A private channel of nitrogen alleviates interspecific competition for an annual legume

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Abstract. The way resource availability predictably alters interspecific interactions and may favor one resource-acquisition strategy over another is critical for understanding context dependency. The ubiquity of nitrogen (N) limitation across terrestrial environments is a driver of plant competition and the association of some plants with N-fixing bacteria (rhizobia) may alleviate competition with nonfixing plants. Conversely, when available soil N is elevated, competitive advantages imparted by rhizobia are hypothesized to decline because nonfixing species are able to acquire those nutrients readily. We manipulated competition, soil N, and soil microbial inoculation, employing the ground bean *Amphicarpaea bracteata*, a native annual N-fixing legume, and jewelweed *Impatiens capensis*, a native co-occurring nonfixing annual. We found that legume performance was negatively impacted by interspecific competition, but less so under lower soil N in both the greenhouse and field. The legume invested a greater proportion of resources in rhizobia when competing, but only under low N. Also consistent with predictions, a competition-by-microbial-inoculation interaction demonstrated that negative effects of competition were alleviated by rhizobia. Finally, we detected an interaction between inoculation and fertilization, whereby N addition resulted in increased performance for uninoculated legumes, but a small decline in performance for inoculated plants, the latter likely representing a cost of mutualism. Thus, several lines of evidence point to the legume–rhizobia mutualism being more beneficial under competition and limited soil N. Competing *I. capensis*, in contrast, benefited from N addition regardless of the addition of soil microbes. In a survey of natural populations, legume and rhizobia growth were positively correlated at population edges (where interspecific competition is expected to be higher, the mutualism is stronger), whereas at population centers we found no association. Isotopic evidence confirmed a higher degree of rhizobial N-fixation at population edges compared to centers. Taken together, our results demonstrate an important role for the largely private channel of nitrogen in legume competitive performance, but with the benefits imparted by rhizobia being predictably weaker at higher soil fertility. We speculate that alleviation of competitive impacts through resource partitioning is an important and yet largely overlooked aspect of the evolutionary ecology of legume–rhizobia interactions.

Key words: context dependency; Fabaceae; mutualism; nitrogen fertilization; plant competition; plant–microbe interactions; rhizobia; trade-offs.

INTRODUCTION

The mutualistic association between legumes and nitrogen-fixing bacteria is widely considered to be a key adaptation allowing legumes to cope with nutrient limitation (Gutschick 1981, Sprent 2001, Werner et al. 2014). Because of this symbiosis, legumes are often colonizers of early successional communities where nitrogen (N) is limiting (van der Heijden et al. 2008). Additionally, having a largely “private channel” of N, which is mostly unavailable to neighboring plants, allows legumes to avoid competition for nitrogen with other plants, and potentially promotes coexistence within communities through resource partitioning (Schoener 1974). However, costs of hosting rhizobia (e.g., provisioning of carbon) can make the mutualism highly context dependent. High availability of soil N can cause a dissociation of plants from their rhizobial (Kiers and Denison 2008, Regus et al. 2017) and mycorrhizal mutualists (Hoesema et al. 2010, Kiers et al. 2011, Putten et al. 2013), leading to changes in resource allocation and the evolution of less cooperative partners (Weese et al. 2015).

Although experiments have elucidated predictable legume–rhizobia interactions under variable soil N, which are typically mutualistic even if weakened in highly fertile soil, many studies focus on the bipartite association of legumes and rhizobia (Bartholomew and
Allos 1955, Mahon and Child 1979, Xia and Wan 2008). Here we argue that interspecific plant competition is an important context for understanding legume–rhizobia interactions. If the association with rhizobia can alleviate competition for a key limiting resource, any reduction in N fixation could result in increased in niche overlap with non-N-fixing competitors, generating stronger interspecific competition (Parker 2001, Temperton et al. 2007). Indeed, a large body of work has reported nitrogen-induced reductions in plant community diversity, driven in part by the disappearance of legumes and other plants adapted to low N environments (Tilman 1987; Berendse et al. 1993, Pannek et al. 2015, Simkin et al. 2016). These studies suggest that legumes may be competitively inferior in fertile environments, a hypothesis that has rarely been directly tested (Bobbink et al. 2010, Skogen et al. 2011). Conversely, it is expected that under low soil N, legumes will enjoy benefits of N through the largely private channel of rhizobia, and thus should possess a competitive advantage relative to plants lacking a N-fixing symbiont. Although non-N-fixing neighbors may acquire some of the legume’s N though various mechanisms, this is typically a fraction of fixed N, and often occurs at a delay with the decomposition of legume roots (Temperton et al. 2007, Thilakarathna et al. 2016).

In this study we manipulate interspecific competition, soil N, and soil microbial inoculation for a wild legume, directly testing the role of different types of available resources in the outcome of interspecific plant competition. Our experiments focused on competition between two native herbaceous annuals of temperate forests in the eastern United States, *Amphicarpaea bracteata* (Fabaceae), and *Impatiens capensis* (Balsaminaceae). These species are likely frequent competitors, and often co-occur in canopy gaps where light and water are relatively abundant (JDE, personal observations, Struijk and Curtis 1962). *Amphicarpaea bracteata* forms mutualistic associations with N-fixing *Bradyrhizobium* bacteria (hereafter “rhizobia” [Sterner and Parker 1999]) giving it a mostly private channel of N available via nodulation. Previous work in this system has revealed variation in symbiotic compatibility even at kilometer-level local scales (Wilkinson et al. 1996), suggesting variation in effects of rhizobia, from positive to negative, in different environments. From the competitor’s perspective, in an experimental study of N deposition, a different species of *Impatiens, Impatiens parviflora*, was the only species that responded positively to nitrogen addition (Falkengren-Gerup 1993). Given similarities in growth habit and environmental requirements, this suggests *I. capensis* may also be a competent competitor for available nitrogen in soil.

We conducted field and greenhouse experiments and surveyed natural populations to assess the impact of soil N and rhizobial symbiosis on the outcome of competitive interactions between focal *A. bracteata* and *I. capensis*. In the greenhouse, we performed a fully factorial experiment in which we manipulated the presence and absence of three variables: competition with *I. capensis* (C), soil nitrogen availability (F), and inoculation with soil microbes (I). With this design, we tested three predictions, each based on a two-way interaction of those variables. First, we predicted that negative effects of competition on legume performance should be stronger when fertilized (C × F), because nonlegume competitors are better able to acquire available soil N. Second, we hypothesized weaker negative effects of competition on the legume when inoculated with microbes and nodulating (C × I), because legumes hosting rhizobia have a largely private channel of N that is unavailable to their nonrhizobial competitors, especially in the first year of their interaction. Finally, we tested the prediction that fertilization would be more beneficial in noninoculated *A. bracteata* compared to inoculated plants, irrespective of competition, because nitrogen-fixing associates impose costs to their host (F × I). We also report on the performance of competing *I. capensis*, which we predicted to be improved by fertilization, regardless of the inoculation status of the legume. We replicated our test of the first prediction in an independent field experiment. Finally, using field surveys of 16 natural populations of *A. bracteata* across New York State and western Massachusetts (United States), we collected measures of plant and rhizobia performance in the centers of populations (where interspecific competition is typically low) compared to the edges of populations (where competition was presumed to be stronger). Based on our mechanistic hypotheses and manipulative experiments, we expected that within natural populations, N fixation would be stronger at population edges, resulting in stronger mutual benefit between the rhizobia and plant host. Accordingly, we quantified the strength of mutualistic interactions at population centers and edges and used the natural abundance of stable N isotopes (Skogen et al. 2011) to determine relative rates of N fixation.

**Materials and Methods**

Ground bean (*Amphicarpaea bracteata*) is an annual legume native to eastern North America that produces subterranean cleistogamous (nonopening) flowers as well as aerial open-pollinated chasmogamous (opening) and cleistogamous flowers. Self-pollinated belowground seeds are on average 34 times larger than aboveground seeds (Trapp and Hendrix 1988), and are responsible for the majority of local reproduction in the system, resulting in highly inbred (but locally adapted) populations (Parker 1986). In order to control for variation in seed size, we exclusively used aboveground seeds from chasmogamous flowers for the experiments described below. *Amphicarpaea bracteata* associates with facultative bacterial mutualists in the genus *Bradyrhizobium* that possess genetic similarities to *Bradyrhizobium elkanii* (Sterner and Parker 1999).
For all experiments, rhizospheric soil, *A. bracteata* seeds, and *I. capensis* seedlings were collected from a population in which the two species co-occur in Danby State Forest near Ithaca, New York, United States (42°18′53.9″ N, 76°29′46.7″ W). Soil was collected by digging up random *A. bracteata* individuals, and retrieving roughly 100 g of soil per plant in the immediate root zone at a depth of 5–10 cm, where we have observed the majority of nodulation occurring. *Amphicarpaea bracteata* seeds were collected from random plants throughout this population, in excess of what was needed for the experiment; seeds were also randomly selected from this collection. Field-collected *A. bracteata* seeds were sterilized in 3% bleach for 15 min, then repeatedly rinsed before being mechanically scarified (small nick in seed coat opposite the site of radicle emergence).

Scarified seeds imbibed tap water overnight, and were subsequently placed in sealed petri plates with a wet paper towel.

For the greenhouse experiment, *A. bracteata* seedlings were planted after radicles emerged 2–3 mm (4–5 d). We timed the germination of *A. bracteata* with the natural spring emergence of *I. capensis*. Because *I. capensis* requires multiple extended stratification periods, and can take two years to germinate, we collected recently emerged seedlings from the source population at the cotyledonary stage. By harvesting at this early stage, we intended to limit the potential root microbial contamination of experimental plants. *Impatiens capensis* seedlings’ roots were also washed with water before transplanting, although we certainly recognize that this would not removal all microbes. *Impatiens capensis* seedlings were harvested in excess of the number needed for the experiment, and experimental plants were randomly chosen from those that did not experience transplant shock or mortality. Plants were grown in Lambert LM-111 soil (Lambert, Rivière-Ouelle, Québec, Canada) in 500-mL pots. Our greenhouse experiment was conducted in a shaded greenhouse (30% light reduction) to mimic light and temperature conditions in the forest understory.

For the field experiment, *A. bracteata* was started as above in the greenhouse experiment and transplanted into a natural population of *I. capensis* after both species had developed their first true leaves. In particular, although we did not manipulate soil microbial inoculation in the field, tests of our hypotheses in their natural community are an important check on the relevance of the findings.

**Treatments**

For both the greenhouse and field experiments we used a factorial, randomized block design, with all factors involving a presence or absence treatment. The greenhouse experiment (*n = 200*, 25 per treatment combination) included competition, N addition, and microbial inoculation as factors and four blocks. The field experiment included only competition and fertilization (*n = 25* plants per treatment, five blocks), as all plants had access to soil microbes.

*Amphicarpaea bracteata* in the inoculated treatment in the greenhouse were provided with 15 g field soil collected from the site of the seed source population. Noninoculated plants received soil from the same source after autoclaving twice at 121°C for 30 min with a 6-h intermittent period to ensure sterility. In both cases, field soil was sprinkled on top of an initial 3 in. of potting soil (LM-111) and covered with another inch of potting soil on the same day seedlings were potted. We used whole soil rather than isolated *Bradyrhizobium* because whole soil is a more realistic representation of the natural microbial community, with important implications for the study of this symbiosis (Heath and Tiffin 2007, Laroner et al. 2014, Ballhorn et al. 2016). Although we expect that most of our results are driven by rhizobial colonization and nodulation, we acknowledge that our method does not exclude roles for arbuscular mycorrhizal fungi (AMF) as well as other bacteria and fungi in the soil used for inoculation. Nodulation in the presence of microbial inoculation was confirmed by examining roots at the end of the experiment, as was the absence of nodules on uninoculated plants.

In both experiments, all plants were watered twice weekly with 200-mL nitrogen-free nutrient solution according to Broughton and Dilworth (1971), and once weekly with 200 mL tap water. To determine a biologically relevant amount of ammonium nitrate (NH₄NO₃) to serve as our fertilization treatment, we conducted a preliminary experiment comparing the growth responses of unnoculated *A. bracteata* grown along a range of soil fertility treatments, from 0 to 30 ppm N, to unfertilized, nodulating plants that were inoculated with field soil as in the present study (Appendix S1: Section S1). We determined that inoculation was equivalent to twice-weekly fertilization with 5–115 ppm N solution for shoot and root mass (Appendix S1: Fig. S1), and accordingly we used the upper range of these values in our experiments. Thus, fertilized plants were given 100 mL of 15 ppm NH₄NO₃ twice weekly in addition to the Broughton and Dilworth solution, whereas plants receiving no additional N were watered with an additional 100 mL of tap water. Fertilization treatments were initiated on the first day seedlings were potted in the greenhouse or planted in the field. Beginning in late June, as plants grew larger, they received an additional 100 mL of tap water at every watering.

For the competition treatment in the greenhouse, each pot was planted with an individual *I. capensis* seedling in addition to the focal *A. bracteata*. In the field experiment, we selectively cleared portions of a natural population of *I. capensis* outside of Ithaca, New York, USA (42°26′49.9″ N, 76°27′02.4″ W) for the competition-free treatment, and *A. bracteata* individuals were planted 30–35 cm from the nearest competitor. For the competition
treatment, individuals were planted within 10 cm of at least two naturally occurring *I. capensis* individuals. *Amphicarpaea bracteata* in the field experiment experienced greater mortality than in the greenhouse; when individuals died, they were replaced with plants grown in pots in the same field conditions. In the field, 28 of 100 of *A. bracteata* plants needed to be replaced. Of these 28, five needed to be replaced after the first 5 weeks of the experiment. In the greenhouse, only 4 of 200 experimental *A. bracteata* plants experienced mortality and were not replaced. In both cases there was no significant effect of treatment on mortality. We note that for both the greenhouse and field experiment, our competition treatments did not control for plant density, but rather focused on adding an interspecific (non-N-fixing) neighbor next to *A. bracteata*. Although adding additional treatments controlling for density could add additional value, here our primary goal was to compare the impact of adding a competitor under different ecological conditions (with and without enhancing soil N, or soil microbial inoculation).

**Fitness and allocation measures**

To maximize the collection of meaningful data, we harvested plants prior to complete senescence when *A. bracteata* disintegrates. Consequently, harvest was often prior to the full realization of seed set. In a preliminary controlled experiment, we grew plants until seeds fully matured, and found a strong correlation between shoot mass and lifetime production of both aerial and underground seeds (*n* = 35, *r* = 0.84, *P* < 0.001). Seeds for this preliminary experiment were from the same source population as other experiments in this study. Thus, we use *A. bracteata* dry shoot mass as a proxy for total reproductive fitness. Although we report seed production in the greenhouse and field experiments when available, we believe shoot biomass is a more accurate fitness metric in light of our harvesting schedule. Furthermore, for seed number, we only analyzed plants inoculated with microbes, as 86% produced seeds (we excluded non-inoculated plants, where only 26% produced seeds, adjusted *G* = 58.977, *P* = 0.001). Although we did not measure belowground biomass or nodulation in the greenhouse experiment, in an additional preliminary experiment (Appendix S1: Section S2) we characterized the effects of manipulating soil N on nodule number, nodule size, and shoot and root biomass. This experiment showed a very strong effect of N fertilization on reducing nodulation (Appendix S1: Fig. S2).

In the field experiment, we assessed the impacts of competition and fertilization on root, shoot, and nodule biomass as an indication of relative allocation to nutrient acquisition, photosynthesis, and investment in the mutualistic partner, respectively. Because rhizobia nodule production is typically positively correlated with shoot mass in this species (JDE, unpublished data; *F*₁,₁₈₈,₃ = 19.346, *R*² = 0.266, *P* < 0.001) and in other herbaceous legumes (Azad et al. 2013), we also analyzed effects on the number of nodules per unit shoot biomass as a test of changes in proportional allocation to rhizobia.

For *I. capensis*, which produces seeds for a longer portion of the growing season, we used seed production as our measure of fitness. Mature seeds of *I. capensis* are dispersed up to several meters from the parent plant as pods explosively dehisce when perturbed. To collect seeds, we intentionally triggered dehiscence and tallied seed production each time we watered plants for the duration of the greenhouse experiment.

**Survey of natural populations and stable isotope analysis**

We conducted a field survey across 16 populations of *A. bracteata* across New York and Massachusetts (between longitude 77.782776 and 73.281421° W and latitude 42.242966 and 44.620083° N), collecting plants, rhizobia, and rhizospheric soil between 5 and 12 September 2016. Site locations and descriptions are provided in Appendix S2: Table 1. For each population we quantified dry mass of roots, shoots, and nodules, and counted nodules on 10 individuals, five each from the centers and edges of each population. Because *A. bracteata* often grows in near-monoculture patches in natural populations, individuals at edges are more likely to experience interspecific competition than those at centers (JDE, personal observations). Thus, our data collection of plant and rhizobial traits represents the outcome of mutualistic interactions under different ecological conditions.

Different fractions of naturally abundant N isotopes exist in plant tissues as a result of N fixation (Unkovich and Pate 2000, Unkovich et al. 2008, Soper et al. 2015). Because N fixed by bacteria originates as atmospheric N₂, the ratio of $\^{15}$N:$^{14}$N in plant material should approach the atmospheric ratio (0.3663 atom% $^{15}$N) as the total proportion of symbiotically fixed nitrogen increases (Unkovich et al. 2008). Variation in $^{15}$N abundance is expressed in units of δ, or deviation from atmospheric values of $^{15}$N in parts per thousand (‰). Smaller absolute values of δ$^{15}$N represent a greater amount of biological nitrogen fixation (BNF [Skogen et al. 2011]). We confirmed this effect in a preliminary controlled experiment with and without inoculation before field sampling (Appendix S1: Fig. 2). From the center and edge of each population, three samples each for shoots and soil were pooled and homogenized. Soil samples were taken by digging up individual plants. After lightly shaking excess soil off roots, roughly 10 g of rhizospheric soil from each plant was collected at a depth of 5–10 cm in a small envelope prior to pooling and analysis. Dry shoot (3 mg) and soil samples (10 mg) were ground to a fine powder using an MM300 Retsch grinder (Retsch GmbH, Haan, Germany) in 2-mL Eppendorf tubes at 27 Hz for 4 min with a 3-mm steel ball. Samples were analyzed by the Cornell Isotope
Laboratory for percent carbon, percent nitrogen, and associated isotopes (δ^{13}C and δ^{15}N), employing a Thermo Delta V isotope ratio mass spectrometer (IRMS) interfaced to an Carlo Erba NC2500 elemental analyzer.

**Results**

In both the greenhouse and field experiments, we found support for the hypothesis that negative competitive effects on *A. bracteata* are stronger when the soil is fertilized (competition by fertilization interaction for effects on shoot mass: greenhouse: $F_{1,130.6} = 10.380, P = 0.002$; field: $F_{1,63.81} = 6.517, P = 0.013$, Fig. 1A, B). When plants were not fertilized, competition decreased shoot mass (~50% reductions), and greater reductions due to competition occurred when fertilized (~73% in both the greenhouse and field, Fig. 1A, B). We note that in the greenhouse experiment this effect of stronger competition only held for uninoculated plants (Appendix S3 Fig. S2). Among inoculated *A. bracteata* (in the greenhouse), we did not find a significant interaction of competition and fertilization on seed number ($F_{1,60.2} = 0.069, P = 0.793$). Instead, the main effects of fertilization ($F_{1,60.27} = 4.924, P = 0.030$) and competition ($F_{1,60.15} = 8.653, P = 0.005$) reduced seed production by 33% and 32%, respectively.

For root biomass of *A. bracteata* (only measured in the field experiment), only the main effect of competition was significant ($F_{1,63.94} = 23.287, P < 0.001$), with competing plants producing roots that were 42% smaller than those growing alone. Nonetheless, competition and fertilization interacted in altering root:shoot ratio ($F_{1,63.34} = 8.491, P = 0.005$, Fig. 1C). Competition did not alter patterns of root:shoot ratio when plants were not fertilized, but with additional N, competition increased root:shoot ratio by a factor of 1.5. We found significant main effects of both competition and fertilization on nodule number ($F_{1,62.48} = 5.222, P = 0.026$; $F_{1,62.48} = 20.934, P < 0.001$, respectively): competition decreased nodule number by 27% and fertilization decreased nodule number by 49%. We also analyzed the effect of competition and fertilization on the number of nodules per unit shoot biomass, where we again detected main effects of both fertilization ($F_{1,61.56} = 14.211, P < 0.001$, Fig. 1D) and competition ($F_{1,61.41} = 6.397, P = 0.014$), but in this case competition resulted in a 103% increase in nodules per unit biomass. Conversely, fertilized plants produced roughly 67% fewer nodules per unit biomass than nonfertilized plants. Effects of fertilization on root traits and nodulation were confirmed in a separate greenhouse experiment, where even moderate fertilization dramatically reduced the number and size of nodules (Appendix S1: Fig. S2).

Consistent with our predictions, we also found that the negative effects of competition were weaker when *A. bracteata* was inoculated with rhizobia compared to not inoculated (57 vs. 88% reductions in biomass due to competition, interaction term for shoot mass in the greenhouse experiment $F_{1,130.8} = 3.944, P < 0.001$; Fig 2). Finally, we found a significant two-way interaction between inoculation and fertilization for *A. bracteata* shoot biomass ($F_{1,130.3} = 20.216, P < 0.001$; Fig 3A) and seed number ($F_{1,130.1} = 17.344, P < 0.001$), with fertilized, noninoculated plants producing 147% more aboveground biomass than unfertilized (noninoculated) plants, whereas inoculated plants were 18.5%...
smaller when fertilized compared to unfertilized (inoculated) plants. In contrast to the effect of additional N on *Amphicarpaea bracteata*, fertilization had a consistent positive effect on *I. capensis* shoot mass ($F_{1,74.04} = 204.908, P < 0.001$) and seed production ($F_{1,98.04} = 159.328, P < 0.001$, Fig 3B), regardless of inoculation status (i.e., no interactive or main effect of inoculation on *I. capensis*).

**Survey of natural populations**

In natural populations of *A. bracteata*, we sampled soil and plants at centers and edges of patches, representing the outcome of interactions in low and high interspecific competition environments, respectively. We predicted higher soil N in the centers of patches because of nodulation and N mineralization over time. Despite a trend of 40% higher soil N in population centers compared to edges, this was not significant (mean percent N/SE, center: 0.404/0.071, edge: 0.287/0.071, paired analysis $F_{1,15} = 1.359, P = 0.262$). Neither shoot mass ($F_{1,160.6} = 0.283, P = 0.594$) nor total nodule mass ($F_{1,152.9} = 1.206, P = 0.274$) varied by location at the center versus edge of *A. bracteata* populations (Appendix S2: Table S1). However, total nodule mass correlated with shoot mass at population edges ($R^2 = 0.366, F_{1,79} = 53.631, P < 0.001$), but not in the center ($R^2 = 0.012, F_{1,72} = 0.897, P = 0.347$), resulting in a significant interactive effect of location and total nodule mass on shoot mass (position * total nodule mass: $F_{1,150.4} = 12.207, P < 0.001$; Fig. 4). This result is consistent with our prediction that rhizobia are an especially important source of nitrogen for individuals experiencing greater interspecific competition (or with less N mineralization). Indeed, edge plant leaves had a $\delta^{15}N$ value that deviated less (lower absolute values) from atmospheric levels than center plants (mean 15 N/SE, edge: $-3.235 \pm 0.124$, center: $-3.573 \pm 0.110$, $F_{1,15.07}$...
suggesting a higher degree of rhizobial N fixation for *A. bracteata* at population edges.

**DISCUSSION**

We borrow the term “private channel” from a body of research considering plant–pollinator mutualisms, where highly specific signals provide information to interaction partners (Friberg et al. 2014). In our case, the private channel reflects nutrient acquisition and allocation (not information) that alters patterns of interspecific competition. Perhaps as is the case with all private channels, that between legumes and rhizobia is somewhat leaky. As reviewed by Thilakarathna et al. (2016) nonleguminous neighbors may gain some benefit from rhizobia-fixed N, be it through root decomposition, exudation into the soil, or transfer by AMF. Nonetheless, there is conceptual overlap between these two cases (pollination and rhizobia) in that the association facilitates evolutionary specialization and thus the potential for higher community diversity and coexistence of species (Agrawal 2020). As a mechanism of exchange in specialized mutualisms, private channels protect the resource, be it informational or nutritional. Coexisting species likely maintain alternative or additional strategies, because the mutualism is unavailable to them.

Our factorial manipulation of interspecific competition, soil nitrogen, and microbial inoculation revealed several results consistent with a stronger legume-rhizobia mutualism under competition and limited soil fertility. In particular, under competition and limited N, plants disproportionally invested in nodules (Fig. 1D), and under the same conditions, the effect of interspecific competition on *A. bracteata* was the weakest (Fig. 1A, B). Overall, the channel of nitrogen facilitated through rhizobial inoculation was critical to minimizing effects of interspecific competition (Fig. 2). Finally, although nitrogen addition was beneficial to
nonrhizobial plants (both uninoculated _A. bracteata_ and its competitor, _I. capensis_), this effect flipped for inoculated _A. bracteata_, suggesting a conflict, or cost, of rhizobia association when N is plentiful. Interestingly, in a community of coexisting legumes in the genus _Trifolium_, rhizobia stabilized competitive interactions between plant species, suggesting that N fixation may also play a role in the competitive outcomes of species that share mutualists (Siefert et al. 2019). Beyond rhizobia, our study was unable to separate additional effects of AMF, which may have been part of the soil microbial symbiotic community.

Our findings are broadly consistent with community-level patterns of fertilization-induced reductions in plant diversity, which are often driven by the exclusion of legumes (Tilman 1987, Berendse et al. 1993, Pannek et al. 2015, Simkin et al. 2016). For example, McPhee et al. (2015) showed that natural recruitment of legumes in N-fertilized restoration sites was substantially lower compared to that in control plots. Thus, in diverse and competitive environments, lower soil nitrogen may be key to the persistence of legumes. Indeed, lower soil nitrogen was the general rule for pre-Anthropocene soils, the conditions in which most nutrient acquisition traits evolved (Aber et al. 1989). At higher soil N levels, the balance tips in favor of nonrhizobial plants, which presumably are better adapted to utilize the available N and do not pay costs associated with maintaining mutualists (Bronstein 2001). Few studies have measured competition between legumes and nonlegumes under varying soil fertility, but Skogen et al. (2011) found results similar to ours for threatened _Desmodium cuspidatum_. In that study, under higher soil N, nonleguminous competitors monopolized mineral nitrogen, forcing the legume to rely on rhizobially fixed N. One less clear-cut aspect of our results is that in the greenhouse experiment, the effect of stronger competition on _A. bracteata_ when fertilized was only evident in uninoculated plants (Appendix S3, Fig. S2); this outcome is indicated in the three-way statistical interaction and suggests a complex interplay between competitive dynamics and the available sources of nitrogen. For the other two-way interactions (Figs. 2, 3A), the strength, but not direction, of the effect varied over the third factor.

**Costs and context-dependency of the mutualism**

Because legumes do not have complete control over the plant–rhizobial association (Kiers and Denison 2008), they may be stuck with rhizobia regardless of the costs of hosting N fixers relative to obtaining available N from the environment. For this reason, we predicted a negative effect of inoculation in high N environments. Not only was this result borne out for individuals, but longer-term studies have also shown that chronic N-addition leads to the evolution of less cooperative rhizobia (Weese et al. 2015). Less studied are evolutionary responses of legumes to altered soil fertility. Nonetheless, available evidence suggests that plant competition and rhizobial availability impact the evolution of these interactions (Keller and Lau 2018, terHorst et al. 2018). Overall, we argue that minimization of competition is an important and largely overlooked aspect in the evolution of legume–rhizobia interactions.

Context dependency is the rule in complex ecological interactions, especially among facultative mutualisms (Ákçay and Simms 2011, Chamberlain et al. 2014). In our system, and for other legumes, spatiotemporal variation in soil N, as well as variation in the competitive environment, should result in context-dependent ecological outcomes and favor the evolution of plasticity. Plasticity in associations and allocation appears to be dependent not only on competition, but also on abiotic factors such as light availability and the genotypic match with particular rhizobia strains (Heath et al. 2020). In our field surveys, we found spatial variation in the outcome of legume–rhizobia interactions that we speculate were dependent on the build-up of soil N in population centers, where rhizobial N-fixation was reduced (based on δ^15N) and where nodule mass was not associated with plant productivity (Fig. 4B). At population edges, where interspecific competition was more likely, the mutualism was stronger, as evidenced by a positive relationship between nodule mass and plant growth (Fig. 4A). Although plasticity in the association is an important trait for surviving in a landscape of heterogeneous nitrogen, plasticity has limits. Strong nitrogen gradients created by anthropogenic inputs are likely already decreasing the amount of suitable habitat for legumes with plastic associations with rhizobia (Skogen et al. 2011, Stevens et al. 2018).

**Conclusion and global change implications**

Nitrogen fertilization and other anthropogenic disruptions have markedly altered the N cycle (Vitousek 1994, Holmgren et al. 2011). Higher soil N appears to increase the cost-to-benefit ratio of legume associations with rhizobia (Regus et al. 2017). Although _A. bracteata_ is known to adapt to rhizobia locally (and vice versa [Parker 1999]) the plant maintains a relatively promiscuous association among strains of rhizobia that is not highly specific (Parker et al. 2004). Thus, the effects of differentiated populations are likely swamped by changing N gradients at local to regional scales. Legumes like _A. bracteata_ with plasticity in their association with rhizobia are more likely to survive anthropogenic increases in nitrogen compared to less-plastic legumes. Nonetheless, many other legumes are declining (Skogen et al. 2011, Humbert et al. 2016), although the specific traits making these species sensitive is not yet clear. As we have argued, resource-hungry competitors will also likely contribute to this outcome as they crowd out legumes when anthropogenic N increases. Although mitigation of interspecific competition could well have been a driving force behind the success of...
legume-rhizobial interactions, nitrogen pollution across the planet may well reverse this effect, reducing the future success of wild legumes.

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REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/ecy.3449/suppinfo

OPEN RESEARCH

Data (Elias and Agrawal 2021) is available from the Cornell University eCommons Repository: (https://doi.org/10.7298/6g8h-da29).

Appendix S1

Section S1. Methods and results from preliminary experiment #1 on the impact of nitrogen fertilization on plant growth and microbial N-fixation compared to microbe-inoculated *Amphicarpaea bracteata*

As in the main experiments, field collected *Amphicarpaea bracteata* seeds were sterilized in 3% bleach and repeatedly rinsed before being mechanically scarified. Seeds imbibed tap water over night, after which point they were placed in sealed petri plates with a wet paper towel. Seedlings were planted after radicles emerged. Plants were grown in Lambert’s potting soil in 4” pots in a growth chamber (16h L, 8h D; PAR=272.5 µEm⁻²s⁻¹; 27°C). Plants in the inoculated treatment (I+) were provided with 15 g field soil collected from within the seed source population. For all of the other (non-inoculated) treatments (I-) field soil was autoclaved at 121 °C for 30 minutes twice. As in the main experiments, field soil was sprinkled on top of an initial 3 inches of Lambert’s mix, and covered with another inch of the potting mix on the same day seedlings were potted. All plants were watered twice weekly with nitrogen-free nutrient solution as in the main experiments, and once weekly with tap water. On days when plants were provided with N-free nutrient solution, that solution was amended with N (NH₄NO₃) according to treatments specified below. Fertilization treatments were initiated on the first day seedlings were potted.

In addition to microbial inoculation, six additional treatments (total n=70) of N were applied (0, 5, 10, 15, 20, 30 ppm N [NH₄NO₃]). Plants were grown for 10 weeks. Upon harvest, successful nodulation was assessed in inoculated plants. Roots (washed of soil) and shoots were weighed (for total dry mass). We employed a linear model to test for differences in total plant mass with one-way ANOVAs with treatment as factor. Pairwise differences among treatments were determined using Tukey’s HSD post hoc.

To confirm a distinct isotopic signature of microbially-based atmospherically fixed N, a leaf sample (each 3 mg dry) was also analyzed for δ¹⁵N as described in the main experiment (n=70).
Figure S1. Nitrogen fertilizer impacts on A) total plant biomass and B) leaf $\delta^{15}$N for *Amphicarpa bracteata*. Lower $\delta^{15}$N indicates greater rhizobial N fixation. Bars are means ± SE. Significant differences among treatments after Tukey’s HSD are indicated with letters above the error bars.
Section S2. Methods and results from preliminary experiment #2 on the impact of nitrogen fertilization on above- and below-ground growth and nodulation of microbially inoculated *Amphicarpa bracteata*

As in the main experiments, field collected *Amphicarpa bracteata* seeds were sterilized in 3% bleach and repeatedly rinsed before being mechanically scarified. Seeds imbibed tap water over night, after which point they were placed in sealed petri plates with a wet paper towel. Seedlings were planted after radicles emerged. Plants were grown in Lambert’s potting soil in 4” pots in a growth chamber (16h L, 8h D; PAR=272.5 µEm⁻²s⁻¹; 27°C). All plants were inoculated with 15 g field soil collected from within the seed source population. All plants were watered twice weekly with nitrogen-free nutrient solution as in the main experiments, and once weekly with tap water. On days when plants were provided with N-free nutrient solution, that solution was amended with N (NH₄NO₃) according to treatments specified below. Fertilization treatments were initiated on the first day seedlings were potted.

Four treatments (total n=70) of N were applied (0, 23, 46, and 70 ppm N [NH₄NO₃]). Plants were grown for 10 weeks. Upon harvest, successful nodulation according to treatment was assessed. Roots (washed of soil) and shoots were weighed (dry mass), and nodules from every plant were counted. Nodule diameter was measured using a Vernier caliper. We employed a linear model to test for differences in shoot mass, root mass, nodule number, and nodule diameter each with one-way ANOVAs with treatment as factor. Pairwise differences among fertilization treatments were determined using Tukey’s HSD post hoc.
Figure S2. Nitrogen fertilizer (shown in ppm N) impacts on above- and below-ground biomass and nodulation of *Amphicarpa bracteata*. When N is not limiting, plants exercise some degree of control over the association, wherein fewer nodules form under higher N conditions, and those that do are smaller. All plants were inoculated with field soil. Bars are means ± SE. Significant differences among treatments after Tukey’s HSD are indicated with letters above error bars.

Appendix S2.

Table S1. Site location, size, and soil information for the field survey.

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<th>longitude</th>
<th>pop size (m²)</th>
<th>elev_m</th>
<th>position</th>
<th>Soil %N</th>
<th>Soil %C</th>
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Appendix S3.

Table S1. Responses of *Amphicarpa bracteata* and *Impatiens capensis* performance to factorial greenhouse manipulations of competition, fertilization, and inoculation. For *I. capensis*, all plants were competing with *A. bracteata*. In this mixed model ANOVA, block was included as a random effect.

I = soil microbial inoculation, C = interspecific competition, and F = nitrogen fertilization.

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<th>F</th>
<th>P</th>
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<td>294.240</td>
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<tr>
<td></td>
<td>C</td>
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<tr>
<td></td>
<td>I*C</td>
<td>1, 130.8</td>
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<td>F</td>
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*I. capensis* seed number

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Figure S2. Illustration of the three-way interaction between competition, nitrogen fertilization, and soil microbial inoculation for impacting shoot growth of *Amphicarpa bracteata* in the greenhouse experiment. Shown are raw means ± SE.