**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 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 different background soil nitrogen availabilities 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, 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, 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**

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 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 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 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). These nitrogen returns 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 case for acquisition strategies that involve other soil micro-organisms. However, the nitrogen return from a given belowground carbon investment may be greater than direct uptake if carbon is exuded to decomposing soil microbial communities who increase inorganic soil nitrogen availability that can be taken up by roots (Bengtson *et al.*, 2012; Meier *et al.*, 2017), 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 bacteria symbionts in exchange for nitrogen that is fixed from the atmosphere (Gutschick, 1981; Vitousek & Field, 1999; Rastetter *et al.*, 2001; Vitousek *et al.*, 2002). The variability in costs to acquire nitrogen may help to 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).

Nitrogen acquisition costs for a given nitrogen acquisition strategy are likely dependent on external environmental factors such as atmospheric CO2, light availability, and soil nutrient availability (Brzostek *et al.*, 2014; Terrer *et al.*, 2018; Taylor & Menge, 2018, 2021; Friel & 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 CO2, as these factors reduce the cost to produce photosynthate (Terrer *et al.*, 2018; Taylor & Menge, 2018; Friel & Friesen, 2019; Perkowski *et al.*, 2021; Waring *et al.*, 2023). However, soil nitrogen availability likely reduces costs for nitrogen acquisition due stronger increases in plant nitrogen acquisition per unit carbon allocated belowground. This pattern increases plant nitrogen uptake efficiency (Perkowski *et al.*, 2021) and is the likely result of a reduction in soil resource mining (by roots or symbionts) needed to meet 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, such as plant species that associate with symbiotic nitrogen-fixing bacteria.

In a recent study, Perkowski *et al.* (2021) show that increasing soil nitrogen fertilization 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 *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 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, 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, as carbon costs to acquire nitrogen through symbiotic nitrogen fixation will be less than the carbon cost to acquire nitrogen via direct uptake. However, there will be no effect of inoculation 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 20,000 ppm sodium hypochlorite for 5 minutes followed by three washes in ultrapure water (Scouten & Beuchat, 2002; Montville & 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 & 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-1 within each treatment combination (Table S2; 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 (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. 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), 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 as the sum of total root carbon biomass and total root nodule carbon biomass. Total root carbon biomass was calculated by multiplying the carbon content of roots by total root biomass, while total root nodule carbon biomass was calculated by multiplying the carbon content of root nodules 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*

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 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.

We used Shapiro-Wilk tests of normality to determine whether linear mixed-effects models satisfied residual normality assumptions. Whole-plant nitrogen biomass and total leaf area were the only models that satisfied residual normality assumptions. 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.

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*

An interaction between 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: *p*<0.001), as there was no inoculation effect on structural carbon costs to acquire nitrogen under high soil nitrogen fertilization (Tukey: *p*>0.05; Fig. 1A). Structural carbon costs to acquire nitrogen also generally decreased with increasing soil nitrogen fertilization (*p*<0.001; Table 1; Fig. 1A)*.*

Inoculation decreased belowground carbon biomass (*p*<0.05; Table 1), a response that was not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction: *p*>0.05; Table 1; Fig. 1B). There was no effect of soil nitrogen fertilization on belowground carbon biomass (*p*>0.05; Table 1).

An 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: *p*<0.001), as there was no effect of inoculation on whole-plant nitrogen biomass under high soil nitrogen fertilization (Tukey: *p*>0.05; Fig. 1C). Whole-plant nitrogen biomass generally increased with increasing soil nitrogen fertilization (*p*<0.001; 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 | 23.340 | **<0.001** | 0.076 | 0.782 | 358.695 | **<0.001** | 292.458 | **<0.001** | 52.427 | **<0.001** |
| Inoculation (I) | 1 | 16.749 | **<0.001** | 4.166 | **0.041** | 24.113 | **<0.001** | 35.095 | **<0.001** | 2.042 | 0.153 |
| N\*I | 1 | 4.833 | **0.028** | 0.265 | 0.607 | 13.515 | **<0.001** | 17.898 | **<0.001** | 1.230 | 0.267 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  |  | **Nodule biomass: root biomass** | | **Nodule**  **biomass** | | **Root**  **biomass** | |  | |  | |
|  | df | χ2 | *p* | χ2 | *p* | χ2 | *p* |  |  |  |  |
| N fertilization (N) | 1 | 1.291 | 0.256 | 1.364 | 0.243 | 0.011 | 0.918 |  |  |  |  |
| Inoculation (I) | 1 | 27.375 | **<0.001** | 30.788 | **<0.001** | 3.268 | *0.071* |  |  |  |  |
| N\*I | 1 | 0.493 | 0.483 | 1.005 | 0.316 | 0.254 | 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**

**A graph of different types of fertilizers

Description automatically generated**

**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*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).

*Whole-plant growth*

An interaction between 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 nitrogen fertilization (Tukey: *p*<0.001), as there was no inoculation effect on total leaf area under high nitrogen fertilization (Tukey: *p*>0.05; Fig. 2A). Increasing soil nitrogen fertilization increased total leaf area (*p*<0.001; Table 1; Fig. 2A).

Increasing nitrogen fertilization increased total biomass (*p*<0.001; Table 1; Fig. 2B), a 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).

**Figure 2**

**A graph of different stages of fertilization

Description automatically generated**

**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).

*Plant investment in symbiotic nitrogen fixation*

Inoculation increased root nodule biomass: root biomass (*p*<0.001; Table 1; Fig 3A), a pattern that was not modified by fertilization (inoculation-by-fertilization interaction: *p*>0.05; Table 1). There was also no effect of soil nitrogen fertilization 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), a pattern that was not modified by fertilization (inoculation-by-fertilization interaction: *p*>0.05; Table 1). There was also no effect of soil nitrogen fertilization 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), a pattern that was not modified by fertilization (inoculation-by-fertilization interaction: *p*>0.05; Table 1). Fertilization had no effect on root biomass (*p*>0.05; Table 1; Fig. 3C).

**Figure 3**

**A diagram of different types of plants

Description automatically generated with medium confidence**

**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).

**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 fully factorial 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 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 as a result of high amounts of plant nitrogen uptake. Interestingly, investment in root nodules in inoculated individuals did not vary with soil nitrogen fertilization, though inoculated individuals did have 46% reduced root nodule biomass under high fertilization that resulted in a 40% decrease in the ratio of root nodule biomass to root biomass. We expand on and contextualize these key results below.

*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 large inoculation effect on carbon costs to acquire nitrogen at low soil nitrogen availability, there was no impact (positive or negative) of inoculation 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 (e.g., Monks *et al.*, 2012), as expected from theory (Vitousek & 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, though recent studies indicate that nitrogen-fixing species may inhibit nitrogen fixation to minimize resource facilitation to neighboring non-fixing species (Nasto *et al.*, 2017; Taylor & Menge, 2021). Additionally, other bottom-up (e.g., soil resources) and top-down (e.g., herbivory) factors may 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 & Field, 1999; Rastetter *et al.*, 2001; Vitousek *et al.*, 2002, 2013). Longer term field and mesocosm experiments (e.g., Finzi & 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*

Reductions in 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, but not due to a change in belowground investment in structural carbon. In fact, belowground structural carbon was not impacted by any of our treatments, suggesting that treatment effects on carbon costs to acquire nitrogen were 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 large stimulation 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 in response to greater surface area for light interception and therefore greater primary productivity. Theory suggests that increasing nitrogen availability (from soil or symbionts) should increase relative plant investment in aboveground tissues (Ågren & Franklin, 2003), as we observed here. And, 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 a possible 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 from 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*

We found that inoculated plants had similar levels of nodulation under both of our soil nitrogen availability treatments. This indicates that, in this study, the level of nitrogen availability did not impact the strength of the symbiosis between *G. max* and *B. japonicum*. This result was counter to 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% lower 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. In fact, if there were greater amounts of unquantified plant carbon going to bacterial respiration, we may have actually observed higher carbon costs for nitrogen acquisition in inoculated plants under high soil nitrogen. 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 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 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 did allow 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. The findings can be used to help improve simulations of carbon-nitrogen economics in terrestrial biosphere models.

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**Author contributions**

EAP conducted data analysis, wrote the first draft of the manuscript with equal contributions from NGS, and made revisions based on collaborator and reviewer feedback with NGS. JT designed the experiment with NGS and EAP, carried out the experiment, and contributed to manuscript revisions. HG 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.

**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 https://github.com/eaperkowski/NxI\_ms\_data ( https://doi.org/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, Friedlingstein P, Brovkin V, Fan Y, Fisher RA, Jones CD, Lee H, Peano D, *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.

**Fay PA, Prober SM, Harpole WS, Knops JMH, Bakker JD, Borer ET, Lind EM, MacDougall AS, Seabloom EW, Wragg PD, *et al.*** **2015**. Grassland productivity limited by multiple nutrients. *Nature Plants* **1**: 15080.

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

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

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

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

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

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

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

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

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

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

**Lawrence DM, Fisher RA, Koven CD, Oleson KW, Swenson SC, Bonan GB, Collier N, Ghimire B, Kampenhout L, Kennedy D, *et al.*** **2019**. The Community Land Model Version 5: description of new features, benchmarking, and impact of forcing uncertainty. *Journal of Advances in Modeling Earth Systems* **11**: 4245–4287.

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

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

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

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

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

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

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

**Meyerholt J, Zaehle S, Smith MJ**. **2016**. Variability of projected terrestrial biosphere responses to elevated levels of atmospheric CO2 due to uncertainty in biological nitrogen fixation. *Biogeosciences* **13**: 1491–1518.

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

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

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

**Oreskes N, Shrader-Frechette K, Belitz K**. **1994**. Verification, validation, and confirmation of numerical models in the Earth sciences. *Science* **263**: 641–646.

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

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

**Phillips RP, Finzi AC, Bernhardt ES**. **2011**. Enhanced root exudation induces microbial feedbacks to N cycling in a pine forest under long-term CO2 fumigation. *Ecology Letters* **14**: 187–194.

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

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

**R Core Team**. **2021**. R: A language and environment for statistical computing.

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

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

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

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

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

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

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

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

**Terrer C, Vicca S, Stocker BD, Hungate BA, Phillips RP, Reich PB, Finzi AC, Prentice IC**. **2018**. Ecosystem responses to elevated CO2 governed by plant–soil interactions and the cost of nitrogen acquisition. *New Phytologist* **217**: 507–522.

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

**Vance CP, Heichel GH**. **1991**. Carbon in N2 fixation: Limitation or exquisite adaptation. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**: 373–392.

**Vitousek PM, Cassman K, Cleveland CC, Crews T, Field CB, Grimm NB, Howarth RW, Marino R, Martinelli L, Rastetter EB, *et al.*** **2002**. Towards an ecological understanding of biological nitrogen fixation. In: The Nitrogen Cycle at Regional to Global Scales. Dordrecht: Springer Netherlands, 1–45.

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

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

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

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

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