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

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**Abstract**

Many plant species form symbiotic associations with nitrogen fixing bacteria. Through this symbiosis, plants give photosynthate 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 different background soil nitrogen availabilities are not well understood. Here, we used a manipulation experiment to examine how the costs of nitrogen acquisition vary under a factorial combination of soil nitrogen availability and nitrogen fixing bacteria inoculation in *Glycine max* L. (Merr.). After a 7-week growth period, we measured root, stem, leaf, and nodule biomass as well as carbon and nitrogen amounts of each organ. We used this information to assess structural carbon costs to acquire nitrogen, plant investments to nitrogen fixation, leaf nitrogen allocation, and whole plant growth. We found that structural carbon costs to acquire nitrogen decreased with inoculation in the low soil nitrogen availability treatment, but were unaffected by inoculation in the high soil nitrogen fertilization treatment. The treatment differences were the result of greater plant nitrogen, rather than any 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. This helps to 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**

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 more realistically simulate past, present, and future atmosphere-biosphere fluxes (Oreskes *et al.*, 1994; Hungate *et al.*, 2003; Prentice *et al.*, 2015). 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 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 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 & Heichel, 1991; Marschner & Dell, 1994; Smith & Read, 2008; Udvardi & Poole, 2013), or root exudates that supply carbon to free-living soil microbial communities (Phillips *et al.*, 2011; Wen *et al.*, 2022).

In principle, plants cannot acquire nitrogen without first allocating carbon belowground, which implies an inherent carbon cost to the plant for acquiring nitrogen. These nitrogen return on carbon invested belowground 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 cases for acquisition strategies that involve other organisms. However, the nitrogen return may be greater is carbon is given to decomposers who produce inorganic nitrogen than can be taken up by roots (CITE), fungal symbionts that mine the soil for nitrogen (CITE), or bacteria symbionts that can provide nitrogen fixed from the atmosphere (CITE). The variability in costs to acquire nitrogen may help to explain the prevalence of different nitrogen acquisition strategies in different environments, but these have not been well quantified outside of a few studies (Terrer et al., 2018, OTHERS??).

Nitrogen acquisition costs for a given nitrogen acquisition strategy is likely dependent on external environmental factors such as atmospheric CO2, light availability, and soil nutrient availability (Brzostek *et al.*, 2014; Terrer *et al.*, 2018; Allen *et al.*, 2020; Perkowski *et al.*, 2021; Lu *et al.*, 2022). For instance, the amount of photosynthate paid for nitrogen may increase with increased light and CO2, as these factors reduce the cost to produce photosynthate (Perkowski et al., 2021, Terrer et al., 2018, OTHERS??). However, soil nitrogen availability is likely to reduce costs for nitrogen acquisition due a reduction in soil resourcing mining (by roots or symbionts) needed to meet plant nitrogen demand. However, this may not play out in plant species with strong and specialized symbiotic relationships with nitrogen-acquiring partners, such as plants that associate with nitrogen fixing bacteria.

In a recent study, Perkowski *et al.* (2021) show that increasing soil nitrogen fertilization generally decreased carbon costs to acquire nitrogen in *Gossypium hirsutum* and *Glycine max*. *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*, a pattern that coincided with a reduction in 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. 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 because the two species are not phylogenetically related and adopt different growth forms and growth durations.

To better understand how nitrogen fixation and soil nitrogen fertilization interact to influence carbon costs to acquire nitrogen, we grew *Glycine max* L. (Merr.) 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 the amount of nitrogen acquired per belowground carbon investment.
2. Inoculation with nitrogen-fixing bacteria will decrease carbon costs to acquire nitrogen under low soil nitrogen availability, as carbon costs to acquire nitrogen will be less than the carbon cost to acquire nitrogen via direct uptake. There will be no effect under high soil nitrogen availability due to all plants shifting toward a similar, direct uptake-dominated mode of nitrogen acquisition.
3. There will be a decrease in nodulation with increasing soil nitrogen availability due to a reduction in carbon costs to obtain nitrogen from direct uptake with increasing soil nitrogen fertilization.

**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 XX% sodium hypochlorite for XX minutes followed by three washes in ultrapure water. The remaining 32 pots were planted with seeds that did not receive any inoculation treatment. Uninoculated seeds were also surface sterilized in XX% sodium hypochlorite for XX 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 & 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 pot size induced growth limitation at the time of biomass harvest, indicated by marginal mean whole plant biomass: pot volume ratios less than 1 g L-1 within each treatment combination (Table S2; Fig. S1; Poorter *et al.*, 2012)

*Plant trait measurements*

We harvested all experimental individuals and separated biomass of each experimental individual 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 (cm2) 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. We also quantified carbon and nitrogen content of each respective organ type 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), we calculated structural carbon costs to acquire nitrogen as the ratio of total belowground carbon biomass to whole plant nitrogen biomass (g C g-1 N). Belowground carbon biomass (g C) was calculated by multiplying the carbon content of roots and root nodules by total biomass of each respective organ type, then adding root carbon biomass and root nodule carbon 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 any 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*

We built a series of linear mixed-effects models to investigate the impacts of soil nitrogen fertilization and inoculation on *G. max* traits. 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 whole plant biomass,structural carbon costs to acquire nitrogen, belowground carbon biomass, whole plant nitrogen biomass, total biomass, total leaf area, root nodule biomass: root biomass, root nodule biomass, and root biomass.

We used Shapiro-Wilk tests of normality to determine whether linear mixed-effects models satisfied residual normality assumptions. All models satisfied residual normality assumptions except structural carbon costs to acquire nitrogen, belowground carbon biomass, total biomass, root nodule biomass: root biomass, root nodule biomass, root biomass, and biomass: pot volume (Shapiro-Wilk: p<0.05 in all cases). We attempted to satisfy residual normality assumptions by fitting 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, we natural log transformed structural carbon costs to acquire nitrogen, belowground carbon biomass, total biomass, root biomass, and biomass: pot volume, and square root transformed root nodule biomass: root biomass and root nodule biomass.

In all statistical models, 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 & Weisberg, 2019) to calculate Type II Wald's χ2 and determine the significance (α=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 & 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*

Structural carbon costs to acquire nitrogen were driven by a strong interaction between nitrogen fertilization and inoculation (Table 1; Fig. 1A). This interaction indicated that inoculated individuals grown under low soil nitrogen fertilization had 63.4% lower structural carbon costs to acquire nitrogen than non-inoculated individuals also grown under low soil nitrogen fertilization (Tukey: p<0.001). There was no difference in structural carbon costs to acquire nitrogen between inoculation treatments under high soil nitrogen fertilization (Tukey: p=0.597). Soil nitrogen fertilization also decreased structural carbon costs to acquire nitrogen, where individuals grown under high soil nitrogen fertilization had 54.1% lower structural carbon costs to acquire nitrogen than those grown under low soil nitrogen fertilization (Tukey: p<0.001). Inoculation decreased structural carbon costs to acquire nitrogen, where inoculated individuals had 91.5% lower structural carbon costs to acquire nitrogen than non-inoculated individuals (Tukey: p<0.001).

Structural carbon costs to acquire nitrogen results were primarily due to treatment impacts on whole plant biomass, rather than belowground carbon biomass. Specifically, whole plant nitrogen biomass was driven by a strong interaction between soil nitrogen fertilization and inoculation (Table 1; Fig. 1C). This interaction indicated that inoculated individuals grown under low soil nitrogen fertilization had 72.4% higher whole plant nitrogen biomass than non-inoculated individuals also grown under low soil nitrogen fertilization (Tukey: p<0.001), with no difference between inoculation treatments under high soil nitrogen fertilization (Tukey: p=0.873). Soil nitrogen fertilization also increased whole plant nitrogen biomass, where individuals grown under high soil nitrogen fertilization had 119.0% higher whole plant nitrogen biomass than those grown under low soil nitrogen fertilization (Tukey: p<0.001). Inoculation increased whole plant nitrogen biomass, where inoculated individuals had 17.4% higher whole plant nitrogen biomass than those that were not inoculated (Tukey: p<0.001).

Belowground carbon biomass was not impacted by the treatments nearly as much as whole plant nitrogen biomass. However, inoculation had a slight affected belowground carbon biomass (Table 1; Fig. 1B). Specifically, inoculated individuals had 29.9% less belowground carbon biomass than those that were not inoculated (Tukey: p=0.050). There was no effect of soil nitrogen fertilization or any observable interaction between soil nitrogen fertilization and inoculation on belowground carbon biomass (Table 1).

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

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | **Carbon cost to acquire nitrogen** | | **Belowground carbon biomass** | | **Whole plant nitrogen biomass** | | **Total**  **leaf area** | | **Whole plant biomass** | |
|  | df | χ2 | *p* | χ2 | *p* | χ2 | *p* | χ2 | *p* | χ2 | *p* |
| N fertilization (N) | 1 | 23.34 | **<0.001** | 0.08 | 0.782 | 358.69 | **<0.001** | 292.46 | **<0.001** | 52.43 | **<0.001** |
| Inoculation (I) | 1 | 16.75 | **<0.001** | 4.17 | **0.041** | 24.11 | **<0.001** | 35.09 | **<0.001** | 2.04 | 0.153 |
| N\*I | 1 | 4.83 | **0.028** | 0.265 | 0.607 | 13.52 | **<0.001** | 17.90 | **<0.001** | 1.23 | 0.267 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  |  | **Nodule biomass: root biomass** | | **Nodule**  **biomass** | | **Root**  **biomass** | |  | |  | |
|  | df | χ2 | *p* | χ2 | *p* | χ2 | *p* |  |  |  |  |
| N fertilization (N) | 1 | 0.99 | 0.320 | 1.36 | 0.243 | 0.01 | 0.918 |  |  |  |  |
| Inoculation (I) | 1 | 31.13 | **<0.001** | 30.79 | **<0.001** | 3.27 | *0.071* |  |  |  |  |
| N\*I | 1 | 0.76 | 0.383 | 1.01 | 0.316 | 0.25 | 0.614 |  |  |  |  |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold and *P*-values between 0.05 and 0.1 are italicized.

**Figure 1**

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**Figure 1** Effects of soil nitrogen fertilization and inoculation on *G. max* structural carbon costs to acquire nitrogen (“*N*cost”; panel A), belowground carbon biomass (“*C*bg”; panel B), and whole plant nitrogen biomass (“*N*ag + *N*bg”; panel C). Soil nitrogen fertilization is represented categorically 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. Grey dots are individual data points, jittered for visibility. The lettering over each box indicates the results from post-hoc Tukey’s tests with different lettering indicating statistically different groups (p<0.05).

*Whole plant growth and plant investments to nitrogen fixation*

There was an interaction between nitrogen fertilization and inoculation on total leaf area (Table 1; Fig. 2A). This interaction indicated that inoculated individuals grown under low nitrogen fertilization had 59.7% higher total leaf area than non-inoculated individuals also grown under low nitrogen fertilization (Tukey: p<0.001), with no difference between inoculation treatments under high nitrogen fertilization (Tukey: p=0.631). Nitrogen fertilization also increased total leaf area, where individuals grown under high nitrogen fertilization had 77.4% higher total leaf area than those grown under low nitrogen fertilization (Tukey: p<0.001). Inoculation also increased total leaf area, where inoculated individuals had 21.4% higher total leaf area than non-inoculated individuals (Tukey: p<0.001).

Whole plant biomass was positively effected by nitrogen fertilization (Table 5; Fig. 5B), where individuals grown under high nitrogen fertilization had 55.5% higher whole plant biomass than those grown under low nitrogen fertilization (Tukey: p<0.001). There was no inoculation effect nor was there any interaction between inoculation and nitrogen fertilization (Table 1; Fig. 2B).

Root nodule biomass: root biomass was positively effected of inoculation (Table 1; Fig. 2C). Specifically, inoculated individuals had 323.5% greater root nodule biomass: root biomass than non-inoculated individuals when averaged across nitrogen fertilization treatments. There was no effect of fertilization or any observable interaction between fertilization and inoculation on root nodule biomass: root biomass (Table 1). Root nodule biomass: root biomass patterns were driven by a strong increase in root nodule biomass and a marginal decrease in root biomass with inoculation.

**Figure 2**

**Chart, box and whisker chart

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**Figure 2** Effects of soil nitrogen fertilization and inoculation on *G. max* total leaf area (panel A), whole plant biomass (panel B), and nodule biomass: root biomass (panel C). Soil nitrogen fertilization is represented categorically 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. Grey dots are individual data points, jittered for visibility. The lettering over each box indicates the results from post-hoc Tukey’s tests with different lettering indicating statistically different groups (p<0.05).











**Discussion**

[**Main point #1**: The impact of inoculation on plant carbon costs to acquire nitrogen depend on soil nitrogen availability]

[**Main point #2**: Soil nitrogen availability and inoculation modify whole plant nitrogen, but not belowground structural carbon]

[**Main point #3**: Soil nitrogen fertilization does not significantly reduce plant investment in nitrogen fixing bacteria symbiosis]

*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 such as the one done here 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 suite of nitrogen fertilization treatments than what is presented here. Additionally, this study used a single plant species and an inoculant comprising a single bacterial species. While this did allow us to isolate mechanisms that drove *G. max* responses to nitrogen fertilization and inoculation independent of phylogeny or genetic diversity, future work should consider conducting similar experiments using a suite of leguminous species, as well as a suite of different *Rhizobium* or other *Actinobacteria* mixtures. Doing so would better allow us to generalize patterns observed here, and better replicate soil microbial communities observed in nature.

*Conclusions*

[add concluding paragraph here]

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**Author contributions**

EAP coordinated leaf physiological measurements, conducted data analysis, wrote the first draft of the manuscript, and made revisions based on collaborator and reviewer feedback. JT designed the experiment with NGS and EAP, carried out the experiment, and contributed to manuscript revisions. HG assisted with post-experiment harvest and contributed to manuscript revisions. NGS oversaw experiment progress, assisted with the post-experiment harvest, and contributed to manuscript revisions. All authors support publication of this manuscript to XX.

**Data Availability Statement**

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 <insert URL here> (<insert DOI from Zenodo here>).

**References**