**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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**Manuscript compilation details**

**Abstract:** 284 words

**Main text word count**:

Introduction: 1379 words

Methods: 2017 words

Results: 1522 words (not including text in figures or tables)

Discussion: XX words (XX % of total word count)

**References**: XX

**Tables and Figures**: 5 tables, 6 figures

**Supplemental Information**: This manuscript reports XX tables and XX figures as supplemental information

**Abstract**

Many terrestrial biosphere models formulate photosynthesis based on positive linear relationships between soil nutrient availability, leaf nutrient allocation, and photosynthetic capacity. While commonly observed in nature, this formulation does not account for plant acclimation responses to the environment, which can modify leaf nutrient allocation and photosynthetic capacity independent of soil nutrient availability. Photosynthetic least-cost theory could be a useful solution for including acclimation responses in next generation terrestrial biosphere models. However, few direct tests of the theory exist aside from a few global gradient analyses, which limits our ability to evaluate underlying assumptions of the theory. In this study, we measured leaf and whole plant traits in *Glycine max* L. (Merr.) grown under two soil nitrogen fertilization treatments both with and without *Bradyrhizobium japonicum* inoculation through a full factorial greenhouse experiment. After a seven-week growth period, we found that increasing nitrogen fertilization increased leaf nitrogen allocation, leaf nitrogen per stomatal conductance, total leaf area, and total biomass. These patterns corresponded with a reduction in leaf photosynthesis, photosynthetic nitrogen use efficiency, stomatal conductance, and structural carbon costs to acquire nitrogen. Interestingly, inoculation increased leaf nitrogen allocation and total leaf area and decreased structural carbon costs to acquire nitrogen, but only under low fertilization. There was also no overall effect of inoculation on leaf biochemistry, photosynthetic nitrogen use efficiency, water use efficiency, or whole plant growth. While the effects of nitrogen fertilization on nitrogen-water use tradeoffs observed here support patterns expected from photosynthetic least-cost theory, we speculate that these responses may have been diminished by stronger whole plant growth responses to fertilization. Additionally, we found no evidence to suggest that inoculation modified patterns expected from theory, although did contribute to biomass accumulation and total leaf area under low soil nitrogen that may have further diminished the magnitude of leaf acclimation responses to soil nitrogen fertilization.

**Keywords**

nitrogen fixation; photosynthetic least-cost theory; 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 must accurately represent these complex cycles if they are to accurately simulate past and predict future carbon and nutrient fluxes between the atmosphere and biosphere (Oreskes et al., 1994; Prentice et al., 2015). However, while carbon and nutrient flux simulations generally agree across terrestrial biosphere model products using past climate data, there is considerable variation in the simulation of these fluxes under future environmental change scenarios (Friedlingstein et al., 2014). This could be due to an incomplete understanding of how changing environments modifies important processes that are directly or indirectly regulated by carbon and nutrient fluxes between the atmosphere and biosphere, such as litter decomposition (), soil organic matter decomposition (), photosynthesis (), autotrophic respiration (), and heterotrophic respiration ().

Plants undergo multiple processes that couple carbon and nutrient fluxes between the atmosphere and biosphere. For example, plants acquire nitrogen from the soil via direct uptake or through symbioses with nitrogen-fixing bacteria or mycorrhizal fungi

For example, plants use a series of photosynthetic reactions with nitrogen-dense enzymes such as Ribulose-1,5-bisphosphate carboxylase/oxygenase (“Rubisco”) to fix carbon dioxide and create simple sugars that are either accumulated as biomass (), sequestered to secondary metabolites (), lost through autotrophic respiration (), or shuttled belowground to acquire soil resources such as nutrients or water ().

The inherent link between carbon and nutrient fluxes in driving photosynthetic processes

As a result, many have conducted experiments attempting to understand photosynthetic responses to environmental change, most notably due to light availability (), nitrogen availability (), increasing temperatures (), water stress (), or increasing CO2 ().

Nitrogen availability may alter linkages between terrestrial carbon and nitrogen cycles through modifying leaf carbon and nitrogen use. Due to the high nitrogen requirements to build and maintain photosynthetic enzymes, positive relationships between soil nitrogen availability, leaf nitrogen allocation, and photosynthetic capacity are common ().

Nitrogen availability may also alter linkages

Leaf responses to nitrogen availability may depend on whole plant carbon and nitrogen use responses

While these leaf responses are commonly observed, this might depend on whether nitrogen availability also modifies whole plant carbon and nitrogen use. [whole plant level]

Effects of nitrogen availability on leaf or whole plant carbon and nitrogen use may depend on whether a species is capable of associating with symbiotic nitrogen-fixing bacteria. Plants acquire nutrients through different nutrient acquisition strategies such as direct uptake or through symbioses with mycorrhizal fungi and symbiotic nitrogen-fixing bacteria (Smith & Read, 2008). Costs of nutrient use have been shown to vary in species that have different nutrient acquisition strategies (Brzostek et al., 2014; Perkowski et al., 2021; Terrer et al., 2018); ().

In this study, 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 increase whole plant growth as a result of a lower carbon cost to obtain nitrogen needed to build structural tissues and proteins.
2. Inoculation will reduce carbon costs to obtain nitrogen when soil nitrogen is low, as carbon costs to obtain nitrogen via nitrogen fixing bacteria will be lower than the cost needed for direct uptake. As a result, inoculation will increase whole plant growth when nitrogen availability is low.
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.
4. Soil nitrogen fertilization will increase leaf nitrogen per stomatal conductance through an increase in leaf nitrogen allocation and reduction in stomatal conductance. This response will be driven by a reduction in the carbon cost of acquiring nitrogen versus water, causing individuals to sacrifice inefficient nitrogen use for more efficient water use. We expect that inoculation will increase the magnitude of nitrogen-water tradeoffs but will only be observed in the low soil nitrogen treatment due to shifts away from nitrogen fixation with increasing fertilization.
5. Effects of soil nitrogen fertilization and inoculation on leaf nitrogen-water use tradeoffs will depend on whole plant acclimation responses to soil nitrogen availability. Weak or null whole plant responses to either soil nitrogen fertilization or inoculation will enhance leaf nitrogen-water use tradeoffs. However, if soil nitrogen fertilization or inoculation elicit strong whole plant growth responses, then we expect either weak or no effect of these treatments on leaf nitrogen-water use tradeoffs

**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 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 a span of 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 period of darkness 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; g g-1) through elemental combustion analysis (Costech-4010, Costech, Inc., Valencia, CA, USA). Leaf nitrogen mass per unit leaf area (*N*area; g 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).

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 Kattge & Knorr, 2007):

(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 Kattge & Knorr (2007) 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 duration of 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 temperature standardized respiration rate, *T* is the temperature in which a given respiration rate is being standardized, set to 25C, and *T*ref is the 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). Finally, we calculated the ratio of *R*d25 to *V*cmax25 (*R*d25: *V*cmax25; unitless).

*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, *R*d25:*V*cmax25,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, *R*d25:*V*cmax25, *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 first attempted to satisfy residual normality assumptions for these dependent variables using Bonferroni outlier tests to indicate any data points that were statistical outliers. This was done using the ‘OutlierTest’ function in the car R package (Fox & Weisberg, 2019), which uses a Bonferroni-corrected t-distribution to evaluate whether residuals of a given data point shift the mean of the sampling population. We removed any data points where Bonferroni: p<0.05, which resulted in one data point being removed from each of *N*area, *g*s, and *PNUE,* and two data points being removed from each of *J*max25:*V*cmax25, *R*d25, and *R*d25:*V*cmax25. The removal of these statistical outliers satisfied residual normality assumptions for *N*area, *J*max25:*V*cmax25, *R*d25:*V*cmax25, *g*s, and *PNUE* (Shapiro-Wilk: p>0.05 in all cases).

For any dependent variables where statistical outlier removal did not satisfy residual normality assumptions, we then 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 |
| *R*d25: *V*cmax25 | unitless | dark respiration per maximum Rubisco carboxylation rate, 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**

*Leaf nitrogen allocation*

*N*area and *N*mass were both driven by an interaction between inoculation and nitrogen fertilization (Table 2; Figs. 1A-B). This interaction indicated that inoculated individuals (marginal mean ± SE; *N*area: 0.98 ± 0.04 g m-2; *N*mass: 4.64 ± 0.12 g m-2) had 19.5% and 41.9% higher respective *N*area and *N*mass under low nitrogen fertilization than non-inoculated individuals, (*N*area: 0.82 ± 0.04 g m-2; *N*mass: 3.27 ± 0.12 g m-2;Tukey: p<0.001 in both cases), with no difference between inoculation treatments under high nitrogen fertilization (*N*area Tukey: p=0.623; *N*mass Tukey: p=0.941). Individuals grown under high nitrogen fertilization (*N*area: 1.17 ± 0.03 g m-2; *N*mass: 5.39±0.09 g m-2) also had 30.0% and 38.2% higher *N*area and *N*mass than those grown under low nitrogen fertilization (*N*area: 0.90 ± 0.03 g m-2; *N*mass: 3.9 ± 0.09 g m-2), respectively (Table 2; Figs. 1A-B).

*SLA* increased with inoculation and marginally increased with increasing soil nitrogen fertilization, with no observable interaction between fertilization and inoculation (Table 2; Fig. 1C). Specifically, inoculated individuals (578.6 ± 10.9 cm2 g-1) had 6.3% higher *SLA* than non-inoculated individuals (544.1 ± 10.9 cm2 g-1; Tukey: p=0.014), while individuals grown under high nitrogen fertilization (572.8 ± 10.9 cm2 g-1) had 4.2% higher *SLA* than those grown under low nitrogen fertilization (549.8 ± 10.9 cm2 g-1; 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 1**

**Chart, box and whisker chart

Description automatically generated**

**Figure 1** 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 negative effect on *A*net (Table 3; Fig. 2A-B), where individuals grown under high nitrogen fertilization (9.79 ± 0.46 μmol m-2 s-1) had 20.8% lower *A*net than individuals grown under low nitrogen fertilization (*A*net: 12.36 ± 0.46 μmol m-2 s-1; Tukey: p<0.001). There was a marginal negative effect of increasing nitrogen fertilization on *V*cmax25, but no observable fertilization effect on *J*max25 or *J*max25: *V*cmax25 (Table 3; Fig. 2C). There was also no effect of inoculation or any interaction between fertilization and inoculation on *A*net, *V*cmax25, *J*max25: *V*cmax25, but there was a marginal positive effect of inoculation on *J*max25 (Table 3; Fig. 2A-C).

*R*d25 was determined through a weak interaction between nitrogen fertilization and inoculation (Table 3; Fig. 2D). 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).

We also observed a marginal interaction between fertilization and inoculation on *R*d25: *V*cmax25 (Table 3). This interaction indicated that inoculated individuals growing under high nitrogen fertilization (0.0163 ± 0.0015) had 66.3% higher *R*d25: *V*cmax25 than inoculated individuals growing under low nitrogen fertilization (0.0098 ± 0.0015; Tukey: p=0.004), with no fertilization effect observed in non-inoculated individuals (Tukey: p=0.770). Additionally, increasing nitrogen fertilization had a positive effect on *R*d25: *V*cmax25 (Table 3), where individuals grown under high nitrogen fertilization (0.0146 ± 0.0012) had 40.3% higher *R*d25: *V*cmax25 than those grown under low nitrogen fertilization (0.0104 ± 0.0012; Tukey: p=0.003). There was no individual inoculation effect on *R*d25: *V*cmax25 (Table 3).

Increasing nitrogen fertilization generally decreased *g*s, but did not change *C*i: *C*a (Table 3). Specifically, individuals grown under high nitrogen fertilization (0.132 ± 0.009 mol m-2 s-1) had 22.8% lower *g*s than those grown under low nitrogen fertilization (0.171 ± 0.010 mol m-2 s-1), respectively (Tukey: p=0.002). There was no effect of inoculation or any observable interaction between nitrogen fertilization and inoculation on either response variable (Table 3).

**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** | 2.87 | *0.090* | 2.38 | 0.123 | 0.77 | 0.381 | 8.61 | **0.003** |
| Inoculation (I) | 1 | 0.46 | 0.498 | 1.50 | 0.221 | 2.86 | *0.091* | 2.20 | 0.138 | 1.51 | 0.219 |
| N\*I | 1 | 0.39 | 0.533 | 0.18 | 0.668 | 0.45 | 0.502 | 0.97 | 0.607 | 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 | 10.54 | **0.001** | 9.97 | **0.002** | 0.01 | 0.913 |  |  |  |  |
| Inoculation (I) | 1 | 0.65 | 0.421 | 0.34 | 0.561 | 0.28 | 0.597 |  |  |  |  |
| N\*I | 1 | 3.06 | *0.080* | 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 2**

**Chart, box and whisker chart

Description automatically generated**

**Figure 2** Effects of soil nitrogen fertilization and inoculation on *G. max* net photosynthesis (panel A), dark respiration standardized to 25C (panel B), maximum Rubisco carboxylation rate standardized to 25C (panel C), and the maximum electron transport for RuBP regeneration rate 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. 3A). This interaction indicated that inoculated individuals grown under low nitrogen fertilization (12.60 ± 0.62 μmol CO2 g-1 N s-1) had 17.3% lower *PNUE* than non-inoculated individuals also grown under low nitrogen fertilization (15.23 ± 0.64 μmol CO2 g-1 N s-1; 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 (8.34 ± 0.45 μmol CO2 g-1 N s-1) had 40.1% lower *PNUE* than those grown under low nitrogen fertilization (13.92 ± 0.45 μmol CO2 g-1 N s-1; Tukey: p<0.001). There was no individual inoculation effect on *PNUE* (Table 4; Fig. 3A).

There was no effect of nitrogen fertilization, inoculation, or any observable interaction between fertilization and inoculation on *iWUE* (Table 4; Fig. 3B).

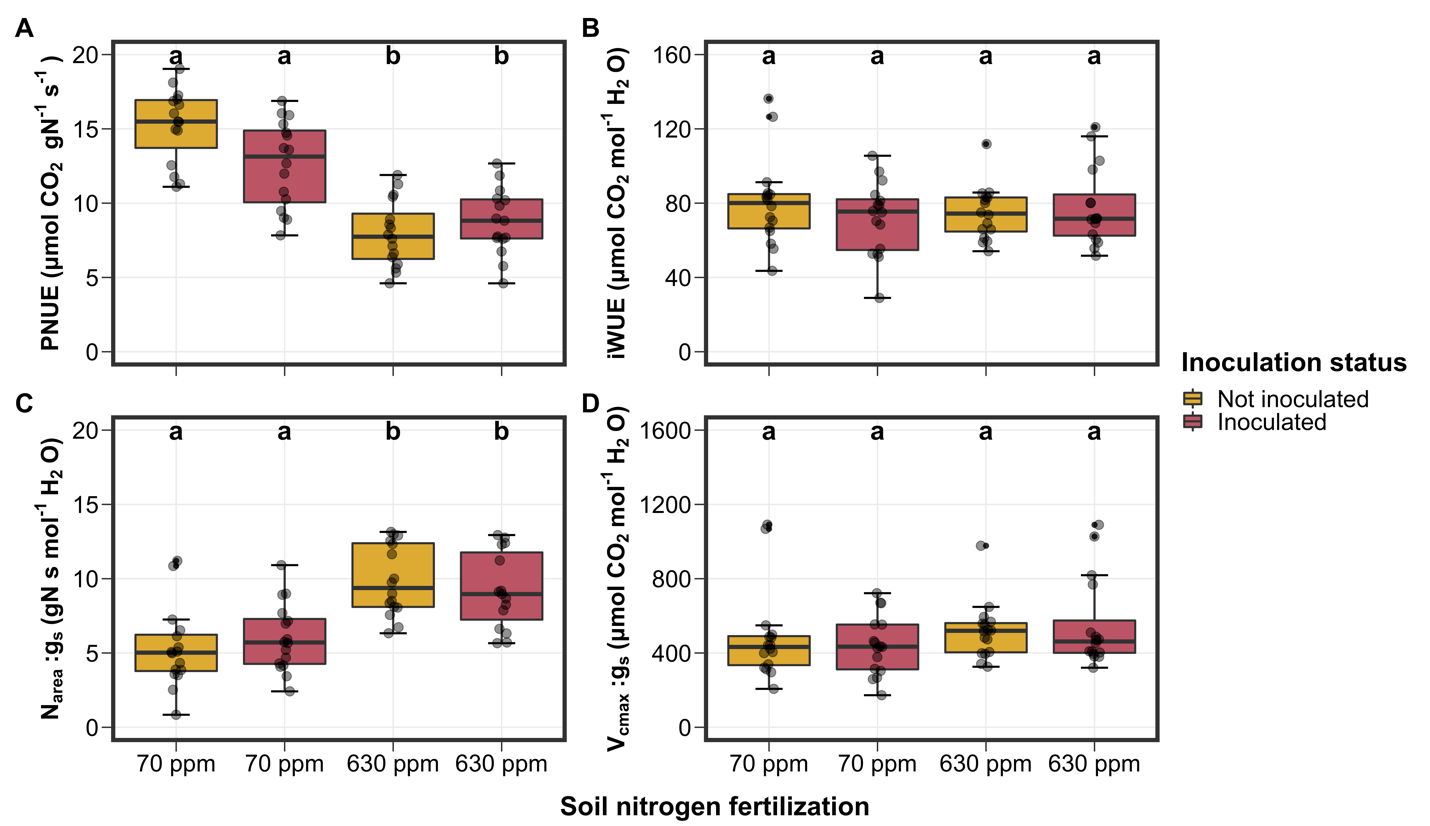
Increasing nitrogen fertilization generally increased *N*area: *g*s (Table 4; Fig 3C) and marginally increased *V*cmax: *g*s (Table 4; Fig 3D). Individuals grown under high nitrogen fertilization (9.54 ± 0.45 g N s mol-1 H2O) had 68.3% higher *N*area: *g*s than those grown under low nitrogen fertilization (5.67 ± 0.0.44 g N s mol-1 H2O; Tukey: p<0.001). 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 | 38.07 | **<0.001** | 3.46 | *0.063* |
| Inoculation (I) | 1 | 2.06 | 0.152 | 0.30 | 0.586 | <0.01 | 0.967 | 0.06 | 0.811 |
| N\*I | 1 | 7.42 | **0.006** | 1.36 | 0.243 | 1.20 | 0.274 | 0.25 | 0.619 |

\*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 3**

****

**Figure 3** 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).

*Structural carbon costs to acquire nitrogen*

Structural carbon costs to acquire nitrogen were driven by a strong interaction between nitrogen fertilization and inoculation (Table 5; Fig. 4A). This interaction indicated that inoculated individuals grown under low nitrogen fertilization (2.77 ± 0.44 g C g-1 N) had 63.4% lower structural carbon costs to acquire nitrogen than non-inoculated individuals also grown under low nitrogen fertilization (7.56 ± 1.20 g C g-1 N; 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 (2.10 ± 0.25 g C g-1 N) had 54.1% lower structural carbon costs to acquire nitrogen than those grown under low nitrogen fertilization (4.58 ± 0.52 g C g-1 N; Tukey: p<0.001). Inoculation decreased structural carbon costs to acquire nitrogen, where inoculated individuals (2.24 ± 0.26 g C g-1 N) had 91.5% lower structural carbon costs to acquire nitrogen than non-inoculated individuals (4.29 ± 0.49 g C g-1 N; Tukey: p<0.001).

Inoculation negatively affected belowground carbon biomass (Table 5; Fig. 4B). Specifically, inoculated individuals (0.295 ± 0.037 g C) had 29.9% less belowground carbon biomass than non-inoculated individuals (0.421 ± 0.053 g C; Tukey: p=0.050). There was no effect of nitrogen fertilization or any observable interaction between nitrogen fertilization and inoculation on belowground carbon biomass (Table 5).

Whole plant nitrogen biomass was driven by a strong interaction between fertilization and inoculation (Table 5; Fig. 5C). This interaction indicated that inoculated individuals grown under low nitrogen fertilization (0.100 ± 0.005 g N) had 72.4% higher whole plant nitrogen biomass than non-inoculated individuals also grown under low nitrogen fertilization (0.058 ± 0.005 g N; 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 (0.173 ± 0.004 gN) had 119.0% higher whole plant nitrogen biomass than those grown under low nitrogen fertilization (0.079 ± 0.003 gN; Tukey: p<0.001). Inoculation increased whole plant nitrogen biomass, where inoculated individuals (0.138 ± 0.004 gN) had 17.4% higher whole plant nitrogen biomass than non-inoculated individuals (0.114 ± 0.004 gN; Tukey: p<0.001).

**Table 5** 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 4**

**Chart, box and whisker chart

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**Figure 4** 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 processes*

Total leaf area was driven by a strong interaction between nitrogen fertilization and inoculation (Table 5; Fig. 5A). This interaction indicated that inoculated individuals grown under low nitrogen fertilization (829.2 ± 30.5 cm2) had 59.7% higher total leaf area than non-inoculated individuals also grown under low nitrogen fertilization (519.2 ± 30.5 cm2; 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 (1196.3 ± 21.6 cm2) had 77.4% higher total leaf area than those grown under low nitrogen fertilization (674.2 ± 21.6 cm2; Tukey: p<0.001). Inoculation also increased total leaf area, where inoculated individuals (1025.7 ± 21.6 cm2) had 21.4 higher total leaf area than non-inoculated individuals (844.8 ± 21.6 g C g-1 N; Tukey: p<0.001).

Whole plant biomass was driven by nitrogen fertilization (Table 5; Fig. 5B), where individuals grown under high nitrogen fertilization (5.24 ± 0.23 g) had 55.5% higher whole plant biomass than those grown under low nitrogen fertilization (3.37 ± 0.14 g; Tukey: p<0.001). There was no observable inoculation effect nor was there any interaction between inoculation and nitrogen fertilization (Table 5; Fig. 5B).

**Figure 5**

**Chart, box and whisker chart

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**Figure 5** Effects of soil nitrogen fertilization and inoculation on *G. max* total leaf area (panel A) and whole plant biomass (panel B). 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).

*Plant investment in nitrogen fixation*

Root nodule biomass: root biomass and root nodule biomass were driven by a positive inoculation effect (Table 5; Figs. 6A-B). Specifically, inoculated individuals (root nodule biomass: root biomass: 0.144 ± 0.023 g; root nodule biomass: 0.0148 ± 0.0043 g) had 323.5% and 2366.7% greater root nodule biomass: root biomass and root nodule biomass than non-inoculated individuals (root nodule biomass: root biomass: 0.034 ± 0.023 g; root nodule biomass: 0.0006 ± 0.0009 g), respectively (Tukey: p<0.001 in both cases). There was also a marginal negative effect of inoculation on root biomass, which indicated that inoculated individuals (0.671 ± 0.084 g) had 26.9% lower root biomass than non-inoculated individuals (0.918±0.114 g; Tukey: p=0.081). There was no observable effect of nitrogen fertilization or interaction between nitrogen fertilization and inoculation on root nodule biomass: root biomass, root nodule biomass, or root biomass (Table 5).

**Figure 6**

**Chart, box and whisker chart

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**Figure 6** Effects of soil nitrogen fertilization and inoculation on the root nodule biomass: root biomass ratio (panel A), root nodule biomass (panel B), and 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**

Photosynthetic least-cost theory suggests that plants acclimate to growing conditions by maximizing photosynthetic carbon gain at the lowest summed costs of nitrogen and water use (Prentice et al., 2014; Wright et al., 2003). All else equal, the theory predicts that an increase in soil nitrogen availability should increase in water use efficiency and decrease in nitrogen use efficiency through an increase in leaf nitrogen allocation per stomatal conductance (Paillassa et al., 2020). However, the cost of nutrient use, and therefore the magnitude of nitrogen-water use tradeoffs, might vary in species different nutrient acquisition strategies due to differential costs of nutrient acquisition (Brzostek et al., 2014; Perkowski et al., 2021; Terrer et al., 2018) and may also depend on whole plant nutrient demand to build and maintain structures that support whole plant growth (LeBauer & Treseder, 2008; Liang et al., 2020). In this experiment, we grew *G. max* under two soil nitrogen fertilization treatments and two inoculation treatments levels in a full factorial greenhouse experiment to better understand how acquisition strategy and whole plant nutrient demand might modify expected photosynthetic least-cost patterns.

[**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 Perkowski et al. (2021), 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 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* 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]

**Acknowledgements**

We would like to thank Jeffrey Chieppa and Ezinwanne Ezekannagha for their extensive help with generating CO2 response curves and dark respiration measurements, and assistance with the experiment harvest. We would also like to thank Gwendolyn Wagner and Garrison Garza for their help with the experiment harvest, and members of the Schwilk and van Gestel lab for analysis feedback. NGS acknowledges funding support from the NSF (DEB-2045968), Eric and Wendy Schmidt and the Schmidt Futures VESRI program, and Texas Tech University.

**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>).

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