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IMPACT OF RHIZOBIA PRESENCE AND NITROGEN ABUNDANCE ON COMPETITION BETWEEN LEGUMES AND NON-LEGUMES

by

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A THESIS

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IMPACT OF RHIZOBIA PRESENCE AND NITROGEN ABUNDANCE ON COMPETITION BETWEEN LEGUMES AND NON-LEGUMES

Approved: [redacted]

Jeff Diez

The mutualism between legume species and rhizobia has long been acknowledged as a vital part of plant community dynamics in both natural and agricultural systems. Increasing anthropogenic nitrogen inputs can alter this mutualism and in turn plant community dynamics. Still, few studies have explored the dependencies of these resource-based mutualistic microbes, rhizobia, on ambient nitrogen levels, and how this may affect competition between hosts and neighboring plants. To test the potential nitrogen-dependent role of rhizobia in mediating coexistence between legumes and non-legumes, we conducted a competition experiment in the greenhouse replicated across rhizobial inoculation and nitrogen fertilization treatments. Here, we employ contemporary ecological theory to assess how rhizobia and nitrogen fertilization influence niche differences and fitness inequalities determining competitive outcomes. We measured the fecundity of plants across a competition gradient to parameterize annual plant population models and to use these parameter estimates to predict the outcomes of competition between our plant species. We found that both nitrogen and rhizobia shift the probability of coexistence, and further that the effect of rhizobia was dependent on nitrogen concentration. We also found that these results differed when legumes were competing with either other legumes or non-legumes. These results suggest that these context-dependent mutualisms are important in determining competitive outcomes. Further, this study sheds light on the ways anthropogenic nitrogen inputs are likely to shift community composition and diversity of native plants and microbial communities alike.

Impact of Rhizobia Presence and Nitrogen Abundance on Competition Between Legumes and Non-Legumes

Environmental Studies Honors Thesis

Macy Patel, Spring 2023

Introduction

Microbial resource mutualists are an integral part of plant communities and can alter coexistence outcomes of their host organisms (Johnson and Bronstein, 2019). Rhizobia are an important species-specific mutualistic soil bacterium that forms a symbiotic relationship with legume hosts by inducing nodule formation (Figure 1) on the hosts roots, inducing positive feedback that affects competitive outcomes (Bever et al., 2010). Within these nodules, rhizobia fix atmospheric nitrogen to ammonia for the host plant in exchange for energy sources, primarily carbon and other photosynthesis products (Wang et al., 2019). Interactions between plant roots and soil organisms have the potential to alter growth and biomass of above-ground plant parts (van der Putten et al., 2013). As a result, these leguminous plants play an important role in plant community dynamics and agricultural practices alike. Associations with these species-specific resource mutualists provide competitive advantages to the host species by supplying resources they otherwise may have to compete for.

Figure 1: Root nodulation induced by rhizobia and the exchange between the bacteria and plant.

Interactions between plants and rhizobia are not static and instead dependent on available nitrogen quantities in the soil, which makes them vulnerable to the increase in anthropogenic inputs of synthetic nitrogen in soil. Differences in available soil nitrogen has been found to influence plant-rhizobial interactions by altering allocation and benefits of rhizobia (Magnoli and Lau, 2020), and this surplus of nitrogen may be important for community dynamics. Nitrogen is a limiting resource for plant growth because atmospheric nitrogen is not usable by plants and the natural processes of cycling nitrogen into the soil for plants to utilize is slow. The natural mechanisms of atmospheric nitrogen fixation are lightning strikes, combustion, and by some bacteria and archaea (Wang et al., 2019). The invention of the Haber-Bosch process created synthetic nitrogen fertilizers, which while increasing agricultural outputs and productivity, have detrimental impacts on ecosystems. Excess nitrogen from application of these fertilizers leaches from the soil resulting in water contamination, algal blooms, soil acidification, and atmospheric pollution (Dubrovsky et al., 2010; Scavia et al., 2017; Xu et al., 2017). Average atmospheric nitrogen in this century is twenty times higher than pre-industrial times and soil nitrogen levels vary spatially in relation to anthropogenic nitrogen inputs (Xu et al., 2017). Though the mechanisms by which increased nitrogen levels in soil may impact diversity and competitive dynamics are only beginning to be explored, recent studies have found that under higher soil nitrogen conditions, non-leguminous plants deplete soil nitrogen, leaving legume plants to rely on rhizobially fixed nitrogen (Elias and Agrawal, 2021; Skogen et al., 2010). It is important to understand how these nitrogen-dependent mutualisms affect competition between leguminous species and non-leguminous species to better predict how plant communities overall may respond to increasing soil nitrogen levels.

One theoretical framework that ecologists are increasingly utilizing is Chesson's Modern Coexistence Theory which accounts for the effects of biotic and abiotic variables on competitive dynamics and coexistence mechanisms. Modern Coexistence Theory quantifies coexistence by the long-term rates at which a competitor recovers from a low density and uses fitness inequalities and niche differences to understand this coexistence (Spaak and Schreiber, 2023). Fitness inequalities are the differences in innate fitness which make it more likely for one species to have a competitive advantage against the other. Niche differences are the ways in which competing species interact with their environment differently, thus increasing the intensity of intraspecific competition relative to interspecific competition. Shifts in environmental contexts might promote coexistence by either lowering fitness inequalities, an equalizing mechanism, or by increasing niche differences, a stabilizing mechanism as species require different resources

(Ke and Wan, 2020). Calculations of fitness inequalities and niche differences are powerful tools to make predictions about coexistence, as these mechanisms can account for population density changes, resource-partitioning, and other environmental factors (Chesson 2000).

The role that these nitrogen-dependent rhizobial interactions play in mediating species coexistence between legumes and non-legumes are not well understood. Here we investigate the specific mechanisms and extent to which rhizobia may mediate competition between legumes and non-legumes, and with the important context dependency of soil nitrogen levels. By quantifying niche differences and fitness inequalities for each of our species' pairs in each of our treatments, we hope to gain further understanding in answering these questions: 1) How do rhizobia mediate competition between legumes and non-legumes? 2) How does this ecological process depend on the abundance of nitrogen? We hypothesize that rhizobia will increase fitness inequalities between legumes and non-legumes, thereby decreasing the probability of coexistence. Further, we hypothesize that the magnitude of this effect is dependent on the abundance of nitrogen available in the soil. Thus, we expect that nitrogen fertilization and rhizobia inoculation will act synergistically to alter coexistence outcome probabilities. We conducted a fully factorial greenhouse experiment in which we manipulated rhizobia presence and nitrogen fertilization and assessed the impacts on competition between two leguminous and one non-leguminous plant species.

Methods

a) *Experimental Design*

We collected data through a two-factor competition greenhouse study at the University of Oregon. Three native annual plants were selected for this study, two legumes (*Lupinus bicolor* and *Acmispon americanus)* and one non-legume (*Collinsia grandiflora)*, all which cooccur in upland prairies in the Willamette Valley. All seeds were sourced from Friends of Buford Park and Mt. Pisgah in Eugene, Oregon. Seeds were sown into a 1:1 sand and soil mixture that was homogenized and sterilized in an autoclave for three 40-minute liquid cycles at 121 degrees Celsius. Using a two-by-two factorial design, rhizobia inoculation, and nitrogen fertilization either occurred or did not occur establishing four treatments in total. For the four treatments, all pairwise combinations of the three species were included, with two densities. Low-density competition pots had two focal species meaning these pots doubled as low-density data for both species, while high-density competition pots had three to five competitors along with the focal. This basic competition gradient was repeated five times for each combination.

b) *Experimental Procedure*

All seeds were sterilized for fifteen minutes in a 3% hydrogen peroxide solution, then rinsed twice in deionized (DI) water, and left to dry on a sterile tissue. Due to physical and physiological factors, seed dormancy is more common in legume species (Naik and Deshpande 2021); thus, mechanical scarification with sandpaper has been used to rupture the seed coat and promote higher germination rates. We scarified both legume species seeds with two pieces of sterile sandpaper, applying medium pressure for 45 seconds (Pain et al. 2018). We used a commercial peat-based rhizobia inoculant which was a combination of *Bradyrhizobium* sp., *Bradyrhizobium japonicum, Rhizobium leguminosarum biovar phaseoli,* and *Rhizobium leguminosarum biovar viceae* (Exceed Peat for Garden Combination; Visjon Biologics). Half of the seeds were then treated with the rhizobia inoculum, using a slurry method as outlined by the user guide of the commercial inoculant (0.0125g of inoculum for every 1g of seed).

Sterile pots were filled with the sterile soil mixture and seeded with a density of three seeds per desired *C. grandiflora* individual and five seeds per *L. bicolor* and *A. americanus* individual. Seeding densities were based on lower success seen in legumes in germination trials. All pots were watered daily, and artificial greenhouse lights were on from 6 am to 6 pm. After initial germination, half of the pots designated for the nitrogen treatment were fertilized using a solution of granular ammonium nitrate (NH_4NO_3) dissolved in DI water (Ammonium Nitrate, Granular, Reagent, ACS; Spectrum Chemical MFG Group). Synthetic nitrogen fertilizer inputs in Oregon's Willamette Valley range from 74 to 336 kg N ha^{-1} yr^{-1} (Compton et al. 2021). We used the median of this range (156 g N ha^{-1} yr^{-1}) to calculate the amount of solution used to fertilize each pot (4.932 milligrams NH_4NO_3 per ten milliliters DI water). Ten milliliters of DI water were applied to all non-nitrogen pots to account for the water that the fertilizer treatment contained. This fertilization process was repeated a few months after initial treatment to account for plants that were transplanted and did not receive initial nitrogen fertilization.

c) *Data Collection and Measurement*

To ensure the correct number of plants were in each pot and to estimate germination rates, we began by collecting initial germination counts of the pots and performed transplants and culls on pots with incorrect densities of plants. We then took recounts of each pot's contents which served as the final competitor densities in our population models. Seed counts were collected to represent the reproductive output for each plant. Flowers per plant were counted and about 100 fruits from each species were collected. We then counted how many seeds where in each fruit and calculated averages of seeds per fruit for each of our three species and finally estimated the seed output of each individual plant.

d) *Analysis*

All statistical analyses were conducted using R (4.2.2, R Core Development Team, 2022). To assess the effect of rhizobia, nitrogen fertilization, and species on germination rates, we fit a Bayesian generalized linear model with a binomial error distribution and logit link function using the package brms (2.19.0, Bürkner, 2017). To estimate pairwise interaction coefficients (α) as well as species' intrinsic growth rates (λ) in each of our treatments, we used each plant's fecundity and number of competitors to fit a Beverton-Holt annual plant population model (Beverton and Holt, 1957; Cervantes-Loreto et al., 2023) in STAN using the package rstan (Stan Development Team, 2023). We then used these estimates to calculate niche differences and fitness inequalities.

We calculated niche differences using a matrix of competition coefficients (α's) which represent the competitive effects of one species on another in each pairwise combination. The term within the square root represents niche overlap between two species, which we then subtract from one to account for how the two niches differ.

$$niche\ differences = 1 - \sqrt{\frac{\alpha_{AB}\alpha_{BA}}{\alpha_{AA}\alpha_{BB}}} \quad (1)$$

We calculated the fitness ratio as the product of demographic ratio (intrinsic growth rates) and the competitive ratio, which is the square root term of competition experienced by one species over competition experienced by the other species. Because the fitness ratio is an asymmetric quantity, we define the fitness inequality as the fitness ratio where the competitively dominant takes the spot on the numerator.

$$fitness\ inequalities = \sqrt{\frac{\alpha_{AA}\alpha_{AB}}{\alpha_{BB}\alpha_{BA}}} * \frac{\lambda_B}{\lambda_A} \quad (2)$$

To propagate the uncertainty in our estimates of α and λ, we extracted all samples from their posterior distributions to use in subsequent calculations. Thus, when calculating niche and fitness differences, we calculated a distribution of these quantities for each species pair. We assessed individual effects of each of our two treatments on $\alpha,\ \lambda$, niche differences, and fitness inequalities by subtracting the distribution of each parameter in the experimental treatment from that of the control treatment. To estimate a potential interactive effect between nitrogen fertilization and rhizobia we then subtracted the difference between parameter distributions in the nitrogen treatments from the difference in parameter distributions in the rhizobia treatments. Significant effects of our treatments on any of these parameter estimates are thus indicated by distributions of these contrasts that deviate from zero. Finally, we made predictions about the outcome of competition on our species and further, to reflect uncertainty in these predictions, we also investigated the probability of each estimate falling into each coexistence outcome.

Results

a) *Germination*

Our analysis revealed no effects of rhizobia and nitrogen fertilization on the observed differences in germination rates. However, there were differences found between species germination rates. *Collinsia grandiflora* displayed the highest germination rates, followed by *Lupinus bicolor,* and then *Acmispon americanus.* Mean percent germination per pot for *L. bicolor* was 14.8% with +/- SE of 0.9% (Figure 2). Mean percent germination per pot for *A. americanus* was 2.2% with +/- SE of 0.4% (Figure 2). Mean percent germination per pot for *C. grandiflora* was 63.3% with +/- SE of 1.8% (Figure 2). Seeds of *C. grandiflora* were 48.5% more likely to germinate than seeds of *L. bicolor* and 61.1% more likely to germinate than seeds of *A. americanus.*

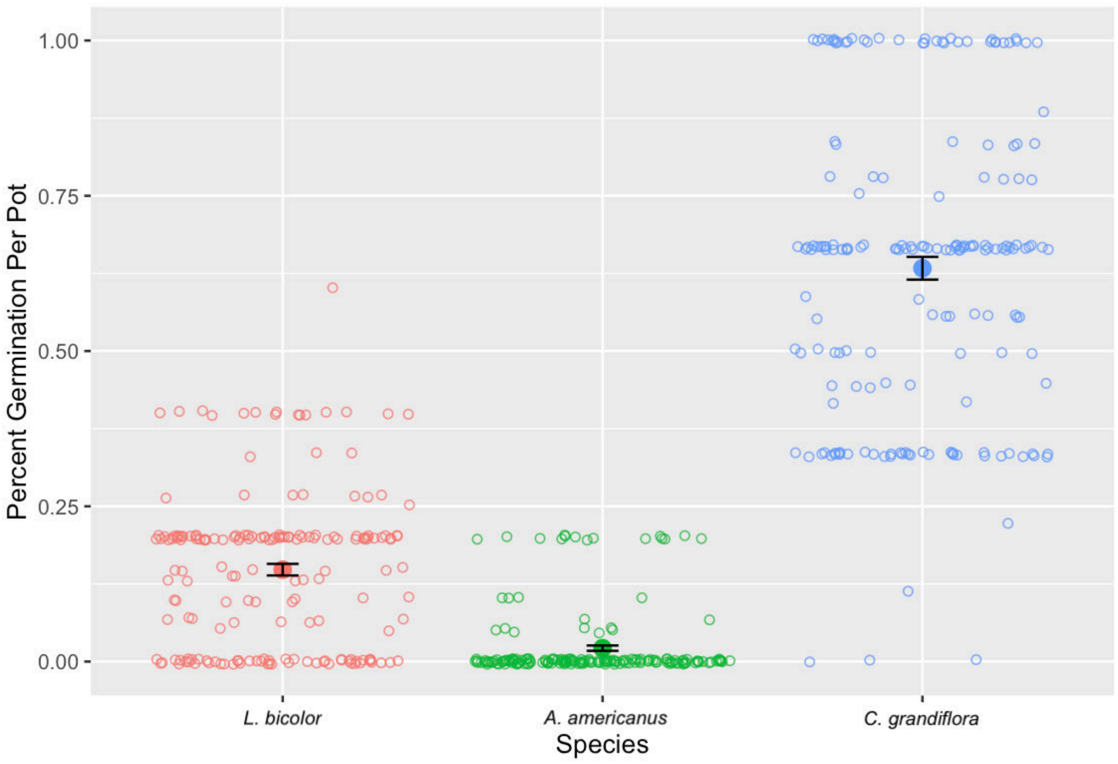

***Figure 2**: Germination rates per pot based off number of seeds sowed. Color indicates species. Solid dots show mean percent germination success per pot and error bars in black show +/- SE.*

b) *Alpha (α) and Lambda (λ) Values*

There were notable differences estimated α and λ values across our four treatments. Rhizobia inoculation tended to increase the λ values of our legume species. Observing contrasts of the sterile control minus the inoculated rhizobia estimates, *L. bicolor* median rhizobial contrast in unfertilized pots was -74.288 (95% CI: -134.812, -5.487) and in fertilized pots was -149.798 (95% CI: -211.660, -87.815). The median of rhizobia contrasts for *A. americanus* was -37.978 (95% CI: -65.239, -19.788) in unfertilized conditions and -18.270 (95% CI: -40.999, 4.496). In both sterile pots and inoculated pots, nitrogen fertilization increased the predicted λ values for non-legume species *C. grandiflora.* Median contrast of fertilization treatments for *C. grandiflora* was -105.663 (95% CI: -172.209, -40.998) in sterile pots and -114.759 (95% CI: -197.621, -37.676) in rhizobia inoculated pots. Rhizobia increased the competitive effect of *C. grandiflora* on *A. americanus* l in unfertilized pots, with a median rhizobia contrast value of -0.649 (95% CI: -1.566, -0.079). We did not observe any significant interaction effects on our α and λ values.

c) *Niche Differences and Fitness Inequalities*

For all species pairs, our four treatments did not affect niche differences, however rhizobia inoculation did affect fitness inequalities and further the effect of rhizobia varied with nitrogen treatment. Though we did observe a pattern of differences between fitness inequalities, these are tendencies (Figure 3), and there is uncertainty observed in the contrasts between median fitness inequalities (Figure 4). For species pair *L. bicolor* versus *A. americanus,* median fitness inequality fertilization contrasts (Figure 4a) are -1.083 (95% CI: -21.910, 12.705) in sterile conditions and 2.361 (95% CI: -0.763, 14.732) in rhizobial conditions. For species pair *L. bicolor* versus *C. grandiflora,* median fitness inequality fertilization contrasts (Figure 4a) are 2.219 (95% CI: -8.660, 51.683) in sterile conditions and -0.079 (95% CI: -3.239, 3.845) in rhizobial conditions. Finally, for species pair *A americanus* and *C. grandiflora,* median fitness inequality fertilization contrasts (Figure 4a) are 13.553 in sterile conditions (95% CI: -1.333, 113.738) and -1.692 (95% CI: -6.889, 2.208) in rhizobial conditions. For species pair *L. bicolor* versus *A. americanus,* median fitness inequality rhizobia contrasts (Figure 4b) are -1.043 (95% CI: -13.872, 13.348) in unfertilized conditions and 2.422 (95% CI: -0.848, 23.102) in fertilized conditions. For species pair *L. bicolor* versus *C. grandiflora,* median fitness inequality rhizobia contrasts (Figure 4b) are 4.758 (95% CI: -1.669, 53.873) in unfertilized conditions and 2.307 (95% CI: -1.937, 11.763) in fertilized conditions. Finally, for species pair *A americanus* and *C. grandiflora,* median fitness inequality rhizobia contrasts (Figure 4b) are 14.238 (95% CI: -0.141, 114.316) in unfertilized conditions and -1.431 (95% CI: -6.604, 5.078) in fertilized conditions.

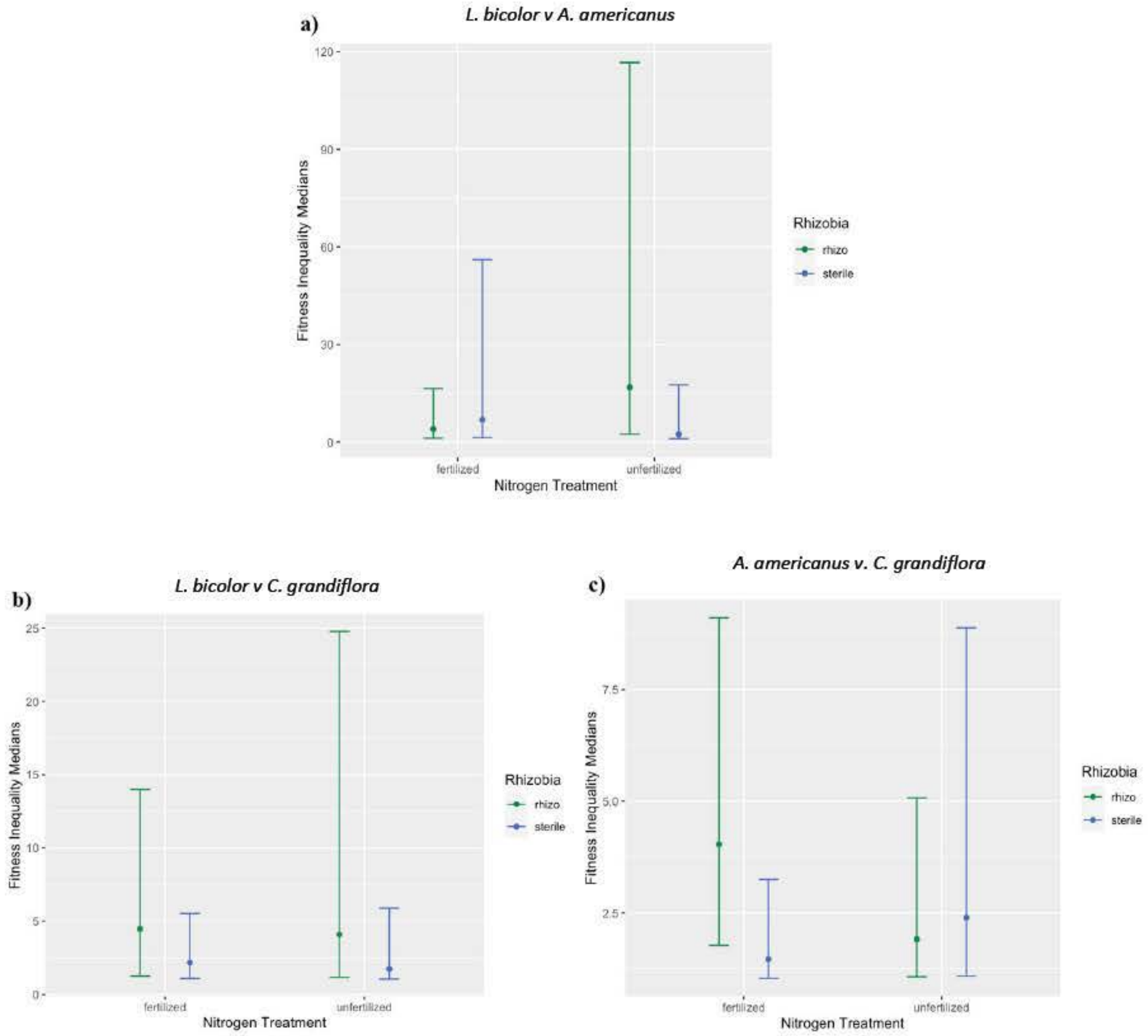

***Figure 3**: Median fitness inequalities by nitrogen treatment colored by rhizobia with error bars showing +/- SE between L. bicolor and A. americanus (3a), L. bicolor and C. grandiflora (3b), and A. americanus and C. grandiflora (3c).*

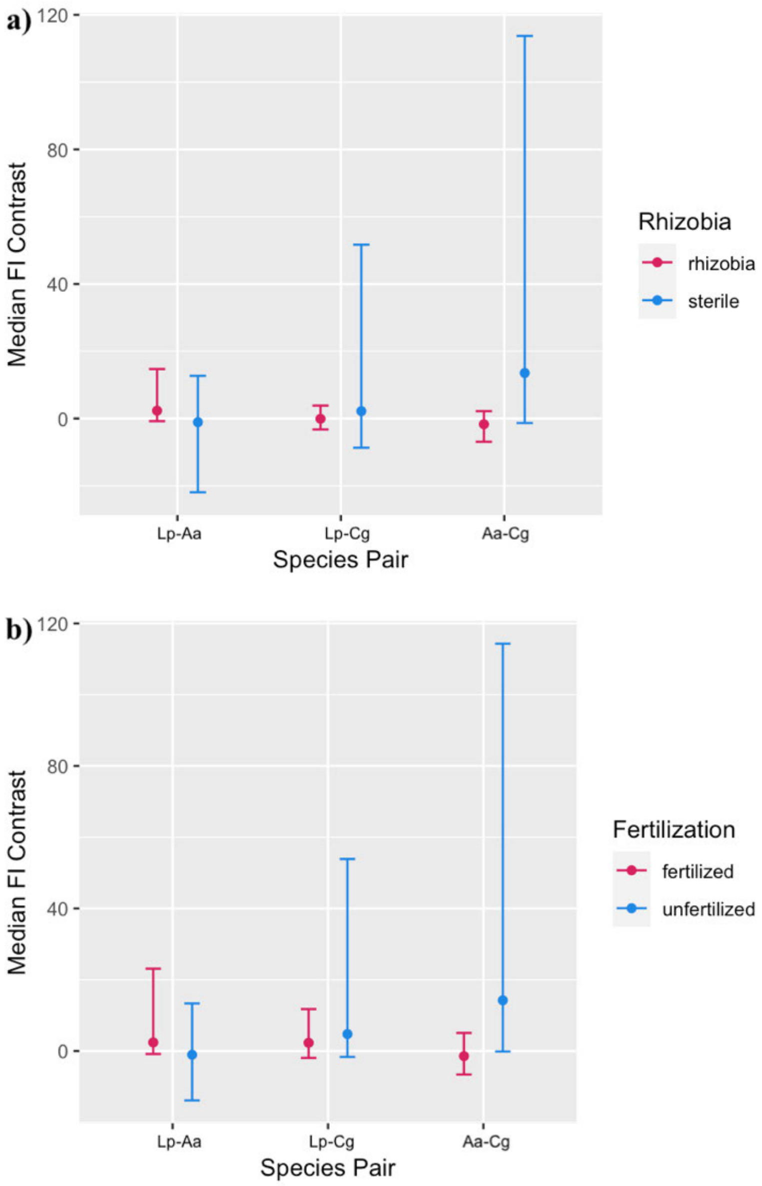

Figure 4: *Median contrasts between fitness inequalities for each species pair based on nitrogen fertilization and colored by rhizobia (4a) and based on rhizobia treatment and colored by nitrogen fertilization (4b) with error bars showing +/- SE.*

d) *Coexistence Outcomes*

Although none of the species' pairs were predicted to coexist in any of the treatment combinations based on median parameter estimates (Figure 5), the probabilities associated with each competitive outcome did vary by treatment combination (Figure 6). Based off the median parameter estimates, priority effects occur most often between species *L. bicolor* and *A. americanus* (Figure 5).

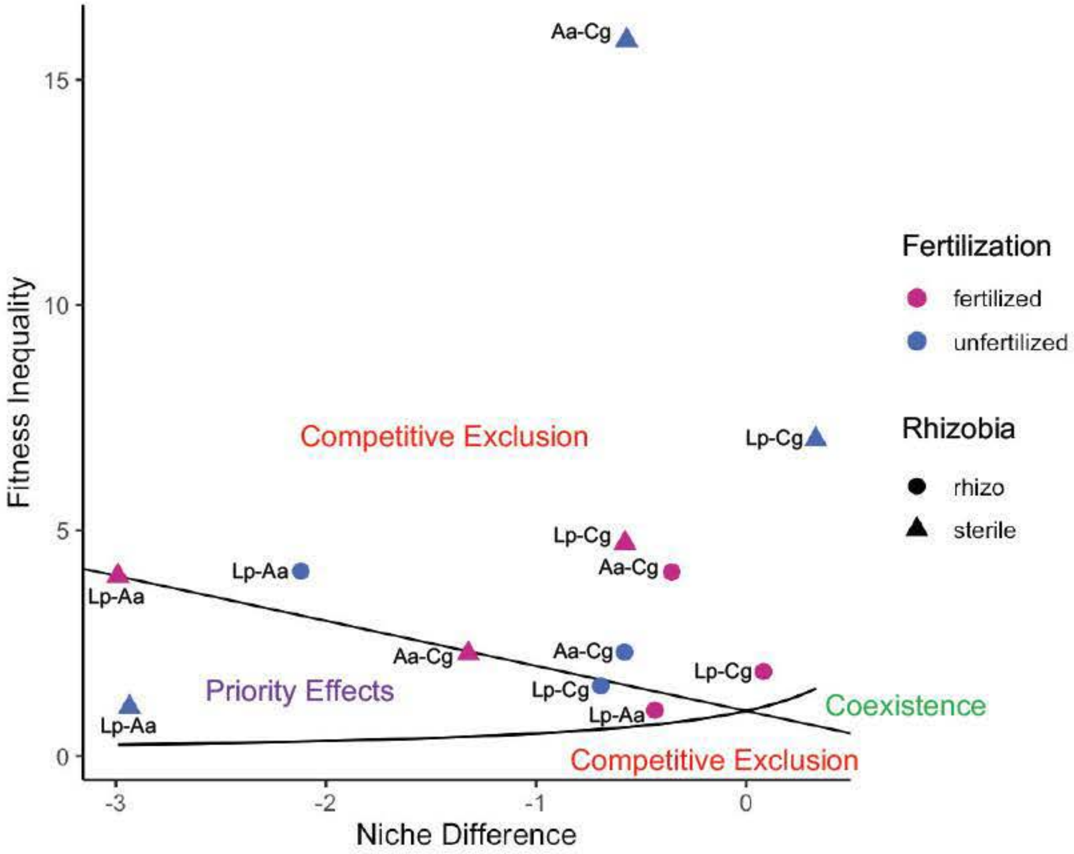

Figure 5: *Niche differences versus fitness inequalities with each species pair in each of treatment at a different point. The species pair that represents each point is listed next to each point. Lines and curves represent relational functions which create these coordinate sections that each represent a different coexistence outcome. To the right of the intersection, coexistence would occur, to the left of the intersection priority effects would occur, and above and below the intersection competitive exclusion would occur.*

Rhizobia presence and nitrogen fertilization increased the probability of coexistence between competition between *L. bicolor* and both *A. americanus* and *C. grandiflora* (Figure 6). In sterile fertilized conditions, the probability of priority effects occurring between *A. americanus* and both *L. bicolor* and *C. grandiflora* became more likely (Figure 6). Pots that were not fertilized with nitrogen but did contain plants inoculated with rhizobia generally displayed increased chances of priority effects occurring between a legume and a non-legume (species pairs Lp-Cg and Aa-Cg). Further, the probability of competitive exclusion occurring was greatest in the sterile unfertilized pots.

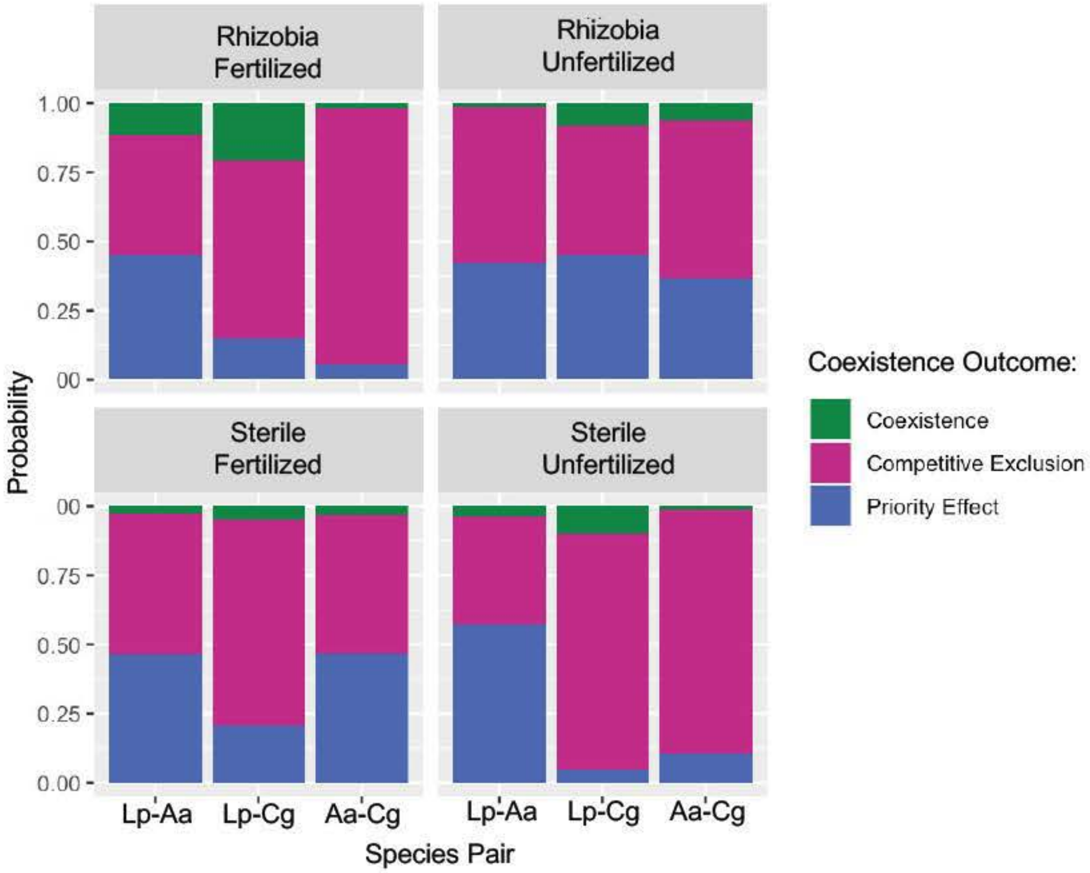

Figure 6: *Probability of each coexistence outcome occurring between each species pair in each treatment. This figure shows not just the medians, but the entire range of calculations of fitness inequalities and niche differences.*

Discussion

Overall, our results revealed both rhizobia and nitrogen affected individual plant species as well as shifted competitive dynamics. Generally, rhizobia significantly increased the λ values of the two legume species *A. americanus* and *L. bicolor*. These results suggest that rhizobia provide legumes with an innate advantage. Further, nitrogen fertilization significantly increased the λ values of non-legume *C. grandiflora* in both sterile and rhizobia inoculated settings. Rhizobia caused an increase in competitive effect (α) of *C. grandiflora* on *A. americanus* in unfertilized pots. No significant interaction effects occurred between nitrogen and rhizobia on α and λ values. We did not observe any effect of nitrogen and rhizobia on niche differences, however there were observed tendencies of nitrogen-dependent shifts of the effect of rhizobia on fitness inequalities (Figure 3). However, it is important to acknowledge that the contrasts between posterior distributions of estimates for both rhizobia and nitrogen do not reveal differences, meaning though there are observable patterns in effect of rhizobia on the fitness inequalities, the extent to which these are widely applicable is not yet understood (Figure 4). Finally, nitrogen and rhizobia do shift the probabilities of competitive exclusion, priority effects, and coexistence for each species pair. These differences in probabilities of coexistence outcomes show that rhizobia and nitrogen simultaneously affect plant interactions and community dynamics.

Legume species have innate differences which allow them to associate with rhizobia (Hirsch, 2001). Our results strongly suggest that these differences may have led to variation in germination as well as changes in the ways rhizobia and nitrogen affect competitive interactions. The Fabaceae family is a plant family whose seeds have an impermeable coat, resulting in the need for some disruption of this dormancy for germination (Naik and Deshpande, 2021). In our study, we observed large differences between the germination rates of the two legume species versus the non-legume (Figure 2). Further, we noticed that *A. americanus* had a much longer window of germination than the other species, continuing to germinate for many months after seeding. This difference in germination strategy may play a larger role of competitive dynamics in the natural world than our study accounts for. Differences in phenological overlap may affect the period in which two species are competing, leading to differing competitive outcomes, such as priority effects (Alexander and Levine, 2019). For our greenhouse study, legume germananes were transplanted if no germination occurred; though this was essential for achieving the necessary competitor density gradient, in field systems, these germination differences reveal a new set of information about the competitive dynamics of legumes.

Our results suggest that legumes compete differently with other legumes than they do nonlegumes. Priority effects were predicted to occur the most between *L. bicolor* and *A. americanus* (Fig 5; Fig 6). Using Modern Coexistence Theory, priority effects are a function of similar fitness inequalities and negative stabilization potential (Ke and Letten, 2018). These two criteria may be occurring between our two legume species as the legumes are likely exhibit similar survival strategies, phenology, growth rates, and other contributors to fitness (Adler and Levine, 2007). Another important aspect of the interactions between legumes and non-legumes is the transfer of nitrogen via decomposition of legume tissue releasing nitrogen, root exudation of soluble nitrogen compounds, and the transfer of nitrogen via mycorrhizal networks

(Thilakarathna et al., 2016). Leguminous plant species may provide additional nitrogen in the soil for neighboring plant species. Although some of these mechanisms may have been accounted for in our study, decomposition of plant tissue would likely affect the next generation of plants. Further, mycorrhizal impacts were not accounted for in our study, but non-legume plants with strong mycorrhizal associations may gain a different advantage by neighboring legumes.

Nitrogen gradients within the soil created by anthropogenic causes are already occurring and decreasing the competitive advantage leguminous plants gain by association with rhizobia (Regus et al., 2017; Skogen et al., 2011). The results of our study show that for some species pairs, nitrogen fertilization changed the effect rhizobia had on competitive dynamics (Fig 3; Fig 6). In our experiment, differences in fitness inequalities caused by rhizobia changed differently based on nitrogen fertilization and species pairs (Figure 3). Regus et al. (2017) found that legume species in California differed in responses to increased nitrogen levels, with some depending solely on rhizobially fixed nitrogen, and others utilizing nitrogen from anthropogenic depositions. High soil nitrogen levels may decrease the relative benefit of the legume-rhizobia mutualism, with the costs of hosting rhizobia become greater when access to nitrogen increases in the soil (Keller and Lau, 2018). Further, nitrogen may affect the competitive dynamics of these legumes indirectly by affecting the competitive ability of non-legume species. If the non-legume monopolizes the anthropogenic supply of nitrogen runoff, the legume may be forced to rely on rhizobially fixed nitrogen (Elias and Agrawal, 2021), but if this does not occur, the legume may utilize the anthropogenic supply of nitrogen to save energy in returning resources to the rhizobia, thus decreasing the need for association with rhizobia (Skogen et al., 2011).

Increasing nitrogen deposition has the potential to be a major anthropogenic driver of loss of biodiversity and changes in ecosystems overall. Our study remains consistent with this idea and suggests that nitrogen levels will impact competitive impacts and community dynamics of these Oregon prairie species communities. The Willamette Valley is a unique study system because of the way agricultural sites have developed around and in these prairie areas. Historically, majority of the vegetation of the inner Willamette Valley has been prairie land, however a lot of this area has been developed into agricultural land in the last century, resulting in not only loss of these ecosystems, but major overlap between remaining Oregon prairie lands and agricultural sites

(Rao and Stephen, 2010; Christy and Alverson, 2011). The conversion of native prairies to agricultural land has already had significant impact on the overall habitat loss of prairies in the Willamette Valley (Fickas et al., 2016). As a result, remaining native prairies are in proximity to agricultural land, making the leaching of synthetic nitrogen fertilizers a realistic risk to the biodiversity and functioning of these already high conservation value remnant prairie systems.

Though there is early research understanding the effects of increased nitrogen levels on the mutualists and plant communities, the extent to which synthetic nitrogen fertilizer runoff is occurring has not yet been investigated extensively, and further the role of these context-dependent mutualists has not been understood in the conservation of Oregon prairie systems. In the conservation efforts of these native plant species and prairies, context-dependent mutualists like rhizobia must be included due to the role that they play in community dynamics, especially the maintenance of soil nitrogen levels. Future studies may investigate the differences between Oregon prairies based on their distance from agricultural sites that utilize synthetic nitrogen fertilizers extensively. The mutualism between legumes and rhizobia have long played a vital role in plant communities and it is largely possible that synthetic nitrogen fertilizer runoff may cause damage to this mutualism and its role in prairie ecosystems.

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