsampled leaf tissues (N = 32). Plants were grown under different light, nitrogen and inoculation treatments. White and grey bars indicate control and rhizobium-inoculated treatments, respectively. Error bars are standard error of the means.

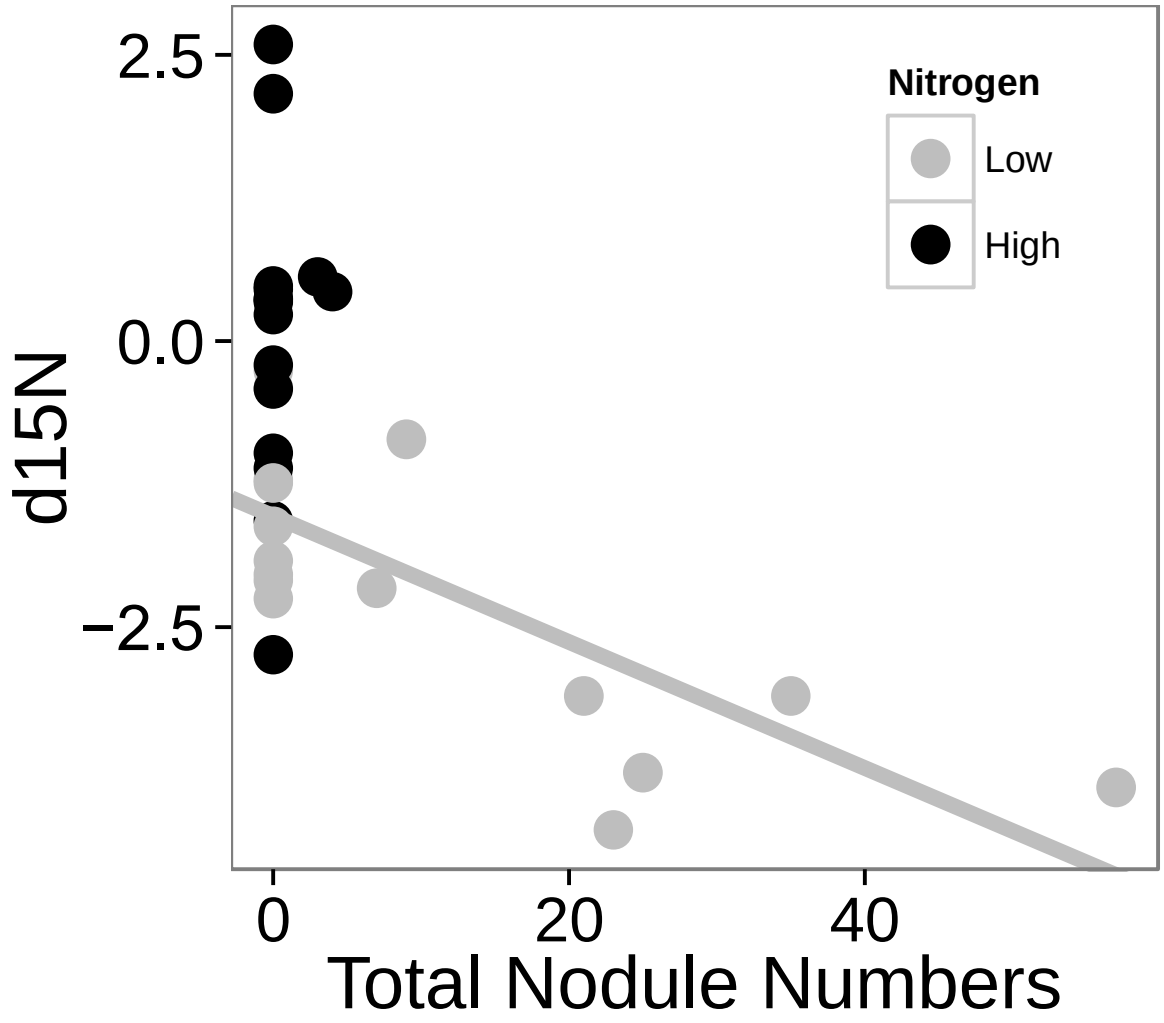

Figure 4.A3. Correlation between total nodule numbers and δ15N of plants growing under low nitrogen (grey points) and high nitrogen (black points). A negative correlation between was detected for plants grown under low N (R^2 = 0.54, $F_{1,14}$ = 112.79, P < 0.001).

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