Effect of variation in nitrogen environment and legume and rhizobia genetics on the outcome of the legume-rhizobium mutualism.

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Abstract

The legume-rhizobium mutualism has been studied for its agricultural importance from the nitrogen that the rhizobia fix in exchange for carbon from the plant, and additionally used as a model to understand the evolution of mutualisms. The objective of this research was to further understand the variation present in natural populations of legumes and rhizobia, and to use a population perspective to build upon the work done with inbred plant lines and single strains of rhizobia. I applied a gradient of nitrogen (N) to a single cultivar of *Lotus corniculatus* inoculated with a population of rhizobia to develop expectations of how *L. corniculatus* responds to N addition. I then used a full-factorial greenhouse experiment with natural populations of *L. corniculatus* and their associated rhizobia to assess the amount of variation present in natural populations, and how they respond to N addition. From this, I found that plant populations did not show variation in nodule traits that could affect rhizobial fitness, whereas rhizobial populations showed variation in all traits measured. The effect of N addition on *L. corniculatus* in general causes a decrease in nodule size, although when tested in the context of natural populations, there was a plant population-dependent effect, as some populations increased, decreased, or did not alter the size of their nodules. This work underscores the importance to incorporate population scale information in how this mutualism responds to varying environmental conditions. Furthermore, considering the amount of variation found in rhizobial populations, future work should focus on sampling legumes and their associated rhizobia in order to have a more accurate measure of the amount of variation present in the mutualism.

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Introduction

Plants in the legume family (such as beans, peas, soybeans, and alfalfa) participate in a facultative mutualism with soil-dwelling bacteria called rhizobia. In this mutualism the rhizobia and plant exchange chemical signals, which if recognized, allow the rhizobia to enter into the plant root and live in organs called nodules. Inside these nodules, the rhizobia fix atmospheric dinitrogen into ammonia, a plant usable form of nitrogen (N). The plant benefits by gaining fixed N, while the rhizobia are provided with sugars and an environment to multiply inside the nodule. This mutualism has been studied not only for its agronomic importance, but also as a system to understand how resource mutualisms evolve.

Both legumes and rhizobia show variation in traits that affect all stages of the mutualism, from the initial association to the benefit that each partner gives the other. The initial chemical exchange between legumes and rhizobia (Oldroyd 2013) has led to broad patterns of host specificity, where species of legumes associate only with certain species of rhizobia (Masson-Boivin et al. 2009; Gyaneshwar et al. 2011). Furthermore this specificity is also found at an intraspecific level, with certain legume genotypes only pairing with others, e.g. *Amphicarpaea bracteata-Bradyrhizobium* (Spoerke et al. 1996). Once compatible pairs are made, there is variation in how much fitness benefit each partner gains from the mutualism. Legumes show intraspecific variation in the amount of resources that they allocate to the mutualism, by forming variable numbers of nodules (in Soybean, Serraj & Sinclair 1998), or nodules of different sizes (in Soybean, Kiers et al. 2006). Rhizobia show both inter- and intraspecific variation in the amount of benefit that they give a plant (Thrall et al. 2011), and even intraspecific variation in the amount of fixed N that they provide to the plant (Steele et al. 1983). Rhizobia show both inter- and intraspecific variation in the amount of benefit that they give a plant (Thrall et al. 2011), and interspecific variation in the amount of fixed N that they provide to the plant (Steele

et al. 1983). Additionally, the benefits to the plant and the rhizobia taking part in the mutualism depend on the genotypes of the interacting legumes and rhizobia, a genotype-by-genotype (GxG) interaction (Lieven-Antoniou & Whittam 1997; Heath & Tiffin 2007; Porter et al. 2011).

Differences in legume and rhizobial traits could be driven by available soil N, as variation in the concentration of plant available soil N potentially alters the benefit that a plant gains from the mutualism. Symbiotically fixed N takes more resources for a plant to acquire than N acquired from the soil (Phillips 1980; Layzell et al. 1981), as a plant must invest resources in nodule formation and allocating C to these organs, resources which could otherwise be invested in plant growth. If a plant is growing in low N soil, then it benefits greatly from symbiotically fixed N, but growing in high soil N reduces the benefits of this association (Regus et al. 2014; Menge et al. 2015). Additionally, soil N availability can cause legumes to alter their interactions with rhizobia. Legumes tend to decrease the number and size of nodules they form when there is high N availability (Day et al. 1989; Streeter & Wong 1988), although there is variation among species in the legume response to N addition (Menge et al. 2015), with some legume species showing no reduction in nodule number under N addition (*Acmispon strigosus,* Regus et al. 2014). Theory predicts that increasing soil N availability would favor lower quality rhizobia that fix less nitrogen (West et al. 2002; Akçay & Simms 2011), and Weese et al. (2015) found that rhizobia evolved in response to increased N addition by decreasing the amount of benefit that they gave plants, and therefore were lower quality mutualists. The benefit that the plant and rhizobia partners receive depend on both N availability and the genetics of the interacting partners, but the relative importance of these factors has not yet been thoroughly evaluated.

The overall objective of my work was to assess the relative importance of environmental variation and genetic variation in natural populations of a legume and its associated rhizobia in the outcome of this mutualism. This will build on research done with inbred plant lines and rhizobia strains to understand the variation present in the

legume-rhizobium mutualism, by looking at how populations of mutualists interact, and if there is variation among them. The specific objectives were to i) determine the effect of N addition on mutualism traits of a legume species and their associated rhizobia, and ii) investigate how much plant and rhizobial populations vary in traits that are expected to affect the fitness of either partner in the mutualism. To address these aims I used *Lotus corniculatus* and its rhizobial partners to conduct two greenhouse experiments. In the first I applied varying amounts of N to a single *L. corniculatus* cultivar inoculated with a rhizobial population to determine the effect of N addition on *L. corniculatus*' mutualism traits. In the second experiment I grew combinations of natural *L. corniculatus* and rhizobial populations, along with a N addition treatment, in a full factorial greenhouse experiment.

Methods

Lotus corniculatus is a perennial legume native to Eurasia that primarily associates with rhizobia in the genus *Mesorhizobium* (Sánchez et al. 2014; Ampomah & Huss-Danell 2011), forming determinate nodules that senesce at the end of every growing season. *L. corniculatus* was introduced into the US on both the east and west coasts sometime before 1753 (Turkington & Franko 1980), and is now widely established across the US and Canada, in part due to its use as a forage crop and roadside erosion control.

During July-August 2014 I surveyed locations in Minnesota for potential plant and rhizobial populations to study based on distribution data from EDDMapS (2017). Ten sites were surveyed, and *L. corniculatus* seeds, nodules and soil samples were collected at each site. I took soil samples by bulking five ¾ inch diameter x 6 inch deep soil cores per site (approximately 43 mL per core, 215 mL total), and submitting them to the UMN Research Analytical Lab for a standard soil analysis (N, P, K, pH, C). I chose four sites based on distance apart and extremes of soil N content to further study: Moose Lake State Park (MOL), Saint Croix River State Park (SCP), Great River Bluffs State Park (GRB),

and UMore Park (UMR) (See Supplementary Figure S1). The sites were as far as ~380 km apart (MOL and GRB), and as close as ~50 km (SCP and MOL), and soil N content was either low (MOL and SCP both had values of 1 ppm NO_3^-), or high (GRB and UMR had values of 5.43 and 11.27 ppm NO_3^- respectively). It is important to note that soil N varies temporally (Wuest 2015; Taylor et al. 1982), and these values are a rough estimate of the soil N that the plants experience over the growing season. Additionally, even though the N environment was used to select the sites, I did not have enough power to determine if the N environment of the populations affected their traits, as I would have needed greater sampling at the level of sites coming from different N environments.

At each site I collected seeds from individual plants, and exhumed plants to collect nodules from. To culture rhizobia I haphazardly selected 10 plants from each site, and from each plant I haphazardly selected, surface sterilized, and crushed 3 nodules, and plated them onto AG media plates (Somasegaran & Hoben 1994). I streaked cultures until there were single colonies, which I then inoculated into liquid AG media, grew for 48hrs, and stored in glycerol stocks (40% glycerol) at -80C. Due to fungal contamination, I could not recover cultures from all nodules or all plants, although I selected cultures from 5-9 plants to account for potential differences in how plants might be sampling the rhizobial population and capture the most variation in the rhizobial population. To represent the rhizobial population at each site, I selected 10 cultures that I then mixed at equal ratios to form a rhizobial inoculum. Since rhizobia populations were created by mixing strains in equal ratios, this may not reflect their abundance in the site they were sampled from and could lead to greater proportions of rhizobia that are of lower / higher quality than what is truly at the site.

Nitrogen Gradient Experiment

I conducted a greenhouse experiment to characterize the effect of N addition on plant mutualism traits. I used the *L. corniculatus* cultivar Norcen as a single plant genotype. I chose a cultivar in order to reduce the amount of variation in the traits measured due to genetic variation, as the plant is an outcrossing tetraploid with tetrasomic inheritance (Miri & Bubar 1966), and therefore have more power to detect effects of N addition. I scarified seeds by shaking them in sand for 1 hour on a vortex, I then removed them from the sand and gently mixed them in a 50% diluted commercial bleach solution (3% NaClO) for 5 minutes, rinsed them three times with sterile dH_2O, and let them imbibe overnight at 4ºC in darkness. I planted the seeds into bleach sterilized D16 Deepot Conetainers (262 mL volume, Stuewe & Sons), filled with a steam-sterilized mixture of 1:1 Sunshine Mix LC8:MVP Turface (Sun Gro® Horticulture, PROFILE Products LLC).

The rhizobia inoculum used was a mixture of 10 *Mesorhizobium* strains collected from MOL population, and were the same strains used later in the Population Experiment (below). The MOL site was haphazardly chosen to supply 10 Rhizobia strains, which were the same strains used later in the Population Experiment (below). Glycerol stocks of each of the 10 strains were used to inoculate liquid AG media, grown at 28ºC for two days, and then mixed at equal ratios based on cell numbers estimated from OD_{600} values to form the inoculum. I inoculated each plant with approximately $5x10^6$ rhizobial cells in 5mL of sterile phosphate buffered saline (PBS) when the cotyledons had emerged, 4 days after planting. I applied 50 mL of ammonium nitrate (NH_4NO_3) solution to plants once a week, at one of five concentrations: 0 mM, 0.625 mM, 1.25 mM, 2.5 mM, and 25 mM, resulting in an NH_4NO_3 application of 0g, 0.0025g, 0.005g, 0.01g, and 0.1g per week (equivalent to 0, 6.875, 13.75, 27.5, and 275 kg-N/week), and a total of 0g, 0.02g, 0.04g, 0.08g, and 0.8g (0, 55, 110, 220, and 2200 kg-N) applied over the course of the experiment. I chose these levels because they were below growth saturating N levels in a

similar species (*Acmispon strigosus*, Regus et al. 2014). At each N level, I grew 40 replicates.

After 9 weeks of growth I exhumed the plants from their pots, cut the shoots from the roots, dried the shoots at 60^{o}C for a minimum of 48 hours, and weighed them. I washed the roots and counted the total number of nodules per plant, and dried and weighed 10 of the larger nodules from the top 5 cm of the root system.

Statistical analyses were conducted in the R statistical programming environment (R Core Team 2016). For the N Gradient experiment, I tested the effect of N treatments on shoot dry mass, nodule number, average nodule dry mass, and total nodule dry mass (total nodule number * average nodule dry mass) using a series of linear models fit with the lm() function. Plants in the 25mM N treatment resulted in no nodules being formed and was a high leverage point in the models fit, so it was removed from subsequent analysis. After evaluating the residuals for each model fit, nodule number met the assumptions of normality of residuals, whereas average nodule dry mass and total nodule dry mass did not. I used a natural log transformation of the average nodule dry mass based on the results from a Box-Cox transformation (boxCox() in the car package, Fox & Weisberg 2011). Total nodule dry mass was not transformed as the F values of the model terms only changed by a small amount, so I chose the untransformed model for simplicity in interpretation of the results.

Population Experiment

I conducted a greenhouse experiment to look at the variation among natural populations of legumes and rhizobia, and how these populations respond to N addition. Briefly, this experiment was full factorial with 4 plant populations, 5 rhizobial treatments (4 rhizobial populations and uninoculated control), and two N treatments with 40 replicates of each combination for a total of 1600 plants. Pots were placed in racks following a completely randomized design.

Seeds collected from the four sites (MOL, SCP, GRB, and UMR) were used to create the plant populations. GRB and UMR sites were comprised of 5 maternal lines each, although due to poor seed set, MOL was comprised of 4 maternal lines and a bulk seed collection from many individuals, and SCP was comprised of 6 maternal lines and a bulk seed mixture from many individuals. It is important to note that plant populations were estimated using only 5-7 maternal lines, which could lead the observed population trait mean being different from the true mean due to sampling error. That said, since *L. corniculatus* is an obligate outcrossing plant, I consider the minimum of 5 maternal lines to adequately sample the variation within each population in order to estimate a population mean. I prepared the rhizobia mixture as in the Nitrogen Gradient experiment, where I used 10 strains from each site to represent the rhizobial population. I mixed liquid cultures of rhizobia in equal ratios determined by OD_{600} to form each rhizobial population.

The two N treatments were with either 50 mL of 0mM (0 N) or 2.5 mM NH_4NO_3 (+N) dissolved in water applied at a rate of once per week. I prepared pots and racks, the sterile potting media, and scarified seeds as in the N Gradient Experiment. Cotyledons were present 2 - 4 days after planting, and at 4 days after planting I inoculated each plant with $5x10^6$ rhizobial cells in 5 mL of sterile PBS. Three days prior to planting, I applied 50mL of full strength N-free Fahraeus media (Fåhraeus 1957) to each pot. I applied a second application of Fahraeus media at week 5. Failure of seeds to germinate and death of control plants resulted in only a total of 1225 out of 1600 planted. The number of replicates of each treatment ranged from 17 to 37 (excluding uninoculated control treatments), with a median replicate number of 32.

After 21 weeks of growth the majority of the plants were flowering, I considered the plants mature, and I exhumed the plants, washed their roots, and harvested and dried the shoots shoots at 60^{o}C for a minimum of 48 hours. I wrapped roots in paper towels and froze them at -20^{o}C for later nodule counting and collection. Later I thawed the roots and

counted the total number of nodules per plant, and sampled 10 of the larger nodules haphazardly selected from the top 5 cm of the roots to dry and weigh.

I analyzed the data with mixed linear models using the lmer() function from the lme4 package (Bates et al. 2015). I fit models with fixed effects of N addition, plant population, and rhizobial population, including their interactions. I included plant maternal line as a random effect as I collected maternal lines from a haphazard sampling of plants at each site, and since maternal line was included due to experimental design, I did not evaluate it for significance. I evaluated terms in the model using Likelihood Ratio Tests in the anova() function as demonstrated by Bates (2010), using nested models to evaluate the main effects before interaction terms, and the two-way interaction terms before the three way interaction term. I natural log transformed both average nodule dry mass and total nodule dry mass so that the residuals fit a normal distribution. Additionally I fit models for total nodule number, average nodule dry mass, and total nodule dry mass with shoot dry mass as a covariate, as plant size was correlated with nodule traits (See Supplementary Figure S2). This allowed me to test if the treatments were directly affecting nodule traits, or if the treatments were affecting nodule traits by means of altering plant size. The proportion of variance explained (η^2) for each fixed term in the mixed linear models was calculated by dividing the sum of squares of the term over the total sum of squares for all terms and the residuals (term sum of squares / sum of squares of all terms and residuals, Cohen 1973). I used the lsmeans() function from the lsmeans package (Lenth 2016) to calculate least square-means (LS-means), and to conduct pairwise comparisons within groups; the degrees of freedom were calculated using the "Satterthwaite" method from the lmerTest package (Kuznetsova et al. 2016), and P-values were adjusted using the Tukey method for comparing a family of estimates.

Results

N Gradient

L. corniculatus Norcen plants inoculated with 10 rhizobium strains showed the expected response to N addition of having reduced plant investment in the mutualism. Fertilized plants had higher shoot dry mass, formed fewer, smaller nodules, and had lower total nodule dry mass (Table 1). Shoot dry mass was on average 0.74g per plant in unfertilized pots (0mM NH_4NO_3), and increased by 6.6% per mM of NH_4NO_3 added (Figure 1A; P = 0.041, $\eta^2 = 0.021$, df = 190). Without accounting for any effects on nodulation traits due to N addition's effect on plant size, fertilizing with N had a marginally significant effect on nodule number, reducing the number of nodules formed by 5.1% per mM of NH_4NO_3 added (P = 0.090, $\eta^2 = 0.012$, df = 152), from an average of 255 nodules per plant in unfertilized soil. The addition of N reduced the size of nodules by 10% per mM of NH_4NO_3 added (P = 0.0037, $\eta^2 = 0.048$, df = 151) from the 0.37 mg average nodule dry mass in unfertilized soil. In terms of total nodule dry mass, plants showed a 10% reduction per mM of NH_4NO_3 added (P = 0.0022, $\eta^2 = 0.054$, df = 151) from an average of 9.9 mg total nodule dry mass per individual plant in 0 mM NH_4NO_3.

I found that N addition had a larger effect on nodule traits after accounting for the indirect effects of N addition on nodule traits due to plant size by including shoot dry mass as a covariate. Total nodule number was reduced by 9.7% per mM of NH_4NO_3 added (Figure 1B, P < 0.001, $\eta^2 = 0.066$, df = 150), nodule size reduced by 13% per mM of NH_4NO_3 added (Figure 1C, P = 0.0049, $\eta^2 = 0.076$, df = 149), and total nodule dry mass formed reduced by 16% per mM of NH_4NO_3 added (Figure 1D, P < 0.001, $\eta^2 = 0.14$, df = 149).

Population experiment

Using mixed effect linear models to test for differences among plant populations for the traits measured found that plant populations did not show variation in nodule traits that could be involved in rhizobial fitness. There were no significant differences in total number of nodules formed, average nodule dry mass, or total nodule dry mass before accounting for plant size (all three models, plant population term $P > 0.19$, Table 2). Plant populations did vary significantly in shoot dry mass, with a difference of 0.5g (~20%) between the largest and smallest population (Figure 2A, $\chi^2_{(df=3)} = 23.6$, $P < 0.001$, $\eta^2 = 0.024$). There were no significant effects of plant population on average nodule dry mass or total nodule dry mass, and there was no significant interaction of plant and rhizobial populations on any of the traits (all terms $P > 0.10$). However, after accounting for plant size, plant populations showed differences in total nodule number (Figure 2B, $\chi^2_{(df=3)} = 10.2$, $P = 0.017$, $\eta^2 = 0.009$). N addition showed both direct and indirect effects on nodule number, as there was a direct effect of N addition in reducing nodule number ($\chi^2_{(df=1)} = 26.3$, $P < 0.001$, $\eta^2 = 0.022$), although the indirect effect of N addition increasing plant size, and therefore increasing nodule number, caused there to be no differences in the total number of nodules that plants formed as N treatment increased.

Rhizobial populations had a greater effect on traits than the plant populations: the number of nodules that plants formed with the rhizobia ($\chi^2_{(df=3)} = 48.2$, $P < 0.001$, $\eta^2 = 0.050$), the size of nodules plants formed with the rhizobia ($\chi^2_{(df=3)} = 124.3$, $P < 0.001$, $\eta^2 = 0.13$), and the total nodule dry mass that plants formed with the rhizobia ($\chi^2_{(df=3)} = 40.1$, $P < 0.001$, $\eta^2 = 0.042$). Rhizobial populations also varied in the benefit given to plants (shoot dry mass, Figure 3A, $\chi^2_{(df=3)} = 14.7$, $P = 0.002$, $\eta^2 = 0.011$), although this was not correlated to the benefit that the rhizobial populations gained from the plant (total nodule dry mass, Figure 4, Pearson's product moment correlation $t_{(df=2)} = -0.92$, $P = 0.46$). Comparing the results of models with and without shoot dry mass as a covariate allowed me to compare how indirect effects of the rhizobia on plant size, and therefore nodule traits, affected the differences between rhizobial populations. Considering models that were measuring the direct (shoot dry mass covariate included) vs direct and indirect (no

shoot dry mass covariate included) effects showed that the proportion of variance explained remained relatively unchanged (with shoot dry mass covariate: total nodule number Figure 3B, $\chi^2_{(df=3)} = 44.7$, $P < 0.001$, $\eta^2 = 0.050$; average nodule dry mass Figure 3C, $\chi^2_{(df=3)} = 116.9$,$P < 0.001$, $\eta^2 = 0.12$; and total nodule dry mass Figure 3D, $\chi^2_{(df=3)} = 44.9$,$P < 0.001$, $\eta^2 = 0.047$), which means that the differences in these traits are due to direct effects of the rhizobia.

The proportion of variance explained by N addition was lower when applied to natural plant populations than when applied to the cultivar Norcen. The indirect effects of N addition on nodules traits resulted in N addition not showing any effect on nodule traits (all N addition terms $P > 0.13$). However, there were significant direct effects of N addition on nodule traits, as the +N treatment had a 11% reduction in total nodule number ($\chi^2_{(df=1)} = 26.3$, $P < 0.001$, $\eta^2 = 0.022$) and a 2.4% reduction in total nodule dry mass ($\chi^2_{(df=1)} = 8.6$, $P = 0.0034$, $\eta^2 = 0.007$), although there was no significant effect on average nodule dry mass ($P > 0.15$). Plant benefitted the same amount from the rhizobial populations in +N, although in 0N the GRB rhizobial population produced smaller plants than the other three rhizobial populations (Figure 5C, $\chi^2_{(df=3)} = 8.2$, $P = 0.042$, $\eta^2 = 0.006$). Additionally, after including shoot dry mass as a covariate to look for direct effects of the rhizobial populations, there was an interaction of rhizobial population with N addition for total nodule number (Figure 5B, $\chi^2_{(df=3)} = 9.8$, $P = 0.02$, $\eta^2 = 0.008$). Plants inoculated with the MOL rhizobial population did not show a decrease in total nodule number from 0N to +N, although plants inoculated with the three other populations showed a trend of fewer nodules in +N.

Plant populations did not show a general trend of reduced nodule size as would be expected from the N Gradient experiment, but there was a plant population by N addition interaction (Figure 5A). This interaction was significant when tested as a direct effect by including shoot dry mass as a covariate ($\chi^2_{(df=3)} = 13.1$, $P = 0.0044$, $\eta^2 = 0.011$), and this interaction was not strongly altered by effects of plant size, as the proportion of variance explained remained largely unchanged when the shoot dry mass covariate was not included ($\chi^2_{(df=3)} = 11.8$, $P = 0.0081$, $\eta^2 = 0.010$). Plant populations showed differences in the relative size of their nodules, and also changed rank compared to the other

populations. Two out of four populations (UMR and MOL) showed an increase in nodule size in +N, and GRB went from producing nodules of an average size in 0N, to producing the smallest nodules in +N. There was also a marginally significant interaction between plant population and N addition for total nodule dry mass ($\chi^2_{(df=3)} = 6.7$, P = 0.083, $\eta^2 =$ 0.007), suggesting that the benefit rhizobia gain from different plant populations depends on N availability.

Discussion

Applying a gradient of N additions to a cultivar of *L. corniculatus* allowed me to determine the effect of increasing N availability on this mutualism, and develop expectations to base my population comparisons on. I found that N addition caused a decrease in average nodule mass and total nodule mass, while nodule number remained the same. By collecting *L. corniculatus* and their associated rhizobia from four naturally occurring populations and growing them in a full-factorial greenhouse experiment with addition of N, I measured how variation in natural populations of legumes and rhizobia interact with N availability to affect the outcome of the mutualism. N addition showed a more complex plant population-dependent effect on nodule size, where plant populations showed either an increase, decrease, or no change in nodule size in higher N. Also, the plant populations did not show significant variation in traits that could affect rhizobial fitness, whereas rhizobial populations showed variation in all nodule traits, the benefit that they gave plants, as well as a differences in how much benefit the rhizobia gave to the plant depending on the N environment. The genetic variation in rhizobial populations for nodulation traits indicate that the rhizobia play a larger role compared to the plant in the potential adaptation of this mutualism to environmental conditions.

Nitrogen affects rhizobial fitness primarily through nodule size

Applying a gradient of additional N to one plant cultivar that had been inoculated with a single rhizobial population lead to reduced nodule size. These effects of N addition are consistent with previous empirical work (Day et al. 1989; Streeter & Wong 1988), and can be interpreted as a reduction in the investment of the plant toward the mutualism. However, when I used four natural plant populations to test the effect of N addition, I found diverse patterns of plant response to N addition, with plants altering their nodule mass by either increasing, decreasing, or not changing their average in +N (Figure 5A). Similarly, work by Heath et al. (2010) found that nodulation rate in selfed plant lines of *M. truncatula* sampled from natural populations also varied in their response to N addition. Both nodule size and nodule number are correlated with the amount of rhizobia released back into the soil and potentially rhizobial fitness (Simms et al. 2006; Kiers et al. 2006; Heath & Tiffin 2007; Ratcliff et al. 2011). If plant populations differ in how they change either of these traits in response to N addition, then which plant population the rhizobia are partnered with has the potential to alter how N addition affects rhizobial population dynamics. Further, the natural variation that I, and others, have observed between populations underscores the importance of including multiple plant populations in studies aimed at understanding how this mutualism responds to varying environments (Friesen 2012; Kiers et al. 2013; Friesen & Heath 2013).

The levels of N that I applied spanned from 0 to 275 kg-N / ha per week, with the amount that I used in the full-factorial population experiment being equivalent to applying 27.5 kg-N / ha per week. The N treatments used in these experiments were biologically relevant, as they caused changes in shoot dry mass, a proxy for plant fitness. To make a more ecologically relevant comparison, the average *yearly* total N deposition rate across the US is approximately a third of what I applied, with atmospheric deposition rates near agricultural fields often twice as high as the US average (National Atmospheric Deposition Program 2017). Additionally, a manipulative field experiment where 123 kg-N / ha per year was added resulted in evolutionary change in rhizobial populations (Weese et al. 2015). However, the comparison of the rates of N that I applied in my experiments in the greenhouse to natural systems is difficult, due to the increased rate at

which N leaches from potting media (Broschat 1995), as opposed to natural and managed systems.

Variation in plant and rhizobial populations

The result that plant populations did not show significant variation in traits that are correlated with rhizobial fitness was unexpected. Work in two model legumes with a panel of selfed plant lines set up the expectation of finding variation in nodulation traits (Heath & Tiffin 2007; Heath 2010; Sinclair et al. 1991). One possible explanation for the lack of phenotypic variation is that the plant populations I sampled came from one large panmictic population, or one large invading population that has not yet diverged genetically. However, this is not the case as populations did differ in shoot dry mass. Porter et al. (2011) also found a lack of variation in nodulation traits in a legume invading a serpentine–non-serpentine area in California even though the populations studied differed in reproductive output and phenotypic traits, which raises a question of if invading legumes show less variation in nodulation traits than established endemic legumes. A study of *Glycine soja*, the wild progenitor of the domesticated soybean *Glycine max,* also found that plants had diverged in phenotypic traits at a scale of 6 km, but not in nodulation traits (Bult & Kiang 1992). In sum, these data suggest that the degree of variation in plant mutualist quality depends on the legume studied, and the scale of population sampling done.

That I observed variation in nodulation traits due to rhizobial population identity is expected due to the variation previously observed in single strains of rhizobia (Heath et al. 2010; Burdon et al. 1999; Barrett et al. 2015). While some of this variation could be caused by variation between strains of the same species, sequencing work I conducted on four natural populations of *L. corniculatus* associated rhizobia found that *L. corniculatus* can associate with rhizobia of the genera *Mesorhizobium, Rhizobium* and and *Phyllobacterium* (D. Nedveck, unpublished). Thus, some of the variation in nodulation traits could be due to *L. corniculatus* sampling rhizobia from multiple genera that represent very different evolutionary histories, as opposed to the populations of rhizobia

being highly differentiated within a single species or genus. This result is not entirely surprising, as other work on invasive legumes have found that legumes have either invaded with their rhizobial symbiont (Porter et al. 2011; Parker et al. 2006), or have formed new associations with the rhizobia that are already present in the new environment (Klock et al. 2015). Thus, the promiscuous association behavior of *L. corniculatus* could explain why the rhizobial populations show variation in every trait measured.

Comparing the relative importance of the plant and rhizobial natural genetic variation on the outcome of the mutualism, I find that my conclusion that the plant playing a smaller role in the variation in the outcome of the mutualism to agree with other work. For instance, in their work studying a panel of 10 *M. truncatula* inbred lines from a germplasm collection and two rhizobial strains, Heath and Tiffin (2007) found that plant and rhizobial identity explained similar proportions of variance for nodule number, but rhizobial strain explained a much larger proportion of variance on nodule morphology compared to plant lines. In contrast, when Heath et al. (2010) studied *M. truncatula* selfed plant lines and rhizobia strains from their native range, they found that plant and rhizobia identity explained the same proportion of variance in nodule number, although no differences were observed in nodule size in either the plant or rhizobial populations. A study by Porter et al. (2011) shed light on how the invasive legume *M. polymorpha* and its associated rhizobial symbiont adapt to serpentine or non-serpentine soil. What they found was that the plant populations in either soil were different in morphology and reproductive output, but not nodulation traits. On the other hand, the rhizobia from either soil type differed in the number and size of nodules that they formed with plants. Taken together, these studies suggest that there is a trend for the genetic variation in rhizobia to play a larger role in the variation in the outcome of the mutualism.

The observation that rhizobia show more variation in traits than their plant partner could be due to differences in natural history. One difference lies in the larger population size and faster generation time of the rhizobia. Additionally, rhizobia find themselves in two different selective environments, living as a mutualist with a plant and free-living in the soil. The amount of variation that is present within a rhizobial population has

important implications for adaptation through standing genetic variation (Barrett & Schluter 2008). Even though my experiment only showed that there was variation between rhizobial populations, and not necessarily within, previous work on with single strains of rhizobia still point to diversity within populations. This diversity among populations could be due to either selection from abiotic or biotic factors acting on these populations, or that these populations have diverged in traits due to drift. Considering that legumes are often studied without the context of their associated rhizobia, we are losing an important aspect in understanding how much variation is present in this mutualism, and the potential for this mutualism to adapt to varying environments.

Tables

Table 1: Results of linear models for N Gradient experiment

Bold values denote statistical significance at $P < 0.05$.

Table 1.a: results of linear models without shoot dry mass covariate

Shoot dry mass

Term	β	**SE**	η^2	**df**	**t**	**P**
Intercept	0.74	0.035				
N addition	0.049	0.024	0.021	151	2.1	**0.041**

Total nodule number

Term	β	**SE**	η^2	**df**	**t**	**P**
Intercept	255	11.1				
N addition	-13	7.7	0.012	152	-1.7	0.09

log(Average nodule dry mass)

Term	β	**SE**	η^2	**df**	**t**	**P**
Intercept	-1.00	0.053				
N addition	-0.11	0.037	0.048	151	-2.9	**0.0037**

Total nodule dry mass

Term	β	**SE**	η^2	**df**	**t**	**P**
Intercept	94.3	4.6				
N addition	-9.9	3.2	0.054	151	-3.1	**0.0022**

Table 1.b: results of linear models with shoot dry mass covariate

Total nodule number

Term	β	**SE**	η^2	**df**	**t**	**P**
Intercept	85	15.5				
Shoot dry mass	231	18.2	0.46	150	12.7	**< 0.0001**
N addition	-25	5.4	0.066	150	-4.6	**< 0.0001**

log(Average nodule dry mass)

Term	β	**SE**	η^2	**df**	**t**	**P**
Intercept	-1.29	0.105				
Shoot dry mass	0.39	0.124	0.038	149	3.2	**0.0018**
N addition	-0.13	0.036	0.076	149	-3.6	**0.00049**

Total nodule dry mass

Term	β	**SE**	η^2	**df**	**t**	**P**
Intercept	27.5	7.0				
Shoot dry mass	91.0	8.3	0.35	149	11.0	**< 0.0001**
N addition	-15.2	2.4	0.14	149	-6.3	**< 0.0001**

Table 2: Results from mixed-effect linear models for the Population Experiment

Tests of significance are based in χ^2 values from likelihood ratio tests comparing nested models with and without the term of interest. η^2 was calculated as the Sum of Squares of the term of interest divided by the total Sum of Squares. Bold values denote statistical significance at $P < 0.05$.

	Without shoot dry mass covariate				**With shoot dry mass covariate**		
Shoot dry mass							
Term	**df**	***η2***	***χ2***	**P**			
N addition	1	0.290	342.0	**< 0.0001**			
plant	3	0.024	23.6	**< 0.0001**			
rhizobia	3	0.011	14.7	**0.002**			
N addition * plant	3	0.001	1.6	0.067			
N addition * rhizobia	3	0.006	8.2	**0.042**			
plant * rhizobia	9	0.009	13.0	0.16			
N * plant * rhizobia	9	0.005	6.7	0.66			
Total nodule number							
Term	**df**	***η2***	***χ2***	**P**	***η2***	***χ2***	**P**
shoot dry mass					0.107	131.8	**< 0.0001**
N addition	1	0.002	2.2	0.14	0.022	26.3	**< 0.0001**
plant	3	0.004	4.7	0.2	0.009	10.2	**0.017**
rhizobia	3	0.050	48.2	**< 0.0001**	0.040	44.7	**< 0.0001**
N addition * plant	3	0.000	0.4	0.94	0.001	1.0	0.8
N addition * rhizobia	3	0.004	4.0	0.26	0.008	9.8	**0.02**
plant * rhizobia	9	0.010	9.9	0.36	0.007	7.8	0.56
N * plant * rhizobia	9	0.013	13.5	0.14	0.010	11.5	0.24
log(Average nodule dry mass)							
Term	**df**	***η2***	***χ2***	**P**	***η2***	***χ2***	**P**
shoot dry mass					0.025	19.4	**< 0.0001**
N addition	1	0.002	1.6	0.21	0.003	2.0	0.16
plant	3	0.002	2.5	0.48	0.004	4.1	0.25
rhizobia	3	0.126	124.3	**< 0.0001**	0.116	116.9	**< 0.0001**
N addition * plant	3	0.010	11.8	**0.0081**	0.011	13.1	**0.0044**
N addition * rhizobia	3	0.002	2.1	0.54	0.003	3.2	0.37
plant * rhizobia	9	0.013	14.5	0.11	0.011	12.0	0.21
N * plant * rhizobia	9	0.012	12.5	0.19	0.011	12.2	0.2
log(Total nodule dry mass)							
Term	**df**	***η2***	***χ2***	**P**	***η2***	***χ2***	**P**
shoot dry mass					0.008	17.7	**< 0.0001**
N addition	1	0.001	0.5	0.48	0.007	8.6	**0.0034**
plant	3	0.001	0.9	0.82	0.001	0.7	0.88
rhizobia	3	0.042	40.1	**< 0.0001**	0.047	44.9	**< 0.0001**
N addition * plant	3	0.007	6.7	0.083	0.006	6.0	0.11
N addition * rhizobia	3	0.005	4.6	0.21	0.005	5.2	0.16
plant * rhizobia	9	0.010	10.2	0.33	0.012	11.7	0.23
N * plant * rhizobia	9	0.005	4.8	0.85	0.004	4.4	0.88

Figures

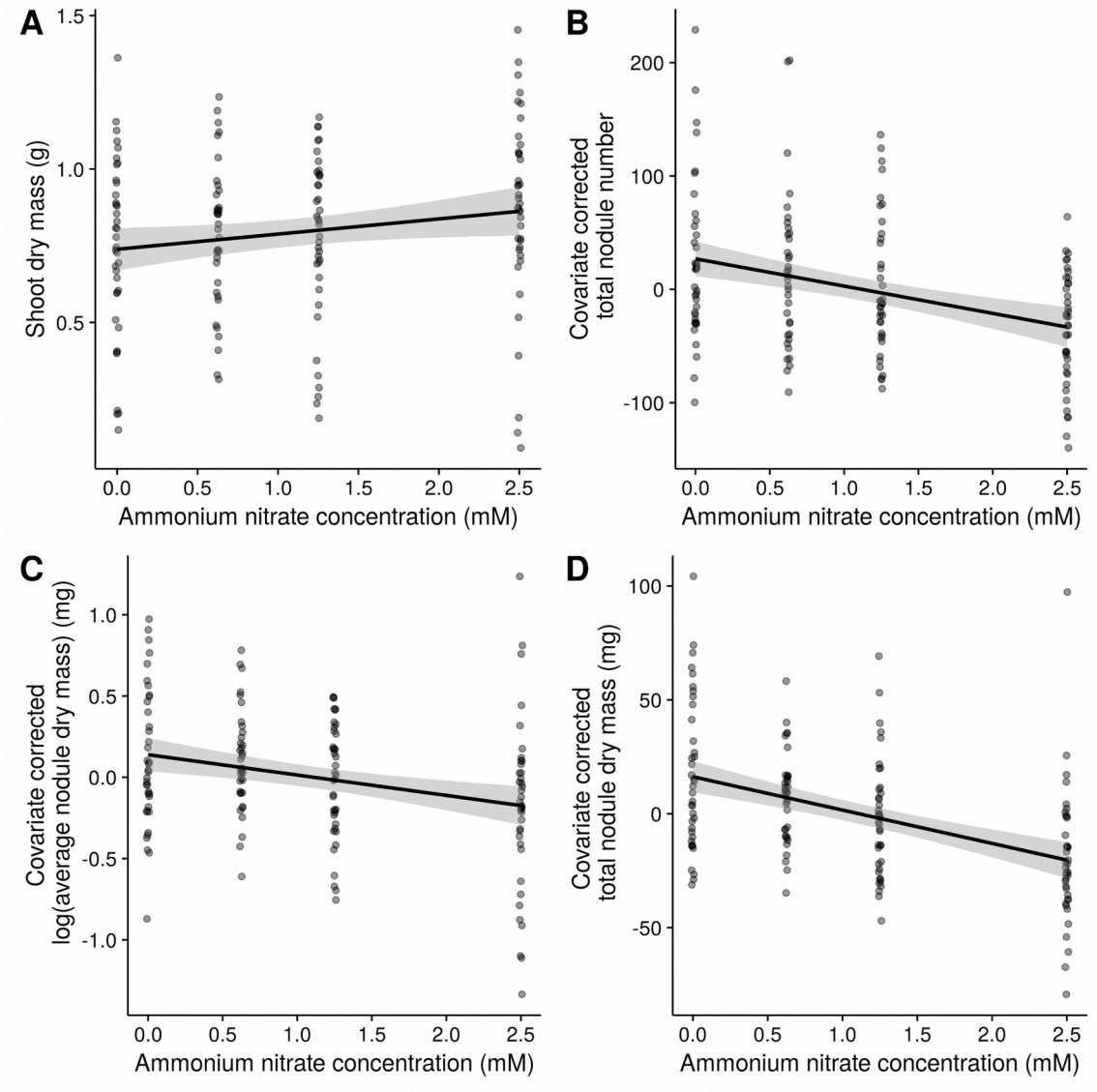

Figure 1: Effect of Nitrogen on traits of 8 week old Lotus corniculatus, showing (A) an increase in shoot dry mass as N level increased, (B) a decrease in total nodule number as N level increased, (C) a decrease in average nodule mass as N level increased, and (D) a decrease in total nodule mass as N level increased. Shaded grey area denotes 95% confidence interval of the slope. Nodule traits were covariate-corrected for shoot dry mass before being plotted by fitting a model of just the trait given shoot dry mass, and plotting the resulting residuals from that model.

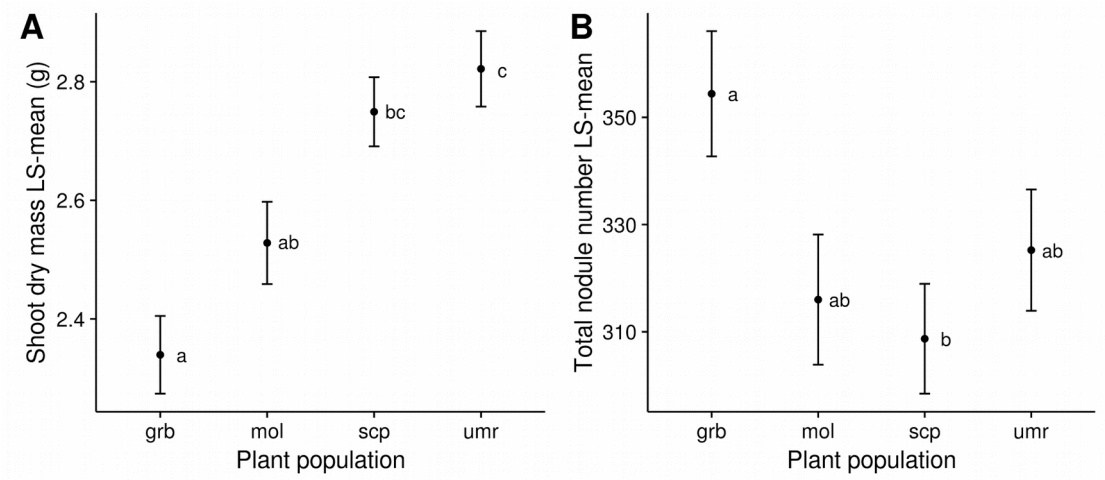

Figure 2: Least square means for (A) shoot dry mass and (B) total nodule number. Total nodule number LS-mean is from a model with shoot dry mass as a covariate. Letters denote group membership at $P < 0.05$, determined by Tukey method for comparing a family of estimates. Error bars denote +/- 1 SE from the LS-mean.

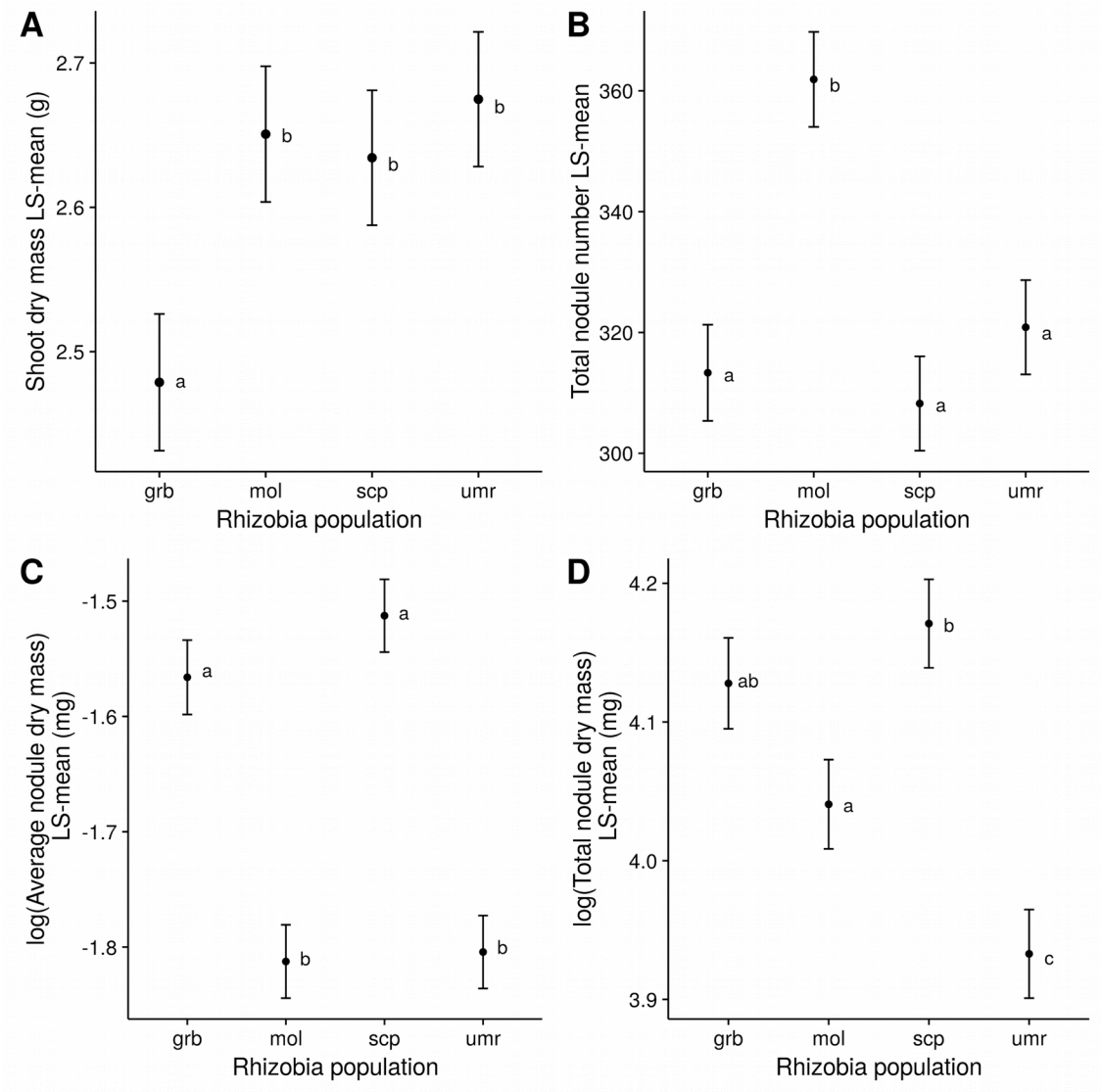

Figure 3: Least Square Means for (A) shoot dry mass, (B) total nodule number, (C) average nodule mass, and (D) total nodule mass. Total nodule number, average nodule mass, and total nodule mass LS-mean estimates are from models including shoot dry mass as a covariate. Letters denote group membership at $P < 0.05$, determined by Tukey method for comparing a family of estimates. Error bars denote +/- 1 SE from the LS-mean.

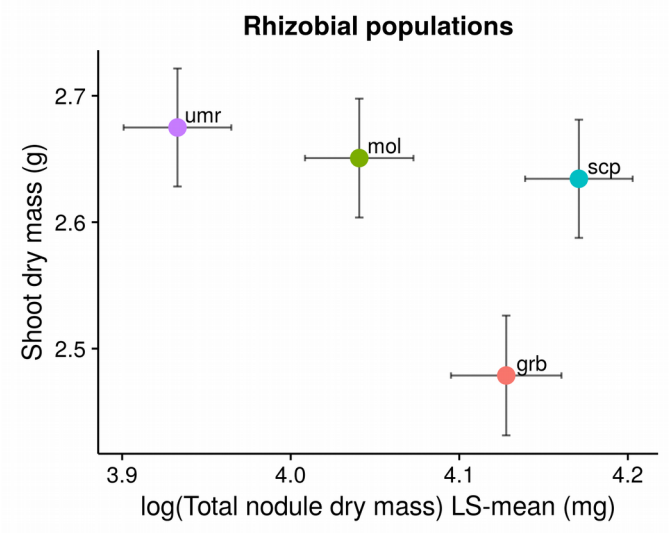

Figure 4: Plot showing the total nodule dry mass formed by plants, and their shoot dry mass, when inoculated with the four rhizobial populations. LS means of total nodule mass and shoot dry mass for each rhizobial population. Error bars denote +/- 1 SE.

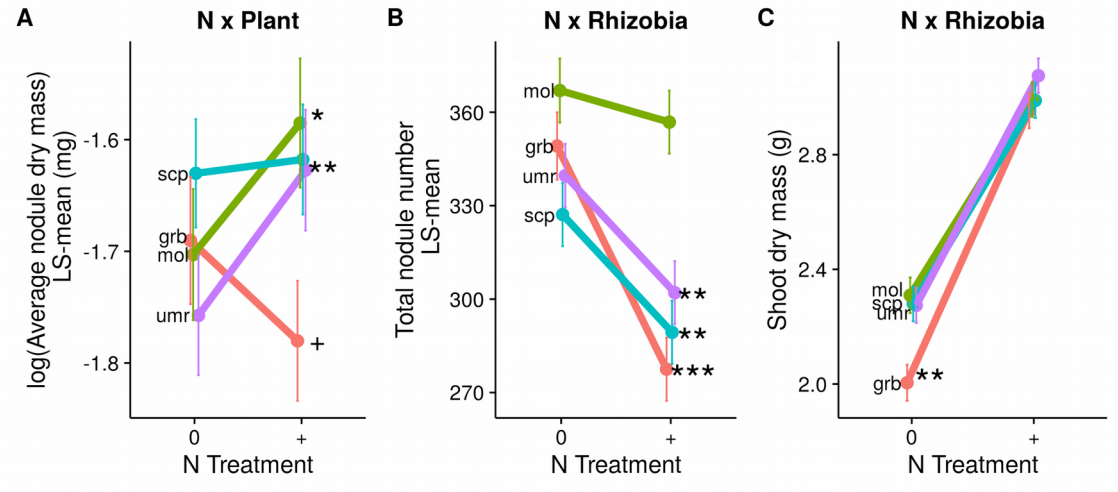

Figure 5: Plots showing an interaction between (A) N treatment and plant population for average nodule mass, (B) N treatment and rhizobial population for total nodule number, and (C) N treatment and rhizobial population for shoot dry mass.Values shown are LS mean values from mixed effects linear models, error bars denote +/- 1 SE from the LS mean. Significant differences between N treatments denoted by ∗∗∗P <0.001, ∗∗ P<0.01, ∗ P<0.05, + P<0. 1 in A and B, stars in C denote difference between GRB and the other three populations in 0 N.

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Supplementary Figures

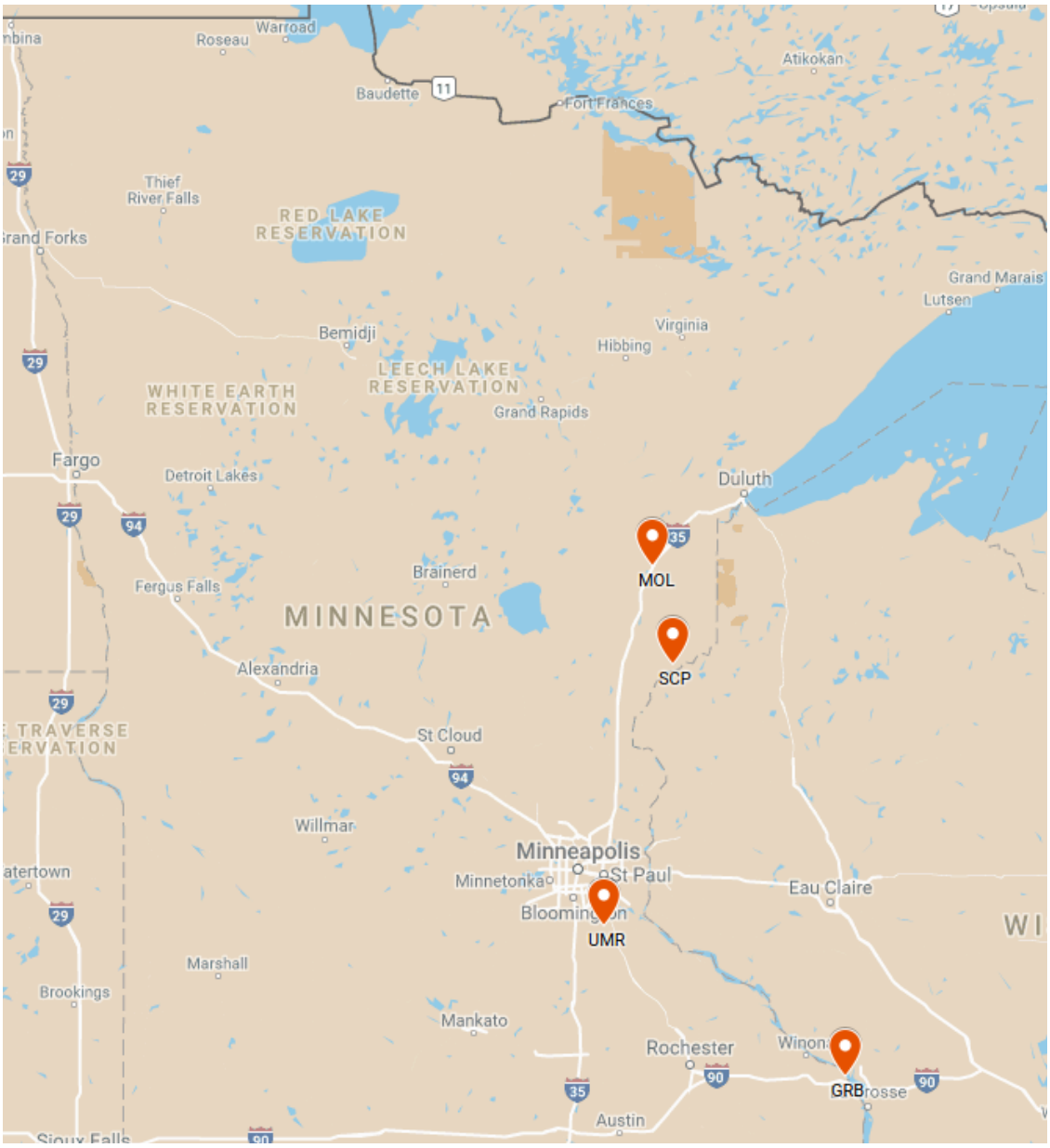

Figure S1: Map of sampling locations where populations were collected. MOL = Moose Lake State Park; SCP = Saint Croix State Park; UMR = The University of Minnesota Outreach, Research and Education (UMore) Park; GRB = Great River Bluffs State Park

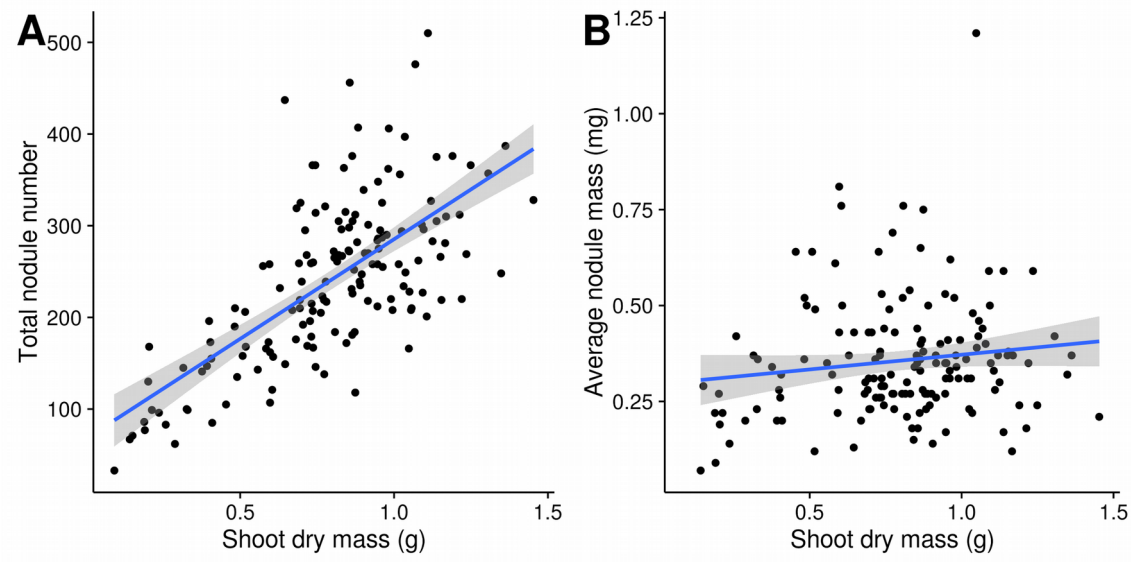

Figure S2: Plot of linear regression of total nodule number (A) and average nodule mass (B) onto shoot dry mass. Blue lines are regression lines, and the shaded regions are the 95% SE of the mean estimate. Data is from the Nitrogen Gradient experiment, and filtered to remove data from the 25mM NH_4NO_3 treatment as those plants did not form nodules.