**Title**: Soil nitrogen fertilization and inoculation with *Bradyrhizobium japonicum* shapes tradeoffs between whole plant growth and leaf resource use in *Glycine max* L.

**Running Head:**

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**Abstract**

Plant nitrogen acquisition and photosynthesis link ecosystem carbon and nitrogen cycles. These two processes are themselves linked – plants must allocate recent photosynthetically derived carbon belowground to acquire nitrogen through direct uptake pathways or associations with microbial symbionts, while nitrogen must be acquired to build and maintain enzymes that drive photosynthetic reactions forward. To date, we do not fully understand mechanisms that link plant nitrogen acquisition and photosynthesis, and how these mechanisms vary by nitrogen acquisition strategy. Here, we grew *Glycine max* L. (Merr.) under two soil nitrogen fertilization treatments both with and without inoculation with *Bradyrhizobium japonicum* in a full-factorial greenhouse experiment. After a 7-week growth period, we measured structural carbon costs to acquire nitrogen, plant investments to nitrogen fixation, leaf nitrogen allocation, photosynthetic capacity, and whole plant growth to understand whether nitrogen acquisition strategy modified linkages between plant nitrogen acquisition and photosynthetic processes. We found that structural carbon costs to acquire nitrogen were lower in

**Keywords**

nitrogen fixation; whole plant growth; greenhouse; crops; nutrient acquisition strategy

**Introduction**

Terrestrial ecosystems are regulated by complex carbon and nitrogen biogeochemical cycles. As a result, terrestrial biosphere models, which are beginning to include coupled carbon and nitrogen cycles, must accurately represent these cycles under different environmental scenarios to reliably simulate past, present, and future carbon and nutrient 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 biogeochemical cycles (Wieder *et al.*, 2015; Fay *et al.*, 2015; Meyerholt *et al.*, 2016).

Plant nitrogen acquisition is one process in terrestrial systems where carbon and nitrogen cycles are linked. 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 prime 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 regardless of nitrogen acquisition strategy. These carbon costs to acquire nitrogen vary in species with different nitrogen acquisition strategies and are depend 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).

Resource optimization theory suggests

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* is a woody perennial species that can acquire nutrients via direct uptake pathways or through symbioses with arbuscular mycorrhizal fungi, while *G. max* is a herbaceous annual species that 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. This was because the two species are not phylogenetically related and adopt different growth forms and growth durations.

To better understand how nitrogen fixation modifies the effect of soil nitrogen fertilization on 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 . The reduction in carbon will increase the amount of nitrogen acquired per belowground carbon investment, which will maximize both nitrogen and carbon allocation to growth and storage because there will be higher demand to allocate nitrogen to whole plant growth than to photosynthetic enzyme production and maintenance
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. This will result in a positive effect of inoculation on whole plant growth and total leaf area, but will only be apparent under low soil nitrogen availability.
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 4 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 three washes in deionized 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)

*Leaf gas exchange and leaf trait measurements*

Six weeks after experiment initiation, we sampled one random, fully expanded leaf with little to no visible external damage for gas exchange measurements. Leaves were attached to a Li-COR LI-6800 (Li-COR Biosciences, Lincoln, Nebraska, USA) portable photosynthesis machine to measure net photosynthesis (*A*net; μmol m-2 s-1), stomatal conductance (*g*s; mmol mol-1), and intercellular CO2 concentration (*C*i; µmol mol-1) at different atmospheric CO2 (*C*a; µmol mol-1) concentrations (i.e., an *A*net/*C*i curve). *A*net/*C*i curves were conducted under saturating light conditions (1,500 μmol m-2 s-1), 50% relative humidity, and with the cuvette temperature set to 25°C. We measured *A*net, *g*s, and *C*i at each of the following reference CO2 concentrations (*C*a; μmol mol-1): 400, 300, 200, 100, 50, 400, 400, 600, 800, 1000, 1200, and 1500. Finally, we subjected individuals to at least a 30-minute dark period and quantified dark respiration (*R*d; μmol m-2 s-1), again using a Li-COR LI-6800 with relative humidity set to 50% and cuvette temperature set to 25°C, with incoming radiation set to 0 μmol m-2 s-1 and*C*a set to 400 μmol mol-1.

Leaf trait measurements were collected on the same focal leaf used to generate each CO2 response curve. Images of each leaf were curated using a flat-bed scanner to determine wet leaf area using the 'LeafArea' R package (Katabuchi, 2015), which automates leaf area calculations using ImageJ software (Schneider *et al.*, 2012). Each leaf was dried at 65C for at least 48 hours and subsequently weighed and ground until homogenized. Specific leaf area (cm2 g-1) was calculated as the ratio of wet leaf area to dry leaf biomass. Using subsamples of ground and homogenized leaf biomass, we also determined leaf nitrogen content (*N*mass; gN g-1) through elemental combustion analysis (Costech-4010, Costech, Inc., Valencia, CA, USA). Leaf nitrogen mass per unit leaf area (*N*area; gN m-2) was calculated by dividing *N*mass by specific leaf area, then multiplying by 10,000 to convert cm-2 to m-2.

*Curve fitting and parameter estimation*

We fit *A*net/*C*i curves of each individual using the 'fitaci' function in the 'plantecophys' R package (Duursma, 2015). This function estimates the maximum rate of Rubisco carboxylation (*V*cmax; µmol m-2 s-1) and maximum rate of electron transport for RuBP regeneration (*J*max; µmol m-2 s-1) based on the Farquhar, von Caemmerer, and Berry biochemical model of C3 photosynthesis (Farquhar *et al.*, 1980). We removed all data points that were likely to confer TPU limitation and fit each curve without imposing TPU limitation as a rate-limiting step. We also determined kinetic parameters and CO2 compensation points using leaf temperature and equations derived in Bernacchi *et al.* (2001) and described in Medlyn *et al.* (2002). Dark respiration measurements were also included in each curve fit.

For all *A*net/*C*i curve fits, we manually standardized *V*cmax and *J*max estimates to25C using a modified Arrhenius equation as in:

(Eqn. 1)

where *k*25 represents the standardized *V*cmax or *J*max rate at 25C, *k*obs represents the *V*cmax or *J*max estimate at the average leaf temperature measured inside the cuvette during the CO2 response curve. *H*a is the activation energy of *V*cmax (71,513 J mol-1; Kattge & Knorr, 2007) or *J*max (49,884 J mol-1; Kattge & Knorr, 2007) *H*d represents the deactivation energy of both *V*cmax and *J*max (200,000 J mol-1; Medlyn *et al.*, 2002), and R represents the universal gas constant (8.314 J mol-1 K-1). *T*ref represents the standardized temperature of 298.15 K and *T*obs represents the mean leaf temperature (K) during each CO2 response curve. ΔS is an entropy term that XX derived as a linear relationship with average growing season temperature (*T*g; °C), where:

(Eqn. 2a)

and:

(Eqn. 2b)

We estimated *T*g in equations 5 and 6 based on mean air temperature for each block throughout the experiment. Temperature data were collected using HOBO MX2301 data loggers (Onset Computer Corporation, Bourne, MA, USA), which recorded temperature and humidity of each block in the greenhouse on a fifteen-minute timestep. We then used *V*cmax25 and *J*max25 estimates to calculate the ratio of *J*max25 to *V*cmax25 (*J*max25:*V*cmax25; unitless).

Finally, we standardized dark respiration measurements to 25C (*R*d25; μmol m-2 s-1) using the log-polynomial approach explained in Heskel *et al.* (2016), where:

(Eqn. 3)

*R*T is the standardized respiration rate at temperature *T* (set to 25C) and *T*ref is the leaf temperature of the respiration measurement *R*Tref. *b* and *c* are coefficients that Heskel *et al.* (2016) derived from a log-polynomial approach described in O’Sullivan *et al.* (2013)for plant functional types and biomes. We used coefficients set for C3 herbaceous species, where *b* was set to 0.1271 and *c* was set to -0.00110 (Heskel *et al.*, 2016).

*Tradeoffs between nitrogen and water usage*

Photosynthetic nitrogen-use efficiency (*PNUE*; µmol CO2 g-1 Ns-1) was calculated by dividing *A*net measured at 400 μmol mol-1 CO2 by *N*area. We also estimated intrinsic water-use efficiency (*iWUE*; μmol CO2 mol-1 H2O) by dividing *A*net measured at 400 μmol mol-1 CO2 by *g*s measured at 400 μmol mol-1 CO2. Tradeoffs between nitrogen and water use were determined by calculating the ratio of *N*area to *g*s measured at 400 μmol mol-1 CO2 (*N*area:*g*s; g N s mol-1 H2O) and *V*cmax to *g*s measured at 400 μmol mol-1 CO2 (*V*cmax:*g*s; μmol CO2 mol-1 H2O). We used the temperature unstandardized *V*cmax value instead of *V*cmax25 for *V*cmax:*g*s because stomatal conductance values were not standardized to 25°C.

*Whole plant traits*

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, including the focal leaf measured during the CO2 response curve. 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* leaf photosynthesis, tradeoffs between nitrogen and water use, and whole plant growth. 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 *N*area, *SLA*, *N*mass, *A*net, *V*cmax25, *J*max25, *J*max25:*V*cmax25, *R*d25, total leaf area, whole plant biomass, *g*s, *C*i: *C*a, *PNUE*, *iWUE*, *N*area:*g*s, *V*cmax:*g*s, 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 *N*area, *J*max25:*V*cmax25, *R*d25, *g*s, *PNUE, V*cmax:*g*s, *N*cost, *C*bg, 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 *R*d25, *V*cmax:*g*s, *N*cost, *C*bg, 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). All acronyms, acronym descriptions, and units used in this paper are summarized in Table 1.

**Table 1** Summary of all measured leaf and whole plant traits, their associated units, and a description if trait is referenced as an acronym throughout the paper

|  |  |  |
| --- | --- | --- |
| **Trait** | **Units** | **Trait description** |
| *A*net | μmol m-2 s-1 | net photosynthesis rate, measured at 400 μmol mol-1 CO2 |
| *C*bg | g C | belowground carbon biomass (numerator of *N*cost) |
| *C*i:*C*a | unitless | intercellular CO2: atmospheric CO2, measured at 400 μmol mol-1 CO2 |
| *g*s | mol m-2 s-1 | stomatal conductance, measured at 400 μmol mol-1 CO2 |
| *iWUE* | μmol CO2 mol-1 H2O | intrinsic water-use efficiency, measured at 400 μmol mol-1 CO2 |
| *J*max25 | μmol m-2 s-1 | maximum RuBP regeneration rate, standardized to 25°C |
| *J*max25:*V*cmax25 | unitless | maximum RuBP regeneration rate: maximum Rubisco carboxylation rate, standardized to 25°C |
| *N*area | g N m-2 | leaf nitrogen per leaf area |
| *N*area:*g*s | g N s mol-1 H2O | leaf nitrogen per stomatal conductance measured at 400 μmol mol-1 CO2 |
| *N*cost | g C g-1 N | structural carbon costs to acquire nitrogen |
| *N*mass | g N g-1 biomass | leaf nitrogen content |
| Nodule biomass: root biomass | unitless | - |
| *N*wp | g N | whole plant nitrogen biomass (denominator of *N*cost) |
| *PNUE* | µmol CO2 g-1 N s-1 | photosynthetic nitrogen-use efficiency, measured at 400 μmol mol-1 CO2 |
| *R*d25 | μmol CO2 m-2 s-1 | dark respiration, measured at 400 μmol mol-1 CO2 and standardized to 25°C |
| Root biomass | g | - |
| Root nodule biomass | g | - |
| SLA | cm2 g-1 | specific leaf area |
| Total leaf area | cm2 | - |
| *V*cmax:*g*s | μmol CO2 mol-1 H2O | maximum Rubisco carboxylation rate per stomatal conductance |
| *V*cmax25 | μmol CO2 m-2 s-1 | maximum Rubisco carboxylation rate, standardized to 25°C |
| Whole plant biomass | g | - |

**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 nitrogen fertilization had 63.4% lower structural carbon costs to acquire nitrogen than non-inoculated individuals also grown under low nitrogen fertilization (Tukey: p<0.001). There was no difference in structural carbon costs to acquire nitrogen between inoculation treatments under high nitrogen fertilization (Tukey: p=0.597). Nitrogen fertilization also decreased structural carbon costs to acquire nitrogen, where individuals grown under high nitrogen fertilization had 54.1% lower structural carbon costs to acquire nitrogen than those grown under low 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).

Inoculation negatively 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 fertilization or any observable interaction between fertilization and inoculation on belowground carbon biomass (Table 1).

Whole plant nitrogen biomass was driven by a strong interaction between fertilization and inoculation (Table 1; Fig. 1C). This interaction indicated that inoculated individuals grown under low nitrogen fertilization had 72.4% higher whole plant nitrogen biomass 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=0.873). Nitrogen fertilization also increased whole plant nitrogen biomass, where individuals grown under high nitrogen fertilization had 119.0% higher whole plant nitrogen biomass than those grown under low 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).

**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**

**Chart, box and whisker chart

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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*

Total leaf area was driven by a strong interaction between nitrogen fertilization and inoculation (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 driven 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 observable inoculation effect nor was there any interaction between inoculation and nitrogen fertilization (Table 1; Fig. 2B).

Root nodule biomass: root biomass was determined through an individual positive effect 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).

*Leaf nitrogen allocation*

*N*area and *N*mass were both driven by an interaction between inoculation and nitrogen fertilization (Table 2; Figs. 3A-B). This interaction indicated that inoculated individuals had 19.5% and 41.9% higher respective *N*area and *N*mass under low nitrogen fertilization than non-inoculated individuals, (Tukey: p<0.001 in both cases), with no difference between inoculation treatments under high nitrogen fertilization (TukeyNarea: p=0.623; TukeyNmass: p=0.941). Individuals grown under high nitrogen fertilization also had 30.0% and 38.2% higher *N*area and *N*mass than those grown under low nitrogen fertilization, respectively (Table 2; Figs. 3A-B).

*SLA* increased with inoculation and marginally increased with increasing soil nitrogen fertilization, with no observable interaction between fertilization and inoculation (Table 2; Fig. 3C). Specifically, inoculated individuals had 6.3% higher *SLA* than non-inoculated individuals (Tukey: p=0.014), while individuals grown under high nitrogen fertilization had 4.2% higher *SLA* than those grown under low nitrogen fertilization (Tukey: p=0.095).

**Table 2** Analysis of variance results exploring effect of nitrogen fertilization, inoculation with *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation on leaf nitrogen allocation\*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | | ***N*area** | | ***N*mass** | | ***SLA*** | |
|  | df | χ2 | *p* | χ2 | *p* | χ2 | *p* |
| N fertilization (N) | 1 | 104.61 | **<0.001** | 139.51 | **<0.001** | 2.88 | *0.090* |
| Inoculation (I) | 1 | 4.45 | **0.035** | 36.38 | **<0.001** | 6.46 | **0.011** |
| N\*I | 1 | 14.62 | **<0.001** | 27.35 | **<0.001** | 1.27 | 0.260 |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold. Key: *N*area=leaf nitrogen per leaf area (g m-2); *N*mass=leaf nitrogen per leaf mass (g g-1); *SLA*=specific leaf area (cm2 g-1)

**Figure 3**

**Chart, box and whisker chart

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**Figure 3** Effects of soil nitrogen fertilization and inoculation on *G. max* leaf nitrogen per unit leaf area (panel A), leaf nitrogen per unit leaf biomass (panel B), and specific leaf area (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).

*Leaf photosynthesis and gas exchange*

Increasing nitrogen fertilization had a weak negative effect on *A*net, *V*cmax25, and *J*max25 (Table 3; Fig. 4A-C). Specifically, individuals grown under high fertilization had 20.8%, 11.3%, and 10.8% lower *A*net, *V*cmax25, and *J*max25 than those grown under low nitrogen fertilization (TukeyAnet: p<0.001; TukeyVcmax25: p=0.043; TukeyJmax25: p=0.038), respectively. There was no observable inoculation effect or interaction between fertilization and inoculation on *A*net, *V*cmax25, or *J*max25 (Table 3). Similar reductions in *V*cmax25 and *J*max25 with fertilization yielded no observable effect of fertilization on *J*max25:*V*cmax25 (Table 3); however, there was a weak stimulation in *J*max25:*V*cmax25 with inoculation (Table 3). *R*d25 was determined through a weak interaction between nitrogen fertilization and inoculation (Table 3; Fig. 4D). This interaction indicated that inoculated individuals grown under high nitrogen fertilization (0.81±0.07 μmol m-2 s-1) had 47.2% higher *R*d25 values than inoculated individuals grown under low nitrogen fertilization (0.55±0.04 μmol m-2 s-1; Tukey: p=0.004), with no fertilization effect observed in non-inoculated individuals (Tukey: p=0.956).

**Table 3** Analysis of variance results exploring effect of soil nitrogen fertilization, inoculation with *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation on leaf photosynthesis and gas exchange\*

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | ***A*net** | | ***V*cmax25** | | ***J*max25** | | ***J*max25:*V*cmax25** | | ***R*d25** | |
|  | df | χ2 | *p* | χ2 | *p* | χ2 | *p* | χ2 | *p* | χ2 | *p* |
| N fertilization (N) | 1 | 15.82 | **<0.001** | 4.28 | **0.038** | 4.53 | **0.033** | 1.43 | 0.231 | 8.61 | **0.003** |
| Inoculation (I) | 1 | 0.46 | 0.498 | 0.56 | 0.453 | 2.13 | 0.145 | 4.55 | **0.033** | 1.51 | 0.219 |
| N\*I | 1 | 0.39 | 0.533 | 0.90 | 0.343 | 1.43 | 0.231 | 1.27 | 0.260 | 4.34 | **0.037** |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  | | ***R*d25:*V*cmax25** | | ***g*s** | | ***C*i: *C*a** | |  | |  | |
|  | df | χ2 | *p* | χ2 | *p* | χ2 | *p* |  |  |  |  |
| N fertilization (N) | 1 | 13.05 | **0.001** | 9.97 | **0.002** | 0.01 | 0.913 |  |  |  |  |
| Inoculation (I) | 1 | 0.66 | 0.421 | 0.34 | 0.561 | 0.28 | 0.597 |  |  |  |  |
| N\*I | 1 | 2.44 | 0.118 | 0.01 | 0.929 | 1.40 | 0.237 |  |  |  |  |

\*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. Key: *A*net=light saturated net photosynthesis measured at 400 μmol mol-1 CO2; *V*cmax25=maximum rate of Rubisco carboxylation standardized to 25°C; *J*max25=maximum rate of electron transport for RuBP regeneration standardized to 25°C, *J*max25:*V*cmax25=the ratio of *J*max25 to *V*cmax25, both standardized to 25°C; *R*d25=dark respiration rate standardized to 25°C; *R*d25:*V*cmax25= ratio of *R*d25 to *V*cmax25, both standardized to 25°C; *g*s=stomatal conductance measured at 400 μmol mol-1 CO2; *C*i:*C*a=ratio of intercellular CO2 to atmospheric CO2.

**Figure 4**

**Chart, box and whisker chart

Description automatically generated**

**Figure 4** Effects of soil nitrogen fertilization and inoculation on *G. max* net photosynthesis (panel A), maximum Rubisco carboxylation rate standardized to 25C (panel B), and the maximum electron transport for RuBP regeneration rate standardized to 25C (panel C), dark respiration standardized to 25C (panel D). 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).

*Tradeoffs between nitrogen and water usage*

*PNUE* was determined through an interaction between nitrogen fertilization and inoculation (Table 4; Fig. 5A). This interaction indicated that inoculated individuals grown under low nitrogen fertilization had 17.3% lower *PNUE* than non-inoculated individuals also grown under low nitrogen fertilization (Tukey: p=0.024), with no difference in inoculation treatments under high nitrogen fertilization (Tukey: p=0.799). We also observed a strong negative effect of soil nitrogen fertilization on *PNUE*, where individuals grown under high nitrogen fertilization had 40.1% lower *PNUE* than those grown under low nitrogen fertilization (Tukey: p<0.001). There was no individual inoculation effect on *PNUE* (Table 4; Fig. 5A).

There was no effect of nitrogen fertilization, inoculation, or any observable interaction between fertilization and inoculation on *iWUE* (Table 4; Fig. 5B). The null response of *iWUE* to fertilization was likely driven by a similar reduction in *A*net and gs with increasing fertilization (Table 3).

Increasing nitrogen fertilization generally increased *N*area:*g*s (Table 4; Fig 5C) and marginally increased *V*cmax:*g*s (Table 4; Fig 6D). Specifically, individuals grown under high nitrogen fertilization had 68.3% higher *N*area:*g*s than those grown under low nitrogen fertilization (Tukey: p<0.001). The *N*area:*g*s response to fertilization was likely driven by an increase in leaf nitrogen allocation that corresponded with a reduction in gs with increasing fertilization (Table 3). There was no observable inoculation effect or interaction between fertilization and inoculation on *N*area:*g*s and *V*cmax:*g*s (Table 4).

**Table 4** Analysis of variance results exploring effect of soil nitrogen fertilization, inoculation with *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation on tradeoffs between nitrogen and water usage\*

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | ***PNUE*** | | ***iWUE*** | | ***N*area:*g*s** | | ***V*cmax:*g*s** | |
|  | df | χ2 | *p* | χ2 | *p* | χ2 | *p* | χ2 | *p* |
| N fertilization (N) | 1 | 77.73 | **<0.001** | <0.01 | 0.974 | 38.45 | **<0.001** | 2.89 | *0.089* |
| Inoculation (I) | 1 | 2.06 | 0.152 | 0.30 | 0.586 | <0.01 | 0.996 | 0.34 | 0.561 |
| N\*I | 1 | 7.42 | **0.006** | 1.36 | 0.243 | 0.88 | 0.349 | 0.60 | 0.439 |

\*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. Key: *PNUE*=photosynthetic nitrogen use efficiency; *N*area:*g*s=ratio of *N*area to *g*s; *V*cmax:*g*s=ratio of temperature unstandardized *V*cmax to *g*s.

**Figure 5**

**Chart, calendar, box and whisker chart

Description automatically generated**

**Figure 6** Effects of soil nitrogen fertilization and inoculation on *G. max* photosynthetic nitrogen use efficiency (panel A), intrinsic water-use efficiency (panel B), the ratio of leaf nitrogen per leaf area to stomatal conductance (panel C), and the ratio of the maximum Rubisco carboxylation rate to stomatal conductance (panel D). 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**: stronger whole plant than leaf level responses to soil N. Might have diminished expected PLCT nitrogen-water use tradeoffs. Important to consider whole plant responses when leaf acclimation responses deviate from those expected from theory]

[**Main point #2**: strong effects of inoculation on whole plant responses under low soil N, no effect of inoculation on nitrogen-water use tradeoffs except for stimulation in leaf N. Hard to tell if this is driven by PLCT-expected strategy or just a pattern of N-fixation strategy. N-fixers usually seem to have higher leaf N than non-fixers. Stimulation in total leaf area with inoculation under low soil N could have exacerbated diminishing nitrogen-water tradeoffs with increasing soil N]

[**Main point #3**: effects of inoculation on total leaf area/carbon costs to acquire nitrogen/leaf nitrogen allocation diminish with increasing N. This could be driven by shift away from N fixation and toward direct uptake with fertilization, as costs to acquire nitrogen become similar between pathways]

*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, as inferred from root nodulation data in (XX), 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* cocktails. 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**