

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

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

Evan A. Perkowski<sup>1</sup>, Joseph Terrones<sup>1</sup>, Hannah German<sup>1</sup>, Nicholas G. Smith<sup>1,\*</sup>

<sup>1</sup>Department of Biological Sciences, Texas Tech University, Lubbock, TX USA

\*Correspondence to:

Nicholas G. Smith

2901 Main St.

Lubbock, TX 79409, USA

[nick.smith@ttu.edu](mailto:nick.smith@ttu.edu)

ORCIDs

Perkowski: 0000-0002-9523-8892

Smith: 0000-0001-7048-4387

Submission Date: January 31, 2024

Number of Tables: 1

Number of Figures: 3

Abstract: 199 words

Word Count: 4016

## Highlight

Inoculation with symbiotic nitrogen-fixing bacteria reduced carbon costs to acquire nitrogen, but only under low soil nitrogen fertilization. This pattern was driven by an increase in plant nitrogen uptake without a change in belowground carbon allocation.

## 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. 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 structural 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 greater plant nitrogen uptake coupled with 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 growth. This pattern helps explain the prevalence of plants capable of forming these associations in less fertile areas and demonstrates patterns 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 ecosystem processes 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, 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 (Fay *et al.*, 2015; Wieder *et al.*, 2015; Meyerholt *et al.*, 2016).

Plant nitrogen acquisition is one process in terrestrial ecosystems that links carbon and nitrogen cycles. Plants acquire nutrients by allocating 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), symbioses with mycorrhizal fungi or symbiotic nitrogen-fixing bacteria (Vance and Heichel, 1991; Marschner and Dell, 1994; Smith and Read, 2008; Udvardi and Poole, 2013), or through root exudation that supplies carbon to free-living soil microbial communities (Phillips *et al.*, 2011; Wen *et al.*, 2022).

Plants cannot acquire nitrogen without first allocating carbon belowground, which implies that there is 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 in roots for direct nitrogen uptake does not require costs beyond root development, as is the case for acquisition strategies that involve other soil micro-organisms. However, the nitrogen acquired from a given belowground carbon investment may be greater than direct uptake if plants increase root exudation to supply soil microbial communities with substrate needed to decompose organic matter and increase inorganic soil nitrogen availability available for root uptake (Bengtson *et al.*, 2012; Meier *et al.*, 2017). Alternatively, the nitrogen acquired from a given belowground carbon investment may be greater if carbon is allocated to fungal symbionts in exchange for nitrogen that is mined from the soil or converted to inorganic nitrogen from soil organic matter (Phillips *et al.*, 2013; Liese *et al.*, 2018), or 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). Variation 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 well

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).

While carbon costs to acquire nitrogen may vary in species with different nitrogen acquisition strategies, these costs are also likely dependent on external environmental factors such as atmospheric CO<sub>2</sub>, light availability, and soil nutrient availability (Brzostek *et al.*, 2014; Taylor and Menge, 2018, 2021; Terrer *et al.*, 2018; 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 (Taylor and Menge, 2018; Terrer *et al.*, 2018; Friel and Friesen, 2019; Perkowski *et al.*, 2021; Waring *et al.*, 2023). However, increasing soil nitrogen availability may reduce costs for nitrogen acquisition due to stronger increases in plant nitrogen acquisition per unit carbon allocated belowground. This pattern increases plant nitrogen uptake efficiency (Perkowski *et al.*, 2021) and may be the result of reduced soil resource mining (by roots or symbionts) needed to satisfy plant nitrogen demand. This response 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).

In a recent study, Perkowski *et al.* (2021) showed that increasing soil nitrogen fertilization decreased carbon costs to acquire nitrogen in *Gossypium hirsutum* (L.) and *Glycine max* L. (Merr). *Gossypium hirsutum* can acquire nutrients via direct uptake pathways or through symbioses with arbuscular mycorrhizal fungi, while *G. max* can acquire nutrients via direct uptake pathways or through symbioses with nitrogen-fixing bacteria. In the experiment, the authors noted that carbon costs to acquire nitrogen in *G. max* were generally 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 fertilization once costs to acquire nitrogen via direct uptake became less than the costs 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 species nutrient acquisition strategy. 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 carbon costs to acquire nitrogen, *Glycine max* L. (Merr.) seedlings were grown under two soil nitrogen fertilization treatments and two inoculation treatments in a full factorial greenhouse experiment. We used this experiment to test the following hypotheses:

- (1) Soil nitrogen fertilization will decrease carbon costs of nitrogen acquisition in both uninoculated and inoculated individuals. This 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 carbon costs to acquire nitrogen under low soil nitrogen availability. This is because carbon costs to acquire nitrogen through symbiotic nitrogen fixation will be less than the carbon cost to acquire nitrogen via direct uptake. However, inoculation will have no effect on 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 carbon costs to obtain nitrogen from direct uptake with increasing soil nitrogen fertilization.

## **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). 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 doses were received as topical agents to the soil surface and were modified to keep concentrations of other macronutrients and micronutrients equivalent (Table S1). Throughout the experiment, plants were routinely well-watered to minimize any chance of water stress. 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 g L<sup>-1</sup> within each treatment combination (Table S2-3; Fig. S1; Poorter *et al.*, 2012).

#### *Plant trait measurements*

All experimental 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. Leaf areas of all harvested leaves were measured using an LI-3100C (Li-COR Biosciences, Lincoln, Nebraska, USA). Total leaf area (cm<sup>2</sup>) was calculated as the sum of all leaf areas. All harvested material was dried in an oven set to 65°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.

Following the approach explained in Perkowski *et al.* (2021), structural carbon costs to acquire nitrogen were calculated as the ratio of total belowground carbon biomass to whole plant nitrogen biomass (g C g<sup>-1</sup> N). Belowground carbon biomass (g C) was calculated as the sum of total root carbon biomass and total root nodule carbon biomass. Total root carbon biomass was calculated by multiplying root carbon content by total root biomass, while total root nodule

carbon biomass was calculated by multiplying root nodule carbon content by total root nodule biomass. Whole-plant nitrogen biomass (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 plant structural carbon costs to acquire nitrogen and does not include 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* 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. Models with this independent variable structure were constructed to quantify relationships between soil nitrogen fertilization and inoculation on structural carbon costs to acquire nitrogen, belowground carbon biomass, 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. Whole-plant nitrogen biomass and total leaf area were 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 structural carbon costs to acquire nitrogen, belowground carbon biomass, total biomass, root biomass, and biomass: pot volume satisfied normality assumptions when response variables were fit using natural log transformed data, while the models for nodule biomass: root biomass and

root nodule biomass were satisfied 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 and plots were conducted in R version 4.2.0 (R Core Team, 2021).

## Results

### *Structural 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 structural carbon costs to acquire nitrogen were only apparent under low soil nitrogen fertilization (Tukey test comparing the inoculation effect under low soil nitrogen fertilization:  $p<0.001$ ), as there was no inoculation effect on structural 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; Fig. 1A).

Inoculation decreased belowground carbon biomass ( $p<0.05$ ; 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 carbon biomass ( $p>0.05$ ; 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:  $p<0.001$ ), as 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).



### *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:  $p < 0.001$ ), as there was no inoculation effect 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 that 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).

### *Plant investment in 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 also 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).

## **Discussion**

Here, we quantified the interactive effect of soil nitrogen fertilization and inoculation by symbiotic nitrogen-fixing bacteria on *G. max* structural carbon costs to acquire nitrogen using a full factorial greenhouse manipulation experiment. We found that inoculation reduced carbon costs to acquire nitrogen under low, but not high, levels of soil nitrogen fertilization. This pattern

was observed despite no significant differences in belowground carbon allocation across the treatments. Instead, reduced costs of nitrogen acquisition were driven by greater whole-plant nitrogen uptake in inoculated plants than uninoculated plants when soil nitrogen fertilization was low. This pattern suggests that symbioses with nitrogen-fixing bacteria reduce nitrogen acquisition costs under low soil nitrogen availability, allowing plants to increase nitrogen uptake efficiency compared to individuals restricted to direct uptake. That said, structural carbon costs to acquire nitrogen were the lowest under high soil nitrogen availability due to enhanced plant nitrogen uptake coupled with no change in belowground carbon allocation. Interestingly, investment in root nodules in inoculated individuals did not vary with soil nitrogen fertilization, though inoculated individuals did have mean respective root nodule biomass and root nodule biomass:root biomass values under high soil nitrogen fertilization that were 46% and 40% lower compared to those grown under low soil nitrogen fertilization.

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

Our results provide direct evidence that symbioses with nitrogen-fixing bacteria reduce carbon costs to acquire nitrogen when soil nitrogen availability is low. This 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 symbioses with nitrogen-fixing bacteria are the primary driver of this response.

Despite a strong inoculation effect on carbon costs to acquire nitrogen at low soil nitrogen availability, there was no impact (positive or negative) of inoculation at high levels of soil nitrogen availability. Similar results were shown in a previous cross-species study that found that plants with and without symbioses with nitrogen-fixing bacteria had more similar carbon costs to acquire nitrogen when soil nitrogen availability was high, compared to that when it was low (Perkowski *et al.*, 2021). The difference 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 plant carbon costs for acquiring nitrogen and enhancing

nitrogen uptake efficiency. 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 species following recycling. 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; Braghiere *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 structural carbon*

Reduced 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 structural carbon allocation was not impacted by any of our treatments, suggesting that treatment effects on carbon costs to acquire nitrogen were principally driven by an increase in plant nitrogen uptake efficiency.

The increase in nitrogen uptake in our study was predominantly used to support aboveground tissue, which showed a large increase under increasing soil nitrogen availability 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. Indeed, meta-analyses 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 these costs 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. However, it must be noted that, in both studies, we were not able to capture additional carbon costs that resulted from differences in root exudation or respiration under our different treatments. It is unclear whether these unaccounted allocation patterns are proportional to structural belowground carbon costs and future studies should be performed to validate this assumption.

#### *Soil nitrogen fertilization does not significantly reduce plant investment in nitrogen fixing bacteria symbiosis*

Inoculated plants exhibited similar levels of nodulation under both of soil nitrogen fertilization treatments. This indicates 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 greater soil nitrogen availability would reduce plant reliance on nitrogen fixing symbionts (Vitousek et al., 2002; Perkowski et al., 2021). However, there was a negative, albeit nonsignificant, trend in the effect of increasing fertilization on plant investment toward symbiotic nitrogen fixation, where 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 greater amounts of unquantified plant carbon were allocated toward bacterial respiration. Carbon and nitrogen tracing experiments would be useful for further examining this result.

#### *Study limitations*

This study has a few limitations that deserve recognition and limit the generality of our observed responses. First, effects of soil nitrogen fertilization on root nodulation may be nonlinear, and a two-point 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 other *Actinobacteria* species. Doing so would better allow us to generalize patterns observed here and would better replicate soil microbial communities observed in nature.

### Conclusions

Here, we used a single-pair symbiosis to quantify the impact of symbiotic nitrogen fixation on the structural carbon costs to acquire nitrogen under varying soil nitrogen environments. We find that symbiotic nitrogen fixing bacteria reduced structural carbon costs to acquire nitrogen when soil nitrogen availability was low but had no impact when soil nitrogen availability was high. Carbon cost to acquire nitrogen differences between treatment combinations were entirely due to changes in plant nitrogen uptake rather than belowground structural carbon investments, suggesting that symbiotic nitrogen fixation allowed plants to maximize nitrogen uptake efficiency under low soil nitrogen environments. 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 aboveground biomass. These results indicate that symbiotic nitrogen fixation may provide a competitive advantage to plants growing in nitrogen-poor soils by enhancing nitrogen uptake efficiency. 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

## **Acknowledgements**

We thank Jeffrey Chieppa, Ezinwanne Ezekannagha, Gwendolyn Wagner, and Garrison Garza for experiment harvest assistance. We also thank members of the Schwilk and van Gestel lab for analysis feedback and Elizabeth Waring for experiment inspiration. NGS acknowledges funding support from the NSF (DEB-2045968 and DEB-2217353) and Texas Tech University. This research is a contribution to the LEMONTREE (Land Ecosystem Models based On New Theory, obseRvations and ExperimEnts) project, funded through the generosity of Eric and Wendy Schmidt by recommendation of the Schmidt Futures programme.

## **Author contributions**

EAP conducted data analysis, wrote the first draft of the manuscript with equal contributions from NGS and JT, and made revisions based on coauthor feedback with NGS. JT designed the experiment with NGS and EAP and carried out the experiment. HLG assisted with the post-experiment harvest and contributed to manuscript revisions. NGS oversaw experiment progress, assisted with the post-experiment harvest, and contributed to manuscript revisions.

## **Conflicts of Interest**

None.

## **Funding**

This research is was funded by US National Science Foundation awards to NGS (DEB-2045968 and DEB-2217353) and is a contribution to the LEMONTREE (Land Ecosystem Models based On New Theory, obseRvations and ExperimEnts) project, funded through the generosity of Eric and Wendy Schmidt by recommendation of the Schmidt Futures programme.

## **Data Availability**

All statistical analyses and plots were created in R version 4.2.0. All R code and data for this manuscript are available in a GitHub repository at [https://github.com/eaperkowski/NxI\\_ms\\_data](https://github.com/eaperkowski/NxI_ms_data) (doi: 10.5281/zenodo.10267997).

## References

- Ågren GI, Franklin O. 2003. Root:shoot ratios, optimization and nitrogen productivity. *Annals of Botany* **92**, 795–800.
- Allen K, Fisher JB, Phillips RP, Powers JS, Brzostek ER. 2020. Modeling the carbon cost of plant nitrogen and phosphorus uptake across temperate and tropical forests. *Frontiers in Forests and Global Change* **3**, 1–12.
- Barber SA. 1962. A diffusion and mass-flow concept of soil nutrient availability. *Soil Science* **93**, 39–49.
- Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**, 1–48.
- Bengtson P, Barker J, Grayston SJ. 2012. Evidence of a strong coupling between root exudation, C and N availability, and stimulated SOM decomposition caused by rhizosphere priming effects. *Ecology and Evolution* **2**, 1843–1852.
- Bloom AJ, Chapin FS, Mooney HA. 1985. Resource Limitation in Plants-An Economic Analogy. *Annual Review of Ecology and Systematics* **16**, 363–392.
- Braghiere RK, Fisher JB, Allen K, Brzostek E, Shi M, Yang X, Ricciuto DM, Fisher RA, Zhu Q, Phillips RP. 2022. Modeling global carbon costs of plant nitrogen and phosphorus acquisition. *Journal of advances in modeling earth systems* **14**, e2022MS003204.
- Brzostek ER, Fisher JB, Phillips RP. 2014. Modeling the carbon cost of plant nitrogen acquisition: Mycorrhizal trade-offs and multipath resistance uptake improve predictions of retranslocation. *Journal of Geophysical Research: Biogeosciences* **119**, 1684–1697.
- Chapin FS, Bloom AJ, Field CB, Waring RH. 1987. Plant Responses to Multiple Environmental Factors. *BioScience* **37**, 49–57.
- Davies-Barnard T, Meyerholt J, Zaehle S, *et al.* 2020. Nitrogen cycling in CMIP6 land surface models: progress and limitations. *Biogeosciences* **17**, 5129–5148.
- Eisele KA, Schimel DS, Kapustka LA, Parton WJ. 1989. Effects of available P and N:P ratios on non-symbiotic dinitrogen fixation in tallgrass prairie soils. *Oecologia* **79**, 471–474.

457 **Fay PA, Prober SM, Harpole WS, *et al.*** 2015. Grassland productivity limited by multiple  
 458 nutrients. *Nature Plants* **1**, 15080.

459 **Finzi AC, Rodgers VL.** 2009. Bottom-up rather than top-down processes regulate the  
 460 abundance and activity of nitrogen fixing plants in two Connecticut old-field ecosystems.  
 461 *Biogeochemistry* **95**, 309–321.

462 **Fisher JB, Sitch S, Malhi Y, Fisher RA, Huntingford C, Tan S-Y.** 2010. Carbon cost of plant  
 463 nitrogen acquisition: A mechanistic, globally applicable model of plant nitrogen uptake,  
 464 retranslocation, and fixation. *Global Biogeochemical Cycles* **24**, 1–17.

465 **Fox J, Weisberg S.** 2019. *An R companion to applied regression*. Thousand Oaks, California:  
 466 Sage.

467 **Friedlingstein P, Meinshausen M, Arora VK, Jones CD, Anav A, Liddicoat SK, Knutti R.**  
 468 2014. Uncertainties in CMIP5 climate projections due to carbon cycle feedbacks. *Journal of*  
 469 *Climate* **27**, 511–526.

470 **Friel CA, Friesen ML.** 2019. Legumes modulate allocation to rhizobial nitrogen fixation in  
 471 response to factorial light and nitrogen manipulation. *Frontiers in Plant Science* **10**, 1316.

472 **Gutschick VP.** 1981. Evolved strategies in nitrogen acquisition by plants. *The American*  
 473 *Naturalist* **118**, 607–637.

474 **Hoagland DR, Arnon DI.** 1950. The water-culture method for growing plants without soil.  
 475 *California Agricultural Experiment Station*: 347 **347**, 1–32.

476 **Hungate BA, Dukes JS, Shaw MR, Luo Y, Field CB.** 2003. Nitrogen and climate change.  
 477 *Science* **302**, 1512–1513.

478 **Kenward MG, Roger JH.** 1997. Small sample inference for fixed effects from restricted  
 479 maximum likelihood. *Biometrics* **53**, 983.

480 **Kou-Giesbrecht S, Arora VK, Seiler C, *et al.*** 2023. Evaluating nitrogen cycling in terrestrial  
 481 biosphere models: a disconnect between the carbon and nitrogen cycles. *Earth System Dynamics*  
 482 **14**, 767–795.

483 **Lai HR, Hall JS, Batterman SA, Turner BL, van Breugel M.** 2018. Nitrogen fixer abundance  
 484 has no effect on biomass recovery during tropical secondary forest succession. *Journal of*  
 485 *Ecology* **106**, 1415–1427.



486 **Lawrence DM, Fisher RA, Koven CD, et al.** 2019. The Community Land Model Version 5:  
 487 description of new features, benchmarking, and impact of forcing uncertainty. *Journal of*  
 488 *Advances in Modeling Earth Systems* **11**, 4245–4287.

489 **Lenth R.** 2019. emmeans: estimated marginal means, aka least-squares means. *in press*.

490 **Li W, Zhang H, Huang G, Liu R, Wu H, Zhao C, McDowell NG.** 2020. Effects of nitrogen  
 491 enrichment on tree carbon allocation: A global synthesis. *Global Ecology and Biogeography* **29**,  
 492 573–589.

493 **Liese R, Lübke T, Albers NW, Meier IC.** 2018. The mycorrhizal type governs root exudation  
 494 and nitrogen uptake of temperate tree species. *Tree Physiology* **38**, 83–95.

495 **Lu J, Yang J, Keitel C, Yin L, Wang P, Cheng W, Dijkstra FA.** 2022. Belowground carbon  
 496 efficiency for nitrogen and phosphorus acquisition varies between *Lolium perenne* and *Trifolium*  
 497 *repens* and depends on phosphorus fertilization. *Frontiers in Plant Science* **13**, 1–9.

498 **Marschner H, Dell B.** 1994. Nutrient uptake in mycorrhizal symbiosis. *Plant and Soil* **159**, 89–  
 499 102.

500 **Meier IC, Finzi AC, Phillips RP.** 2017. Root exudates increase N availability by stimulating  
 501 microbial turnover of fast-cycling N pools. *Soil Biology and Biochemistry* **106**, 119–128.

502 **Menge DNL, Levin SA, Hedin LO.** 2008. Evolutionary tradeoffs can select against nitrogen  
 503 fixation and thereby maintain nitrogen limitation. *Proceedings of the National Academy of*  
 504 *Sciences* **105**, 1573–1578.

505 **Meyerholt J, Zaehle S, Smith MJ.** 2016. Variability of projected terrestrial biosphere responses  
 506 to elevated levels of atmospheric CO<sub>2</sub> due to uncertainty in biological nitrogen fixation.  
 507 *Biogeosciences* **13**, 1491–1518.

508 **Monks A, Cieraad E, Burrows L, Walker S.** 2012. Higher relative performance at low soil  
 509 nitrogen and moisture predicts field distribution of nitrogen-fixing plants. *Plant and Soil* **359**,  
 510 363–374.

511 **Montville R, Schaffner DW.** 2004. Analysis of published sprout seed sanitization studies shows  
 512 treatments are highly variable. *Journal of Food Protection* **67**, 758–765.

513 **Nasto MK, Osborne BB, Lekberg Y, Asner GP, Balzotti CS, Porder S, Taylor PG,**  
 514 **Townsend AR, Cleveland CC.** 2017. Nutrient acquisition, soil phosphorus partitioning and  
 515 competition among trees in a lowland tropical rain forest. *New Phytologist* **214**, 1506–1517.

516 **Perkowski EA, Waring EF, Smith NG.** 2021. Root mass carbon costs to acquire nitrogen are  
 517 determined by nitrogen and light availability in two species with different nitrogen acquisition  
 518 strategies. *Journal of Experimental Botany* **72**, 5766–5776.

519 **Phillips RP, Brzostek ER, Midgley MG.** 2013. The mycorrhizal-associated nutrient economy:  
 520 a new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytologist*  
 521 **199**, 41–51.

522 **Phillips RP, Finzi AC, Bernhardt ES.** 2011. Enhanced root exudation induces microbial  
 523 feedbacks to N cycling in a pine forest under long-term CO<sub>2</sub> fumigation. *Ecology Letters* **14**,  
 524 187–194.

525 **Poorter H, Bühler J, Van Dusschoten D, Climent J, Postma JA.** 2012. Pot size matters: A  
 526 meta-analysis of the effects of rooting volume on plant growth. *Functional Plant Biology* **39**,  
 527 839–850.

528 **Prentice IC, Liang X, Medlyn BE, Wang Y-P.** 2015. Reliable, robust and realistic: The three  
 529 R's of next-generation land-surface modelling. *Atmospheric Chemistry and Physics* **15**, 5987–  
 530 6005.

531 **R Core Team.** 2021. R: A language and environment for statistical computing. R Foundation for  
 532 Statistical Computing *in press*.

533 **Rastetter EB, Vitousek PM, Field CB, Shaver GR, Herbert D, Ågren GI.** 2001. Resource  
 534 optimization and symbiotic nitrogen fixation. *Ecosystems* **4**, 369–388.

535 **Ritchie ME, Tilman DG, Knops JMH.** 1998. Herbivore effects on plant and nitrogen dynamics  
 536 in oak savanna. *Ecology* **79**, 165–177.

537 **Scouten AJ, Beuchat LR.** 2002. Combined effects of chemical, heat and ultrasound treatments  
 538 to kill *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *Journal of Applied*  
 539 *Microbiology* **92**, 668–674.

540 **Shi M, Fisher JB, Brzostek ER, Phillips RP.** 2016. Carbon cost of plant nitrogen acquisition:  
 541 Global carbon cycle impact from an improved plant nitrogen cycle in the Community Land  
 542 Model. *Global Change Biology* **22**, 1299–1314.

543 **Smith SE, Read DJ.** 2008. *Mycorrhizal Symbiosis*.

544 **Taylor BN, Chazdon RL, Bachelot B, Menge DNL.** 2017. Nitrogen-fixing trees inhibit growth  
 545 of regenerating Costa Rican rainforests. *Proceedings of the National Academy of Sciences of the*  
 546 *United States of America* **114**, 8817–8822.

547 **Taylor BN, Menge DNL.** 2018. Light regulates tropical symbiotic nitrogen fixation more  
548 strongly than soil nitrogen. *Nature Plants* **4**, 655–661.

549 **Taylor BN, Menge DNL.** 2021. Light, nitrogen supply, and neighboring plants dictate costs and  
550 benefits of nitrogen fixation for seedlings of a tropical nitrogen-fixing tree. *New Phytologist* **231**,  
551 1758–1769.

552 **Terrer C, Vicca S, Stocker BD, Hungate BA, Phillips RP, Reich PB, Finzi AC, Prentice IC.**  
553 2018. Ecosystem responses to elevated CO<sub>2</sub> governed by plant–soil interactions and the cost of  
554 nitrogen acquisition. *New Phytologist* **217**, 507–522.

555 **Udvardi M, Poole PS.** 2013. Transport and metabolism in legume-rhizobia symbioses. *Annual*  
556 *Review of Plant Biology* **64**, 781–805.

557 **Vance CP, Heichel GH.** 1991. Carbon in N<sub>2</sub> fixation: Limitation or exquisite adaptation.  
558 *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 373–392.

559 **Vitousek PM, Cassman K, Cleveland CC, *et al.*** 2002. Towards an ecological understanding of  
560 biological nitrogen fixation. *The Nitrogen Cycle at Regional to Global Scales*. Dordrecht:  
561 Springer Netherlands, 1–45.

562 **Vitousek PM, Field CB.** 1999. Ecosystem constraints to symbiotic nitrogen fixers: A simple  
563 model and its implications. *Biogeochemistry* **46**, 179–202.

564 **Vitousek PM, Menge DNL, Reed SC, Cleveland CC.** 2013. Biological nitrogen fixation:  
565 Rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of*  
566 *the Royal Society B: Biological Sciences* **368**.

567 **Waring EF, Perkowski EA, Smith NG.** 2023. Soil nitrogen fertilization reduces relative leaf  
568 nitrogen allocation to photosynthesis. *Journal of Experimental Botany* **74**, 5166–5180.

569 **Wen Z, White PJ, Shen J, Lambers H.** 2022. Linking root exudation to belowground  
570 economic traits for resource acquisition. *New Phytologist* **233**, 1620–1635.

571 **Wieder WR, Cleveland CC, Smith WK, Todd-Brown K.** 2015. Future productivity and  
572 carbon storage limited by terrestrial nutrient availability. *Nature Geoscience* **8**, 441–444.

573

574 **Table 1** Analysis of variance results exploring effect of soil nitrogen fertilization, inoculation with *B. japonicum*, and interactions  
575 between soil nitrogen fertilization and inoculation on structural carbon costs to acquire nitrogen, whole plant growth, and root  
576 nodulation\*

		Carbon cost to acquire nitrogen		Belowground carbon biomass		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

577

578 \*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

579 are italicized.

## Figure legends

**Figure 1** Effects of soil nitrogen fertilization and inoculation on *G. max* structural carbon costs to acquire nitrogen (“ $N_{\text{cost}}$ ”; panel A), belowground carbon biomass (“ $C_{\text{bg}}$ ”; panel B), and whole-plant nitrogen biomass (“ $N_{\text{wp}}$ ”; panel C). Soil nitrogen fertilization is represented 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:  $p < 0.05$ ).

**Figure 2** Effects of soil nitrogen fertilization and inoculation on *G. max* total leaf area (panel A), total biomass (panel B). Soil nitrogen fertilization is represented 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:  $p < 0.05$ ).

**Figure 3** Effects of soil nitrogen fertilization and inoculation on *G. max* nodule biomass: root biomass (panel A), nodule biomass (panel B), and root biomass (panel C). Soil nitrogen fertilization is represented 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:  $p < 0.05$ ).