

**Symbiotic nitrogen fixation reduces belowground biomass carbon costs of nitrogen acquisition under low, but not high, nitrogen availability**

*Running title: Symbiotic N fixation reduces nitrogen acquisition costs under low soil N*

**Abstract**

Many plant species form symbiotic associations with nitrogen-fixing bacteria. Through this symbiosis, plants allocate photosynthate belowground to the bacteria in exchange for nitrogen fixed from the atmosphere. This symbiosis forms an important link between carbon and nitrogen cycles in many ecosystems. However, the economics of this relationship under soil nitrogen availability gradients is not well understood, as plant investment toward symbiotic nitrogen fixation tends to decrease with increasing soil nitrogen availability. Here, we used a manipulation experiment to examine how costs of nitrogen acquisition vary under a factorial combination of soil nitrogen availability and inoculation with *Bradyrhizobium japonicum* in *Glycine max* L. (Merr.). We found that inoculation decreased belowground biomass carbon costs to acquire nitrogen and increased total leaf area and total biomass, but these patterns were only observed under low fertilization. Treatment differences were the result of increased plant nitrogen uptake and no change in belowground carbon allocation. These results suggest that symbioses with nitrogen-fixing bacteria reduce carbon costs of nitrogen acquisition, but only when soil nitrogen is low, allowing individuals to increase nitrogen allocation to structures that support aboveground growth. This pattern may help explain the prevalence of plants capable of forming these associations in less fertile areas and demonstrates responses that can help guide models linking carbon and nitrogen cycles in terrestrial ecosystems.

**Keywords**

carbon-nitrogen interactions; nitrogen fixation; whole plant growth; greenhouse; crops; nutrient acquisition strategy

## Introduction

Terrestrial ecosystems are regulated, in part, by interactions between carbon and nitrogen cycles. As a result, terrestrial biosphere models are beginning to include coupled carbon and nitrogen cycles to simulate past, present, and future atmosphere-biosphere fluxes more realistically (Hungate *et al.* 2003; Prentice *et al.* 2015; Kou-Giesbrecht *et al.* 2023). Carbon and nutrient flux simulations tend to converge across terrestrial biosphere model products using past and present climate scenarios; however, these models often diverge under future environmental change scenarios (Friedlingstein *et al.* 2014; Davies-Barnard *et al.* 2020). This divergence could be due to an incomplete understanding of how changing environments modify processes that link ecosystem carbon and nitrogen cycles (Wieder *et al.* 2015; Fay *et al.* 2015; Meyerholt *et al.* 2016).

Plant nitrogen acquisition is a process in terrestrial ecosystems that links carbon and nitrogen cycles. Plants allocate photosynthetically derived carbon belowground in exchange for nitrogen through different nitrogen acquisition strategies. These nitrogen acquisition strategies can include direct uptake pathways such as mass flow or diffusion (Barber 1962; Fisher *et al.* 2010) or through symbioses with nitrogen-fixing bacteria (Vance and Heichel 1991; Vitousek *et al.* 2002; Udvardi and Poole 2013). Plants cannot acquire nitrogen without first allocating carbon belowground, which implies an inherent carbon cost to the plant for acquiring nitrogen (Chapin *et al.* 1987). This carbon cost for acquiring nitrogen may vary in species with different nitrogen acquisition strategies. For instance, carbon investment toward roots for direct nitrogen uptake does not require costs beyond root development, as is the case for acquisition strategies that involve other soil microbiota. However, the nitrogen acquired from a given belowground carbon investment may be greater than direct uptake if carbon is allocated to bacterial symbionts in exchange for nitrogen fixed from the atmosphere (Gutschick 1981; Vitousek and Field 1999; Rastetter *et al.* 2001; Vitousek *et al.* 2002). Variations in the cost to acquire nitrogen may help explain the prevalence of different nitrogen acquisition strategies in different environments, but these costs have not been quantified outside of a few studies (Terrer *et al.* 2018; Perkowski *et al.* 2021; Lu *et al.* 2022) despite their inclusion in nitrogen uptake models (Fisher *et al.* 2010; Brzostek *et al.* 2014; Allen *et al.* 2020) currently implemented in terrestrial biosphere models (Shi *et al.* 2016; Lawrence *et al.* 2019; Braghiere *et al.* 2022). No studies to our knowledge have directly investigated interactions between nitrogen fixation and nitrogen availability on carbon

costs to acquire nitrogen, which limits our ability to evaluate the ability of models to simulate plant nitrogen uptake patterns across environmental gradients.

Carbon costs to acquire nitrogen are likely dependent on external environmental factors such as atmospheric CO<sub>2</sub>, light availability, and soil nutrient availability (Brzostek *et al.* 2014; Terrer *et al.* 2018; Taylor and Menge 2018, 2021; Friel and Friesen 2019; Allen *et al.* 2020; Perkowski *et al.* 2021; Lu *et al.* 2022). For instance, the amount of photosynthate allocated belowground in exchange for nitrogen may increase with increased light and CO<sub>2</sub>, as these factors reduce the cost to produce photosynthate and increase plant demand for acquiring nitrogen to build and maintain photosynthetic enzymes (Terrer *et al.* 2018; Taylor and Menge 2018; Friel and Friesen 2019; Perkowski *et al.* 2021; Waring *et al.* 2023). However, increasing soil nitrogen availability may alternatively reduce costs for nitrogen acquisition due to increases in plant nitrogen acquisition and reductions in belowground carbon allocation. This pattern may be due to reduced soil resource mining (by roots or symbionts) needed to satisfy plant nitrogen demand under greater nitrogen availability.

Reductions in the cost of nitrogen acquisition due to increasing soil nitrogen availability may not be as robust in plant species with strong and specialized symbiotic relationships with nitrogen-acquiring partners that reduce the sensitivity of plant nitrogen uptake to changes in nitrogen availability (e.g., plant species that associate with symbiotic nitrogen-fixing bacteria) (Perkowski *et al.* 2021). Indeed, plants that form associations with symbiotic nitrogen-fixing bacteria often exhibit reduced responses to changes in nitrogen availability despite reduced investment toward nitrogen fixation with increasing nitrogen availability (Gutschick 1981; Taylor and Menge 2018; Friel and Friesen 2019; McCulloch and Porder 2021; Menge *et al.* 2023; Schmidt *et al.* 2023). While previous work notes that plants can still acquire nitrogen through symbiotic nitrogen fixation under high soil nitrogen availability (Menge *et al.* 2023), resource optimization theory suggests that reduced sensitivity of plant nitrogen uptake to changes in nitrogen availability in nitrogen-fixing plants may stem from preferential investment toward the acquisition strategy that confers the lowest carbon cost and greatest nitrogen gain (Bloom *et al.* 1985; Rastetter *et al.* 2001). If true, similar costs to acquire nitrogen in nitrogen-fixing species may be achieved across nitrogen availability gradients due to shifts away from nitrogen acquisition through nitrogen fixation to direct uptake as costs to acquire nitrogen through direct uptake decrease (Fisher *et al.* 2010; Brzostek *et al.* 2014; Perkowski *et al.* 2021).

In a recent study, Perkowski *et al.* (2021) showed that increasing soil nitrogen fertilization decreased belowground biomass carbon costs to acquire nitrogen in *Gossypium hirsutum* (L.) and *Glycine max* L. (Merr). *Gossypium hirsutum* can acquire nutrients via direct uptake pathways, while *G. max* can acquire nutrients via direct uptake pathways or through symbioses with nitrogen-fixing bacteria. In the experiment, belowground biomass carbon costs to acquire nitrogen in *G. max* were less responsive to increasing soil nitrogen fertilization than *G. hirsutum*. This pattern coincided with reduced *G. max* root nodulation with increasing fertilization. The authors speculated that this response may have been driven by resource optimization, where *G. max* shifted their dominant mode of nitrogen acquisition from nitrogen fixation to direct uptake with increasing nitrogen fertilization once the cost to acquire nitrogen via direct uptake became less than the cost to acquire nitrogen via nitrogen fixation (Bloom *et al.* 1985; Rastetter *et al.* 2001). However, the authors were not able to make robust conclusions about whether the carbon cost to acquire nitrogen responses to soil nitrogen fertilization differed between *G. hirsutum* and *G. max* due to differences in the ability to associate with symbiotic nitrogen-fixing bacteria. This was because the two species are not phylogenetically related and adopt different growth forms and growth durations.

To understand how nitrogen fixation and soil nitrogen fertilization interact to influence belowground biomass carbon costs to acquire nitrogen, *Glycine max* L. (Merr.) seedlings were grown under two soil nitrogen fertilization treatments and were either inoculated or not inoculated with symbiotic nitrogen-fixing bacteria in a full factorial greenhouse experiment. We used this experiment to test the following hypotheses:

- (1) Soil nitrogen fertilization will decrease belowground biomass carbon costs of nitrogen acquisition in both uninoculated and inoculated individuals. This decrease will manifest as an increase in the amount of nitrogen acquired per belowground carbon investment, indexed by a stronger increase in plant nitrogen uptake than belowground carbon allocation.
- (2) Inoculation with nitrogen-fixing bacteria will decrease belowground biomass carbon costs to acquire nitrogen under low soil nitrogen availability. This is because belowground biomass carbon costs to acquire nitrogen through symbiotic nitrogen fixation will be less than the belowground biomass carbon cost to acquire nitrogen via direct uptake. However, inoculation will not affect belowground biomass carbon costs

to acquire nitrogen under high soil nitrogen availability due to all plants shifting toward a similar, direct uptake-dominated mode of nitrogen acquisition.

- (3) Root nodulation and plant investment toward symbiotic nitrogen fixation will decrease with increasing soil nitrogen availability. This pattern will be due to reduced belowground biomass carbon costs to obtain nitrogen from direct uptake with increasing soil nitrogen fertilization that will increase relative plant investment toward nutrient uptake through direct uptake pathways.

## **Materials and methods**

### *Experimental Design*

*Glycine max* seeds were planted in 64, 6-liter pots (NS-600, Nursery Supplies, Orange, CA, USA) containing unfertilized potting mix (Sungro Sunshine Mix #2, Agawam, MA, USA). The experiment used *G. max* seedlings to compare observed responses from previous work that was not able to disentangle species-specific effects on belowground biomass carbon costs to acquire nitrogen from the explicit effects of nitrogen fixation (Perkowski *et al.* 2021). Pots and potting mix were steam sterilized at 95°C for three hours to eliminate any bacterial or fungal growth. Thirty-two randomly selected pots were planted with seeds inoculated with *Bradyrhizobium japonicum* (Verdesian N-Dure™ Soybean, Cary, NC, USA) following a brief surface sterilization in 20,000 ppm sodium hypochlorite for 5 minutes followed by three washes in ultrapure water (Scouten and Beuchat 2002; Montville and Schaffner 2004). The remaining 32 pots were planted with seeds that did not receive any inoculation treatment. Uninoculated seeds were also surface sterilized in 20,000 ppm sodium hypochlorite for 5 minutes followed by three ultrapure water washes to ensure that the only difference between seed treatments was the inoculation treatment.

Upon planting, all pots were immediately placed in one of four random blocks in a greenhouse and received one of two nitrogen fertilization treatments as 150 mL of a modified Hoagland's solution (Hoagland and Arnon 1950) equivalent to either 70 or 630 ppm N twice per week for seven weeks. Nitrogen fertilization levels were chosen based on previous work using a larger number of fertilization treatments (Perkowski *et al.*, 2021). Nitrogen fertilization doses were received as topical agents to the soil surface and were modified to keep concentrations of other macronutrients and micronutrients equivalent across the two treatments (Table S1).

Throughout the experiment, plants were routinely well-watered to minimize any chance of water stress. Greenhouse maximum daytime temperatures averaged  $42.4 \pm 3.9^\circ\text{C}$  (mean  $\pm$  standard deviation) across blocks, while minimum nighttime temperature averaged  $19.8 \pm 1.9^\circ\text{C}$  across blocks. There was no evidence of growth limitation due to pot size at the time of biomass harvest, indicated by total biomass: pot volume ratios less than  $1 \text{ g L}^{-1}$  within each treatment combination (Table S2-3; Fig. S1; Poorter et al., 2012).

#### *Plant trait measurements*

All individuals were harvested, and biomass was separated into major organ types (leaves, stems, roots, and root nodules when present) approximately seven weeks after experiment initiation and before the onset of reproduction. Leaf areas of all harvested leaves were measured using an LI-3100C (Li-COR Biosciences, Lincoln, Nebraska, USA). Total leaf area ( $\text{cm}^2$ ) was calculated as the sum of all leaf areas. All harvested material was dried in an oven set to  $65^\circ\text{C}$  for at least 48 hours, weighed, and ground to homogeneity. Total dry biomass (g) was calculated as the sum of dry leaf, stem, root, and root nodule biomass. Carbon and nitrogen content of each respective organ was quantified through elemental combustion (Costech-4010, Costech, Inc., Valencia, CA, USA) using subsamples of ground and homogenized organ tissue.

Belowground biomass carbon costs to acquire nitrogen were calculated as the ratio of total belowground biomass carbon to whole plant nitrogen biomass ( $\text{g C g}^{-1} \text{ N}$ ; Perkowski *et al.*, 2021). Belowground biomass carbon ( $\text{g C}$ ) was calculated as the sum of total root carbon biomass and total root nodule carbon biomass. Total root biomass carbon was calculated by multiplying root carbon content by total root biomass, while total root nodule biomass carbon was calculated by multiplying root nodule carbon content by total root nodule biomass. Whole-plant nitrogen biomass ( $\text{g N}$ ) was calculated by multiplying the nitrogen content of leaves, stems, roots, and root nodules by biomass of each respective organ type, then calculating the sum of nitrogen biomass of each organ type. This calculation only quantifies belowground biomass carbon costs to acquire nitrogen and does not account for additional carbon costs of nitrogen acquisition associated with root respiration, root exudation, or root turnover. An explicit explanation of the limitations for interpreting this calculation can be found in Perkowski *et al.* (2021) and Terrer *et al.* (2018).

## Statistical analyses

A series of linear mixed-effects models were built to investigate the impacts of soil nitrogen fertilization and inoculation on *G. max* belowground biomass carbon costs to acquire nitrogen and investment toward symbiotic nitrogen fixation. All models included soil nitrogen fertilization, inoculation, and interactions between soil nitrogen fertilization and inoculation as categorical fixed effects. Block number was included as a random intercept term to account for any environmental heterogeneity within the greenhouse room. Individuals were categorized by inoculation treatment and not by whether they had formed nodules. Models with this independent variable structure were constructed to quantify relationships between soil nitrogen fertilization and inoculation on belowground biomass carbon costs to acquire nitrogen, belowground biomass carbon, whole-plant nitrogen biomass, total leaf area, total biomass, root nodule biomass: root biomass, root nodule biomass, and root biomass.

Shapiro-Wilk tests of normality were used to determine whether linear mixed-effects models satisfied residual normality assumptions (Shapiro-Wilk:  $p > 0.05$ ). Models for whole-plant nitrogen biomass and total leaf area satisfied residual normality assumptions without data transformation. We attempted to satisfy residual normality assumptions by fitting the other models using dependent variables that were natural log transformed. If residual normality assumptions were still not met after a natural-log transformation (Shapiro-Wilk:  $p < 0.05$ ), then models were fit using dependent variables that were square root transformed. All residual normality assumptions were met with either a natural log or square root data transformation (Shapiro-Wilk:  $p > 0.05$  in all cases). Specifically, models for belowground biomass carbon costs to acquire nitrogen, belowground biomass carbon, total biomass, root biomass, and biomass: pot volume satisfied normality assumptions when response variables were fit using natural log transformed data, while models for nodule biomass: root biomass and root nodule biomass satisfied such assumptions when response variables were fit using square-root transformations.

We used the ‘lmer’ function in the ‘lme4’ R package (Bates *et al.* 2015) to fit each model and the ‘Anova’ function in the ‘car’ R package (Fox and Weisberg 2019) to calculate Type II Wald's  $\chi^2$  and determine the significance ( $\alpha = 0.05$ ) of each fixed effect coefficient. We then used the ‘emmeans’ R package (Lenth, 2019) to conduct post-hoc comparisons using Tukey's tests, where degrees of freedom were approximated using the Kenward-Roger approach (Kenward and

Roger 1997). All analyses were conducted and plots were created using R version 4.2.0 (R Core Team, 2021).

## Results

### *Belowground biomass carbon costs to acquire nitrogen*

The interaction between soil nitrogen fertilization and inoculation ( $p < 0.05$ ; Table 1) indicated that negative effects of inoculation ( $p < 0.001$ ; Table 1) on belowground biomass carbon costs to acquire nitrogen were only apparent under low soil nitrogen fertilization (Tukey test comparing the inoculation effect under low soil nitrogen fertilization:  $p < 0.001$ ; Fig. 1A). There was no inoculation effect on belowground biomass carbon costs to acquire nitrogen under high soil nitrogen fertilization (Tukey test comparing the inoculation effect under high soil nitrogen fertilization:  $p > 0.05$ ; Fig. 1A). Increasing soil nitrogen fertilization decreased structural carbon costs to acquire nitrogen ( $p < 0.001$ ; Table 1).

Inoculation decreased belowground biomass carbon ( $p < 0.05$ ; Table 1). This response was not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction:  $p > 0.05$ ; Table 1; Fig. 1B). Soil nitrogen fertilization had no effect on belowground biomass carbon ( $p > 0.05$ ; Table 1).

The interaction between soil nitrogen fertilization and inoculation ( $p < 0.001$ ; Table 1) indicated that positive effects of inoculation on whole-plant nitrogen biomass ( $p < 0.001$ ; Table 1) were only apparent under low soil nitrogen fertilization (Tukey test comparing the inoculation effect under low soil nitrogen fertilization:  $p < 0.001$ ; Fig. 1C). There was no effect of inoculation on whole-plant nitrogen biomass under high soil nitrogen fertilization (Tukey test comparing the inoculation effect under high soil nitrogen fertilization:  $p > 0.05$ ; Fig. 1C). Increasing soil nitrogen fertilization generally increased whole-plant nitrogen biomass ( $p < 0.001$ ; Table 1).



**Table 1** Analysis of variance results exploring effects of soil nitrogen fertilization, inoculation with *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation on belowground biomass carbon costs to acquire nitrogen, whole-plant growth, and investment toward symbiotic nitrogen fixation\*

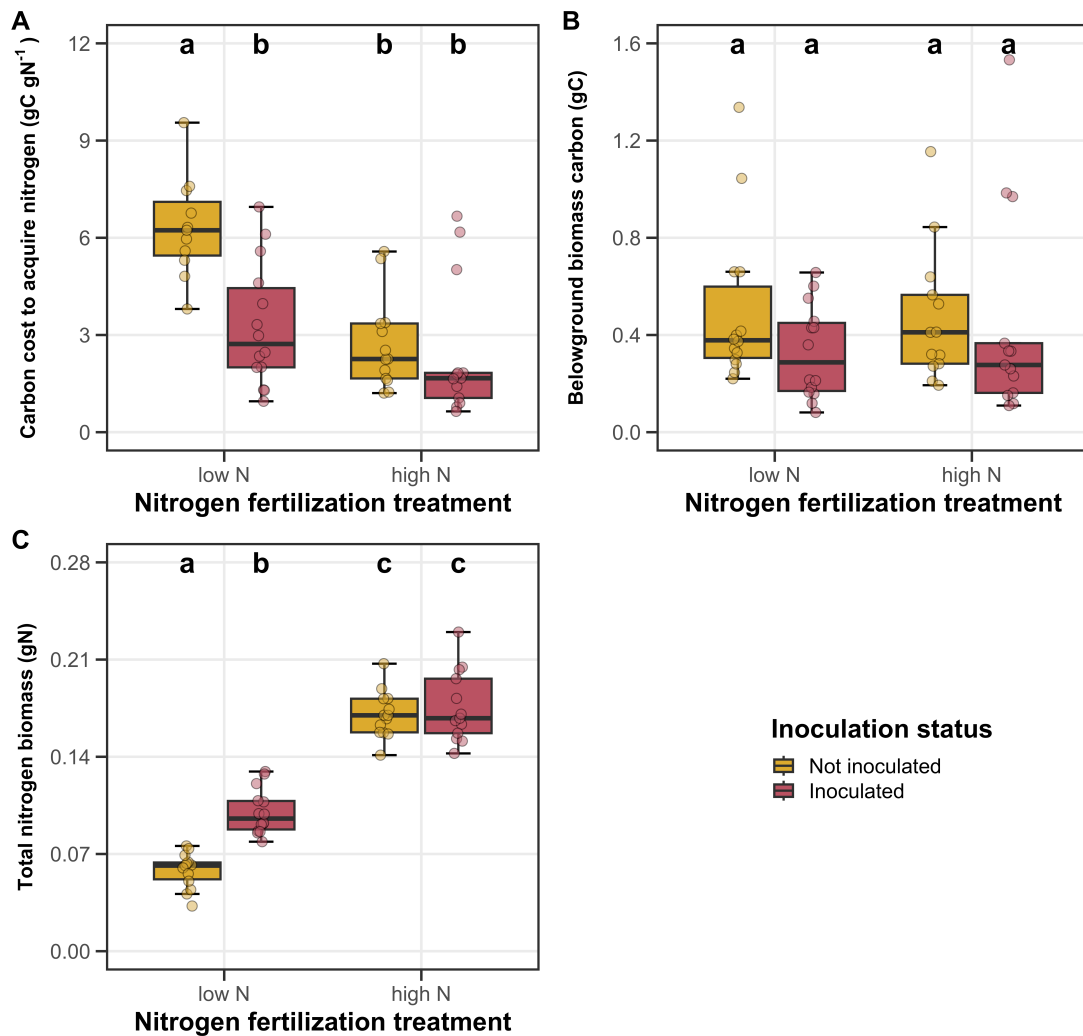
		Carbon cost to acquire nitrogen		Belowground biomass carbon		Whole-plant nitrogen biomass		Total leaf area		Whole plant biomass	
	df	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>
N fertilization (N)	1	23.340	<b>&lt;0.001</b>	0.076	0.782	358.695	<b>&lt;0.001</b>	292.458	<b>&lt;0.001</b>	52.427	<b>&lt;0.001</b>
Inoculation (I)	1	16.749	<b>&lt;0.001</b>	4.166	<b>0.041</b>	24.113	<b>&lt;0.001</b>	35.095	<b>&lt;0.001</b>	2.042	0.153
N*I	1	4.833	<b>0.028</b>	0.265	0.607	13.515	<b>&lt;0.001</b>	17.898	<b>&lt;0.001</b>	1.230	0.267

		Nodule biomass: root biomass		Nodule biomass		Root biomass	
	df	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>
N fertilization (N)	1	1.291	0.256	1.364	0.243	0.011	0.918
Inoculation (I)	1	27.375	<b>&lt;0.001</b>	30.788	<b>&lt;0.001</b>	3.268	<i>0.071</i>
N*I	1	0.493	0.483	1.005	0.316	0.254	0.614

\*Significance determined using Type II Wald  $\chi^2$  tests ( $\alpha=0.05$ ). *P*-values less than 0.05 are in bold and *P*-values between 0.05 and 0.1 are italicized.

Figure 1



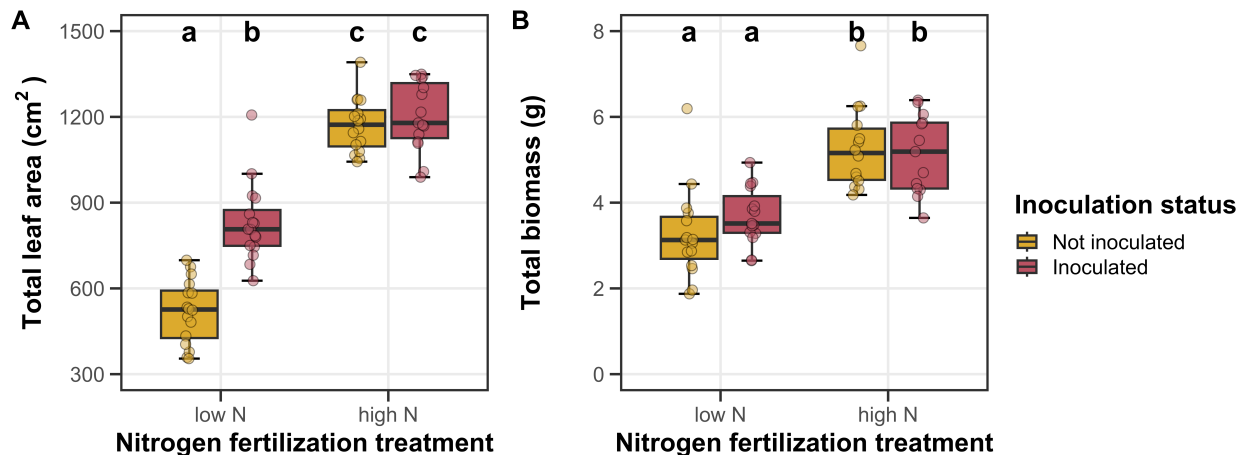
**Figure 1** Effects of soil nitrogen fertilization and inoculation on *G. max* belowground biomass carbon costs to acquire nitrogen (panel A), belowground biomass carbon (panel B), and whole-plant nitrogen biomass (panel C). Soil nitrogen fertilization treatment is on the x-axis, while inoculation treatment is represented by colored boxplots. Yellow shaded boxplots indicate individuals that were not inoculated with *B. japonicum*, while red shaded boxplots indicate individuals that were inoculated with *B. japonicum*. Boxes are the upper (75% percentile) and lower (25% percentile) quartile. The whiskers are the minimum and maximum value, calculated as 1.5 times the upper and lower quartile value. Colored dots are individual data points, jittered for visibility. The lettering above each box indicates the results from post-hoc Tukey's tests with different lettering indicating statistically different groups (Tukey:  $p < 0.05$ ).

*Whole-plant growth*

The interaction between soil nitrogen fertilization and inoculation ( $p < 0.001$ ; Table 1) indicated that positive effects of inoculation on total leaf area ( $p < 0.001$ ; Table 1) were only apparent under low soil nitrogen fertilization (Tukey test comparing the inoculation effect under low soil nitrogen fertilization:  $p < 0.001$ ). There was no effect of inoculation treatment on total leaf area under high soil nitrogen fertilization (Tukey test comparing the inoculation effect under high soil nitrogen fertilization:  $p > 0.05$ ; Fig. 2A). Increasing soil nitrogen fertilization generally increased total leaf area ( $p < 0.001$ ; Table 1; Fig. 2A).

Increasing soil nitrogen fertilization increased total biomass ( $p < 0.001$ ; Table 1; Fig. 2B). This pattern was not modified by inoculation (inoculation-by-fertilization interaction:  $p > 0.05$ ; Table 1). Inoculation had no effect on total biomass ( $p > 0.05$ ; Table 1; Fig. 2B).

**Figure 2**



**Figure 2** Effects of soil nitrogen fertilization and inoculation on *G. max* total leaf area (panel A) and total biomass (panel B). Soil nitrogen fertilization treatment is on the x-axis, while inoculation treatment is represented by colored boxplots. Yellow shaded boxplots indicate individuals that were not inoculated with *B. japonicum*, while red shaded boxplots indicate individuals that were inoculated with *B. japonicum*. Boxes are the upper (75% percentile) and lower (25% percentile) quartile. The whiskers are the minimum and maximum value, calculated as 1.5 times the upper and lower quartile value. Colored dots are individual data points, jittered for visibility. The lettering above each box indicates the results from post-hoc Tukey's tests with different lettering indicating statistically different groups (Tukey:  $p < 0.05$ ).

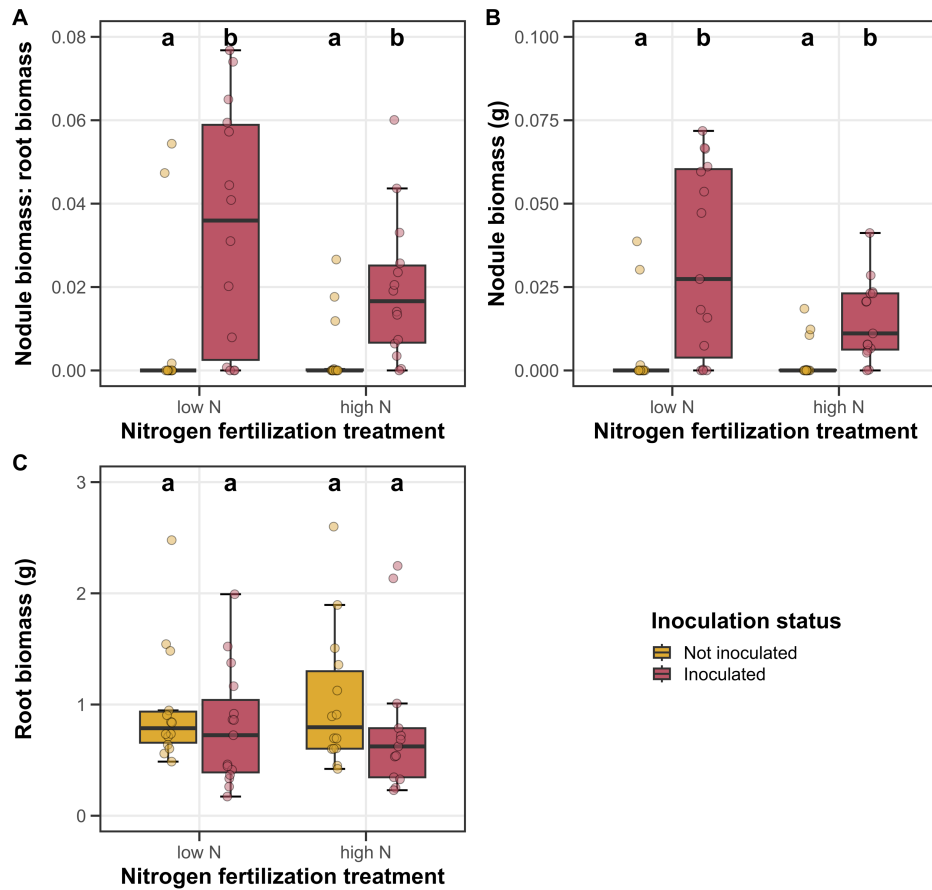
*Plant investment toward symbiotic nitrogen fixation*

Inoculation increased root nodule biomass: root biomass ( $p < 0.001$ ; Table 1; Fig 3A). This pattern was not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction:  $p > 0.05$ ; Table 1). Soil nitrogen fertilization had no effect on root nodule biomass: root biomass ( $p > 0.05$ ; Table 1; Fig 3A).

Inoculation increased root nodule biomass ( $p < 0.001$ ; Table 1; Fig 3B). This pattern was not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction:  $p > 0.05$ ; Table 1). Soil nitrogen fertilization had no effect on root nodule biomass ( $p > 0.05$ ; Table 1; Fig. 3B).

Inoculation had a marginal negative effect on root biomass ( $p < 0.1$ ; Table 1; Fig. 3C). This pattern was not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction:  $p > 0.05$ ; Table 1). Soil nitrogen fertilization had no effect on root biomass ( $p > 0.05$ ; Table 1; Fig. 3C).

291 **Figure 3**



292  
 293 **Figure 3** Effects of soil nitrogen fertilization and inoculation on *G. max* nodule biomass: root  
 294 biomass (panel A), nodule biomass (panel B), and root biomass (panel C). Soil nitrogen  
 295 fertilization treatment is on the x-axis, while inoculation treatment is represented by colored  
 296 boxplots. Yellow shaded boxplots indicate individuals that were not inoculated with *B.*  
 297 *japonicum*, while red shaded boxplots indicate individuals that were inoculated with *B.*  
 298 *japonicum*. Boxes are the upper (75% percentile) and lower (25% percentile) quartile range. The  
 299 whiskers are the minimum and maximum value, calculated as 1.5 times the upper and lower  
 300 quartile value. Colored dots are individual data points, jittered for visibility. The lettering above  
 301 each box indicates the results from post-hoc Tukey's tests with different lettering indicating  
 302 statistically different groups (Tukey:  $p < 0.05$ ).  
 303

## Discussion

Here, we quantified the interactive effect of soil nitrogen fertilization and inoculation with symbiotic nitrogen-fixing bacteria on *G. max* belowground biomass carbon costs to acquire nitrogen using a full-factorial greenhouse manipulation experiment. We found that inoculation reduced belowground biomass carbon costs to acquire nitrogen under the low soil nitrogen fertilization treatment. This pattern was due to similar belowground carbon allocation between inoculation treatments, but significantly greater whole-plant nitrogen uptake in inoculated individuals compared to their uninoculated counterparts. Inoculation effects on belowground biomass carbon costs to acquire nitrogen diminished with increasing nitrogen fertilization, as there was no effect of inoculation treatment on belowground biomass carbon costs to acquire nitrogen under the high soil nitrogen fertilization treatment. That said, belowground biomass carbon costs to acquire nitrogen were the lowest under high soil nitrogen fertilization irrespective of inoculation treatment, a pattern that was also driven by enhanced plant nitrogen uptake coupled with no change in belowground carbon allocation. Overall, results indicate that increased nitrogen supply, either through symbiotic nitrogen under low soil nitrogen fertilization or direct uptake under high soil nitrogen fertilization, reduces the costs of nitrogen acquisition.

### *The impact of inoculation on belowground biomass carbon costs to acquire nitrogen depend on soil nitrogen availability*

Our results provide direct evidence that, under low soil nitrogen availability, nitrogen uptake through symbioses with nitrogen-fixing bacteria reduces belowground biomass carbon costs to acquire nitrogen compared to nitrogen uptake through direct uptake pathways. This result corroborates results from past theory (Vitousek *et al.* 2002), modeling exercises (Brzostek *et al.* 2014), and cross-species experimental studies (Perkowski *et al.* 2021). Here, we used individuals of the same species to confirm that the ability to form symbioses with nitrogen-fixing bacteria are the primary drivers of this response. Despite a strong inoculation effect on belowground biomass carbon costs to acquire nitrogen in the low soil nitrogen fertilization treatment, there was no impact (positive or negative) of inoculation on belowground biomass carbon costs to acquire nitrogen in the high soil nitrogen fertilization treatment. Similar results were shown in a previous cross-species study that observed similar belowground biomass carbon costs to acquire nitrogen under high nitrogen fertilization between a nitrogen-fixing and non-fixing species and

reduced belowground biomass carbon costs to acquire nitrogen in the nitrogen-fixing species under low nitrogen fertilization (Perkowski *et al.* 2021). The differential role of symbiotic nitrogen fixation on belowground biomass carbon costs to acquire nitrogen under the two nitrogen fertilization treatments may help to explain the greater prevalence of plants capable of symbiotic nitrogen fixation where soil nitrogen availability is low (Monks *et al.* 2012), as expected from theory (Vitousek and Field 1999; Vitousek *et al.* 2002; Menge *et al.* 2008) and simulated in plant nitrogen uptake models (Brzostek *et al.* 2014).

Our results indicate that symbiotic nitrogen fixation may provide a competitive advantage in nitrogen-poor soils by reducing belowground biomass carbon costs for acquiring nitrogen relative to direct uptake pathways. However, the longer-term outcomes of this advantage are difficult to predict because nitrogen fixation brings in nitrogen to the ecosystem that may alleviate nitrogen limitation in non-fixing plant species. Additionally, long-term consequences of these dynamics are difficult to predict because nitrogen-fixing species may inhibit nitrogen fixation to minimize resource facilitation to neighboring non-fixing species (Nasto *et al.* 2017; Taylor and Menge 2021). Other bottom-up (e.g., soil resources) and top-down (e.g., herbivory) factors may also limit the competitive ability of species that associate with symbiotic nitrogen-fixing bacteria in terrestrial ecosystems (Eisele *et al.* 1989; Ritchie *et al.* 1998; Vitousek and Field 1999; Rastetter *et al.* 2001; Vitousek *et al.* 2002, 2013). Longer term field and mesocosm experiments (e.g., Finzi and Rodgers, 2009; Taylor *et al.*, 2017; Lai *et al.*, 2018) coupled with targeted model experiments (e.g., Brzostek *et al.*, 2014; Allen *et al.*, 2020; Braghieri *et al.*, 2022) could help to clarify the role of these different drivers.

#### *Soil nitrogen availability and inoculation modify whole-plant nitrogen, but not belowground carbon allocation*

Reduced belowground biomass carbon costs to acquire nitrogen with both increasing soil nitrogen fertilization and inoculation under low soil nitrogen were the result of increased plant nitrogen uptake. Belowground carbon allocation was not impacted by any of our treatments. The increase in nitrogen uptake was predominantly used to support aboveground tissue, which demonstrated a strong increase under increasing soil nitrogen fertilization and with inoculation when soil nitrogen was low. Specifically, increases in plant nitrogen uptake were associated with increased total leaf area, which likely increased total biomass due to greater surface area for light



interception and whole-plant primary productivity. Theory suggests that increasing nitrogen availability (from soil or symbionts) should increase relative plant investment in aboveground tissues (Ågren and Franklin 2003), as was observed here. Meta-analyses also find consistent positive increases in aboveground biomass with increasing soil nitrogen availability but inconsistent impacts on belowground biomass (Li *et al.* 2020).

Our findings provide an empirical benchmark for models that use carbon costs of nitrogen acquisition to simulate terrestrial carbon-nitrogen dynamics (e.g., Brzostek *et al.*, 2014; Shi *et al.*, 2016; Braghiere *et al.*, 2022). Integrating our results with findings presented in Perkowski *et al.* (2021), changes in the belowground cost of nitrogen acquisition due to increasing soil nitrogen availability or ability to associate with symbiotic nitrogen-fixing bacteria should be the result of stronger differences in plant nitrogen uptake than belowground carbon allocation. Thus, models that omit variability in costs to acquire nitrogen are likely to bias estimates of plant carbon-nitrogen economics across environmental gradients. However, it must be noted that, in both studies, additional carbon costs that resulted from differences in root exudation, turnover, or respiration were not quantified. It is unclear whether these unaccounted allocation patterns are proportional to belowground biomass carbon costs and future studies should be performed to validate this assumption.

#### *Soil nitrogen fertilization does not significantly reduce plant investment toward symbioses with nitrogen fixing bacteria*

Inoculated plants exhibited similar levels of nodulation under both soil nitrogen fertilization treatments, indicating that the level of nitrogen availability did not impact the strength of the symbiosis between *G. max* and *B. japonicum*. This result was counter to the expectation that increasing soil nitrogen availability would reduce plant reliance on nitrogen fixing symbionts (Vitousek *et al.* 2002; Perkowski *et al.* 2021), though recent work suggests that plants do still acquire nitrogen through symbiotic nitrogen fixation under high nitrogen availability even if investment in the nitrogen fixation pathway trends in a negative direction (Menge *et al.* 2023). Indeed, we observed a negative, albeit nonsignificant, trend in the effect of increasing fertilization on plant investment toward symbiotic nitrogen fixation, where inoculated individuals grown under high soil nitrogen availability had mean root nodule biomass and root nodule biomass: root biomass values that were 46% and 40% less than individuals grown under

low soil nitrogen availability. Regardless, null effects of soil nitrogen availability on plant investment toward symbiotic nitrogen fixation may imply stronger bacterial control over the symbiosis than previously thought. If true, greater carbon costs for nitrogen acquisition may have been observed in inoculated plants grown under high soil nitrogen if increased amounts of unquantified plant carbon were allocated toward bacterial respiration. Carbon and nitrogen tracing experiments would be useful for examining this hypothesis.

#### *Study limitations*

This study has a few limitations that deserve recognition and limit the generality of the observed responses. First, effects of soil nitrogen fertilization on root nodulation may be nonlinear, and a two-level fertilization experiment is not equipped to address possible nonlinearities that might explain the interaction between soil nitrogen fertilization and root nodulation. Future work should consider conducting similar experiments using a larger number of nitrogen fertilization treatments than presented here. Additionally, this study used a single plant species and an inoculant comprising a single bacterial species. While this allowed us to isolate mechanisms that drove *G. max* responses to nitrogen fertilization and inoculation independent of phylogeny or genetic diversity, a key factor that limited inferences in Perkowski *et al.* (2021), future work should consider conducting similar experiments using a larger number of leguminous species, as well as multi-species mixes of different *Rhizobium* or *Actinobacteria* species. Doing so would better allow us to generalize patterns observed here and would more accurately replicate soil microbial communities that are observed in nature. It should be noted that uninoculated individuals that formed nodules were kept in the analysis. Finally, the belowground biomass carbon cost to acquire nitrogen metric used in this study does not account for changes in belowground carbon allocation due to root turnover, respiration, or root exudation. It is possible that nitrogen fertilization and inoculation with symbiotic nitrogen-fixing bacteria may modify metabolic pathways that alter carbon investment (e.g., bacterial respiration). Future studies should carefully assess whether these carbon pools should be measured as failure to measure these pools could risk underestimating the belowground biomass carbon cost of nitrogen acquisition.

#### *Conclusions*

Here, we used a single-pair symbiosis to quantify the impact of symbiotic nitrogen fixation on belowground biomass carbon costs to acquire nitrogen under varying soil nitrogen environments. Individuals that were inoculated with symbiotic nitrogen-fixing bacteria exhibited reduced belowground biomass carbon costs to acquire nitrogen under the low soil nitrogen fertilization treatment compared to uninoculated counterparts. In contrast, there was no effect of inoculation treatment on belowground biomass carbon costs of nitrogen acquisition under the high soil nitrogen fertilization treatment. Belowground biomass carbon cost to acquire nitrogen differences between treatment combinations were entirely due to changes in plant nitrogen uptake rather than belowground carbon allocation. Treatments that increased plant nitrogen uptake corresponded with enhanced total leaf area and total biomass, suggesting that additional plant nitrogen acquired was being allocated to support aboveground growth. These results indicate that symbiotic nitrogen fixation may provide a competitive advantage to plants growing in nitrogen-poor soils, though these advantages diminish with increasing nitrogen availability as investment in nitrogen uptake through direct uptake pathways increase. These findings can be used to help improve simulations of carbon-nitrogen economics in terrestrial biosphere models.

#### **Supplementary data**

**Table S1** Summary table containing volumes of compounds used to create modified Hoagland's solutions for each soil nitrogen fertilization treatment.

**Table S2** Analysis of variance results exploring effect of nitrogen fertilization, inoculation with *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation status on whole plant biomass: pot volume

**Table S3** Marginal mean, degrees of freedom, and 95% confidence intervals of whole plant biomass: pot volume values across nitrogen fertilization and inoculation treatment combinations

**Figure S1** Effects of soil nitrogen fertilization and inoculation status on whole plant biomass: pot volume

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