**Target Journals:**

**Title**: Soil nitrogen fertilization and inoculation with *Bradyrhizobium japonicum* modifies leaf water-nitrogen economics and structural carbon costs to acquire nitrogen in *Glycine max* L.

**Running Head:**

**Author List:** Evan A. Perkowski1, Joseph Terrones1, Hannah German1, Nicholas G. Smith1

**Author Affiliations:** 1Department of Biological Sciences, Texas Tech University, Lubbock, TX USA

**Manuscript compilation details**

**Abstract:** XX words

**Main text word count**:

Introduction: XX words

Methods: XX words

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

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

**References**: XX

**Tables and Figures**: XX tables, XX figures

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

**Abstract**

**Keywords**

**Introduction**

Photosynthesis represents the largest carbon flux between the atmosphere and biosphere.

[Acclimation is important]

[PLCT basics]

Photosynthetic least-cost theory provides a framework for understanding how plants acclimate to average environmental growing conditions. First principles of this theory predict that plants acclimate to

Photosynthetic least-cost theory provides a framework for understanding how plants acclimate to aboveground and belowground conditions. The theory predicts that plants acclimate to environments by minimizing unit costs of resource use, namely water and nitrogen.

[Tradeoffs between leaf/whole plant allocation. Link in N cost and implications for PLCT]

The relative strength of nitrogen-water tradeoffs may depend on whole plant allocation decisions.

In this study, we grew *G. max* grown under two soil nitrogen fertilization treatments and two inoculation treatments levels in a fully factorial greenhouse experiment. We leveraged this experiment to understand how soil nutrients and nitrogen fixation modify water-nitrogen tradeoffs expected from photosynthetic least cost theory, and whether these responses were dependent on whole plant growth. We hypothesized that (1) soil nitrogen fertilization would increase whole plant growth through an increase in total leaf area, which would allow plants to increase whole plant primary productivity. We expected that inoculation would also increase whole plant growth, but only under the low soil nitrogen treatment due to a reduction in nodulation with increasing fertilization. We also hypothesized that (2) soil nitrogen fertilization would increase leaf nitrogen per stomatal conductance through an increase in leaf nitrogen allocation and reduction in stomatal conductance. We expected this response to be driven by a reduction in the carbon cost of acquiring nitrogen versus water, causing individuals to sacrifice inefficient use of nitrogen for more efficient use of water. We also expected that inoculation would increase the magnitude of nitrogen-water tradeoffs but would only be observed under the low soil nitrogen treatment.

**Methods**

*Experimental Design*

*Glycine max* 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 XX C for XX hours prior to eliminate any bacterial or fungal growth. Thirty-two randomly selected pots were inoculated with *Bradyrhizobium japonicum* (Verdesian N-Dure™ Soybean, Cary, NC, USA) to stimulate root nodulation. The remaining 32 pots did not receive any inoculation treatment. Upon planting, 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 six weeks. Nitrogen fertilization doses were received as topical agents to the soil surface and were modified to keep concentrations of other macro- and micronutrients equivalent (Table S1). Throughout the experiment, plants were also routinely well-watered to eliminate any water stress potential. There was no evidence of pot size induced growth limitation, indicated by marginal mean 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 Bioscience, 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 cuvette temperature set to 25°C. We measured *A*area, *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, 1500, and 2000. We also collected chlorophyll fluorescence measurements for each focal leaf using a MultispeQ (PhotosynQ, East Lansing, MI, USA) device. Finally, we subjected individuals to at least a 30-minute period of no light and quantified dark respiration (*R*d; μmol m-2 s-1), again using a Li-COR LI-6800 under the same relative humidity and temperature settings as the *A*area/*C*i curve with incoming radiation set to 0 μmol m-2 s-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, weighed, and ground until homogenized. Specific leaf area (*SLA*; 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 then calculated by dividing *N*mass by *SLA*, 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). For each curve fit, we included triose phosphate utilization (TPU) limitation avoid underestimating *V*cmax and *J*max (Gregory et al., 2021). We also determined kinetic parameters and CO2 compensation points using leaf temperature and equations derived in Medlyn *et al.* (2002). Dark respiration measurements were included in all curve fits and were first standardized to 25C using a 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 by Heskel *et al.* (2016) for C3 herbaceous species, where *b* was set to 0.1271 and *c* was set to -0.00110.

For all *A*net/*C*i curve fits, we manually standardized *V*cmax and *J*max to25C using a modified Arrhenius equation (as in Kattge & Knorr, 2007):

(Eqn. 4)

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. 5a)

and:

(Eqn. 5b)

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 Corp., 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) and 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 gN-1 s-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; gN 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) approximately seven weeks after experiment initiation. We also harvested root nodules when present. Leaf areas of all harvested leaves were measured using an XX. Total leaf area (*T*L; cm2) was calculated as the sum of all leaf areas, including the leaf area of 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 (*T*bio; g) was calculated as the sum of dry leaf, stem, root, and root nodule biomass. We also quantified carbon and nitrogen content through elemental combustion (Costech-4010, Costech, Inc., Valencia, CA, USA) of each respective organ type using subsamples of ground and homogenized organ tissue.

We determined structural carbon costs to acquire nitrogen following the approach explained in Perkowski *et al.* (2021). Structural carbon costs to acquire nitrogen were calculated as the ratio of total belowground carbon biomass to whole plant nitrogen biomass. Belowground carbon biomass 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. Similarly, whole plant nitrogen biomass was calculated by multiplying the nitrogen content of leaves, stems, roots, and root nodules by biomass of each respective organ type, then taking the sum of nitrogen biomass of each organ type. This calculation only quantifies plant structural carbon costs and does not include any additional carbon costs associated with respiration, exudation, or turnover. An explicit explanation of the limitations of this calculation can be found in Perkowski *et al.* (2021).

*Statistical analyses*

We built a series of linear mixed-effects models to investigate the impacts of soil nitrogen fertilization and inoculation status on *G. max* leaf photosynthesis, tradeoffs between nitrogen and water use, and whole plant growth. All models included soil nitrogen fertilization, inoculation status, and interactions between soil nitrogen fertilization and inoculation status 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 status on *N*area, *SLA*, *N*mass, *A*net, *V*cmax25, *J*max25, *J*max25:*V*cmax25, *R*d25, *R*d25:*V*cmax25, *L*area, *L*mass, *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 and visual assessments of residual distributions (i.e. a histogram) to determine whether linear mixed-effects models satisfied residual normality assumptions. All models satisfied residual normality assumptions except *J*max25:*V*cmax25, *R*d25, *iWUE*, *N*area:*g*s, *V*cmax:*g*s, *N*cost, *C*bg, and *T*L, root nodule biomass: root biomass, and root nodule biomass (Shapiro-Wilk: p<0.05 in all cases). We attempted to satisfy residual normality assumptions for these dependent variables by first 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 *J*max25:*V*cmax25, *R*d25, *iWUE*, *N*area:*g*s, *V*cmax:*g*s, *N*cost, *C*bg, and *T*L. We 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. Finally, we 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 4.1.1 (R Core Team, 2021). All acronyms and acronym descriptions used in this paper are summarized in Table 1.

**Table 1** Trait acronym descriptions and their units

|  |  |  |
| --- | --- | --- |
| **Acronym** | **Units** | **Acronym description** |
| *A*net | μmol m-2 s-1 | net photosynthesis |
| *C*bg | g C | belowground carbon biomass |
| *C*i: *C*a | unitless | intercellular CO2: atmospheric CO2 |
| *g*s | mol m-2 s-1 | stomatal conductance |
| *iWUE* | μmol CO2 mol-1 H2O | intrinsic water-use efficiency |
| *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 |
| *N*cost | g C g-1 N | structural carbon costs to acquire nitrogen |
| *N*mass | g N g-1 biomass | leaf nitrogen content |
| *N*wp | g N | whole plant nitrogen biomass |
| *PNUE* | µmol CO2 g-1 N s-1 | photosynthetic nitrogen-use efficiency |
| Rbio | g biomass | root biomass |
| *R*d25 | μmol CO2 m-2 s-1 | dark respiration, standardized to 25°C |
| *R*d25:*V*cmax25 | unitless | dark respiration per maximum Rubisco carboxylation rate; standardized to 25°C |
| RNbio | g biomass | root nodule biomass |
| RNbio:Rbio | unitless | root nodule biomass: root biomass |
| *SLA* | cm2 g-1 | specific leaf area |
| *T*bio | g biomass | total plant biomass |
| *T*la | cm2 | total leaf area |
| *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 |

**Results**

*Leaf nitrogen allocation*

*N*area and *N*mass were both driven by an interaction between inoculation status and nitrogen fertilization (Table 2; Figs. 1A-B). This interaction indicated that inoculation only increased *N*area and *N*mass under the low soil nitrogen treatment (Tukey: p<0.001 in both cases), with no difference between inoculation treatments under the high soil nitrogen treatment (*N*area Tukey: p=0.623; *N*mass Tukey: p=0.941). Increasing soil nitrogen fertilization generally increased *N*area and *N*mass regardless of inoculation (Table 2; Figs. 1A-B). Specific leaf area increased with inoculation and marginally increased with increasing soil nitrogen fertilization, with no observable interaction between fertilization and inoculation (Table 2; Fig. 1C).

**Table 2** Analysis of variance results exploring effect of nitrogen fertilization, inoculation with *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation status 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 status 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 status 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*. Boxplot indicates the median, first quartile, and third quartile of the data, while whiskers represent the furthest data point that is not more than 1.5 times the inner quartile range. Remaining points outside the whiskers represent outliers that are not statistically different from the rest of the sampling population. Compact lettering above each boxplot indicates treatment combinations that are statistically different from each other, which were determined through Tukey’s tests (Tukey: p<0.050).

*Leaf photosynthesis and gas exchange*

Soil nitrogen fertilization generally decreased *A*net, *V*cmax25, and *J*max25 (Table 3; Fig. 2A-C). There was no effect of inoculation with *B. japonicum* or any interaction between fertilization and inoculation on *A*net, *V*cmax25, and *J*max25 (Table 3; Fig. 2A-C). However, *J*max25: *V*cmax25 was driven by a weak interaction between soil nitrogen fertilization and inoculation. This interaction indicated a negative effect of increasing soil nitrogen fertilization on *J*max25: *V*cmax25 in non-inoculated individuals (Tukey: p=0.008), with no observable effect in inoculated individuals (Tukey: p=0.967).

Interestingly, *R*d25 was determined through a weak interaction between nitrogen fertilization and inoculation (Table 3; Fig. 2D). This interaction indicated a positive effect of soil nitrogen fertilization on *R*d25 in inoculated individuals (Tukey: p=0.004), but not in non-inoculated individuals (Tukey: p=0.956). These patterns were also observed in *R*d25:*V*cmax25, where a marginal interaction between nitrogen fertilization and inoculation status indicated a positive effect of increasing soil nitrogen fertilization on *R*d25:*V*cmax25 in inoculated individuals (Tukey: p=0.009), but not non-inoculated individuals (Tukey: p=0.952).

Increasing soil nitrogen fertilization generally decreased *g*s and the *C*i: *C*a (Table 3). There was no effect of inoculation or any observable interaction between fertilization and inoculation status 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 status 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 | 14.81 | **<0.001** | 4.79 | **0.029** | 8.08 | **0.004** | 7.22 | **0.007** | 8.61 | **0.003** |
| Inoculation (I) | 1 | 1.59 | 0.207 | 0.48 | 0.488 | 0.75 | 0.387 | 1.05 | 0.307 | 1.51 | 0.219 |
| N\*I | 1 | 0.25 | 0.618 | 0.92 | 0.338 | 2.37 | 0.123 | 4.13 | **0.042** | 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 | 7.56 | **0.006** | 23.72 | **<0.001** | 4.06 | **0.043** |  |  |  |  |
| Inoculation (I) | 1 | 0.07 | 0.797 | 0.41 | 0.522 | 0.09 | 0.762 |  |  |  |  |
| N\*I | 1 | 3.71 | *0.054* | 0.06 | 0.804 | 0.56 | 0.452 |  |  |  |  |

\*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 status 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 status 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*. Boxplot indicates the median, first quartile, and third quartile of the data, while whiskers represent the furthest data point that is not more than 1.5 times the inner quartile range. Remaining points outside the whiskers represent outliers that are not statistically different from the rest of the sampling population. Compact lettering above each boxplot indicates treatment combinations that are statistically different from each other, which were determined through Tukey’s tests (Tukey: p<0.050).

*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 inoculation marginally decreased *PNUE* in the low soil nitrogen fertilization treatment (Tukey: p=0.071) but had no effect in the high soil nitrogen fertilization treatment (Tukey: p=0.611). There was also a strong negative effect of soil nitrogen fertilization on *PNUE* regardless of inoculation treatment (Table 3; Fig. 3A).

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

Increasing nitrogen fertilization generally increased *N*area:*g*s (Table 4; Fig 3C) and *V*cmax:*g*s (Table 4; Fig 3D). There was no effect of inoculation or any interaction between fertilization and inoculation (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 status 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 | 82.31 | **<0.001** | 7.06 | **0.008** | 46.17 | **<0.001** | 9.35 | **0.002** |
| Inoculation (I) | 1 | 0.81 | 0.369 | 0.04 | 0.837 | 0.00 | 0.924 | 0.35 | 0.555 |
| N\*I | 1 | 6.97 | **0.008** | 0.09 | 0.758 | 1.38 | 0.241 | 0.09 | 0.764 |

\*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**

**Chart, box and whisker chart

Description automatically generated**

**Figure 3** Effects of soil nitrogen fertilization and inoculation status 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 status 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*. Boxplot indicates the median, first quartile, and third quartile of the data, while whiskers represent the furthest data point that is not more than 1.5 times the inner quartile range. Remaining points outside the whiskers represent outliers that are not statistically different from the rest of the sampling population. Compact lettering above each boxplot indicates treatment combinations that are statistically different from each other, which were determined through Tukey’s tests (Tukey: p<0.050).

*Whole plant processes*

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, while nitrogen fertilization generally decreased structural carbon costs to acquire nitrogen, inoculation only decreased structural carbon costs to acquire nitrogen in the low nitrogen fertilization treatment (Tukey: p<0.001). There was no difference in structural carbon costs to acquire nitrogen between inoculation treatments in the high nitrogen fertilization treatment (Tukey: p=0.597).

Soil nitrogen fertilization had no effect on belowground carbon biomass (numerator of carbon cost to acquire nitrogen calculation; Table 5; Fig. 4B). There was also no observable interaction between soil nitrogen fertilization and inoculation, although there was a weak inoculation effect that indicated a positive effect of inoculation on belowground carbon biomass (Table 5; Fig. 4B).

Whole plant nitrogen biomass was driven by a strong interaction between fertilization and inoculation (Table 5; Fig. 5C). This interaction indicated that, while nitrogen fertilization generally increased whole plant nitrogen biomass, inoculation only increased *N*wp in the low nitrogen fertilization treatment (Tukey: p<0.001). There was no difference in whole plant nitrogen biomass between inoculation treatments in the high nitrogen fertilization treatment (Tukey: p=0.873).

Total leaf area was similarly driven by a strong interaction between nitrogen fertilization and inoculation (Table 5; Fig. 5A). This interaction indicated that, while nitrogen fertilization generally increased total leaf area, inoculation only increased total leaf area in the low nitrogen fertilization treatment (Tukey: p<0.001). There was no difference in total leaf area between inoculation treatments under high soil nitrogen (Tukey: p=0.631).

Increasing nitrogen fertilization generally increased whole plant biomass, with no inoculation or observable interaction between fertilization and inoculation (Table 5; Fig. 5B).

**Table 5** Analysis of variance results exploring effect of soil nitrogen fertilization, inoculation with *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation status on carbon costs to acquire nitrogen and whole plant growth\*

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | ***N*cost** | | ***C*bg** | | ***N*wp** | | ***T*la** | | ***T*bio** | |
|  | 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 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  |  | **RNbio:Rbio** | | **RNbio** | | **Rbio** | |  | |  | |
|  | 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. Key: *N*cost=structural carbon costs to acquire nitrogen; *N*wp=whole plant nitrogen biomass; *C*bg=belowground carbon biomass, *T*la=total leaf area; *T*bio=total biomass; RNbio:Rbio=root nodule biomass: root biomass; RNbio=root nodule biomass; Rbio=root biomass

**Figure 4**

**Chart, box and whisker chart

Description automatically generated**

**Figure 4** Effects of soil nitrogen fertilization and inoculation status on *G. max* structural carbon costs to acquire nitrogen (panel A), belowground carbon biomass (panel B), and whole plant nitrogen biomass (panel C). Soil nitrogen fertilization is represented categorically on the x-axis, while inoculation status 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*. Boxplot indicates the median, first quartile, and third quartile of the data, while whiskers represent the furthest data point that is not more than 1.5 times the inner quartile range. Remaining points outside the whiskers represent outliers that are not statistically different from the rest of the sampling population. Compact lettering above each boxplot indicates treatment combinations that are statistically different from each other, which were determined through Tukey’s tests (Tukey: p<0.050)

**Figure 5**

**Chart, box and whisker chart

Description automatically generated**

**Figure 5** Effects of soil nitrogen fertilization and inoculation status 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 status 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*. Boxplot indicates the median, first quartile, and third quartile of the data, while whiskers represent the furthest data point that is not more than 1.5 times the inner quartile range. Remaining points outside the whiskers represent outliers that are not statistically different from the rest of the sampling population. Compact lettering above each boxplot indicates treatment combinations that are statistically different from each other, which were determined through Tukey’s tests (Tukey: p<0.050).

*Plant investment in nitrogen fixation*

Soil nitrogen fertilization had no effect on root nodule biomass: root biomass, root nodule biomass, and root biomass (Table 5; Fig. 6A-C). Inoculation generally increased root nodule biomass: root biomass, root nodule biomass, and had a marginal negative effect on root biomass. There was no observable interaction between fertilization and inoculation on root nodule biomass: root biomass, root nodule biomass, and root biomass (Table 5).

**Figure 6**

**Chart, box and whisker chart

Description automatically generated**

**Figure 6** Effects of soil nitrogen fertilization and inoculation status 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 status 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*. Boxplot indicates the median, first quartile, and third quartile of the data, while whiskers represent the furthest data point that is not more than 1.5 times the inner quartile range. Remaining points outside the whiskers represent outliers that are not statistically different from the rest of the sampling population. Compact lettering above each boxplot indicates treatment combinations that are statistically different from each other, which were determined through Tukey’s tests (Tukey: p<0.050)

**Discussion**

In this study, we grew *G. max* under two soil nitrogen fertilization treatments and two inoculation treatments levels in a full factorial greenhouse experiment. We used this experiment to better understand how nutrient availability and acquisition strategy modifies leaf water-nitrogen economics and tradeoffs between leaf and whole plant nutrient allocation. In support of our first hypothesis, increasing soil nitrogen fertilization increased total leaf area and whole plant growth, while inoculation increased total leaf area only in the low soil nitrogen fertilization treatment. In support of our second hypothesis, increasing soil nitrogen fertilization increased leaf nitrogen per stomatal conductance, decreased photosynthetic nitrogen-use efficiency, and increased intrinsic water-use efficiency. Structural carbon costs to acquire nitrogen data suggests that these patterns may have been driven by a reduction in costs of acquiring nitrogen relative to water with increasing nitrogen fertilization. However, while inoculation tended to increase leaf nitrogen allocation and decrease photosynthetic nitrogen-use efficiency under low soil nitrogen fertilization, there was no effect of inoculation on stomatal conductance, intrinsic water-use efficiency, or leaf nitrogen allocation per stomatal conductance regardless of soil nitrogen fertilization level despite apparent reductions in structural carbon costs to acquire nitrogen at low soil nitrogen. These findings broadly support nitrogen-water tradeoffs expected from theory in response to soil nitrogen availability. These findings also indicate that nitrogen fixation increased allocation to whole plant processes, which may have reduced the net effect of nitrogen availability on tradeoffs between nitrogen and water use

*Inoculation reduces the positive effect of soil nitrogen availability on whole plant processes*

Total leaf area was driven by a strong interaction between soil nitrogen fertilization and inoculation. This interaction suggested that inoculation decreased the positive effect of soil nitrogen fertilization on total leaf area, indexed by larger total leaf area for inoculated individuals in the low soil nitrogen treatment that was no longer apparent in the high soil nitrogen treatment. Interestingly, we observed no such interaction for whole plant growth, where increasing soil nitrogen fertilization increased whole plant growth with no overall inoculation effect. The stimulation in total leaf area for inoculated individuals under low soil nitrogen was accompanied by a stimulation in leaf dark respiration, leaf dark respiration per maximum Rubisco carboxylation rate and specific leaf area. Together, these results could indicate compensation for increased costs associated with maintaining an association with symbiotic nitrogen-fixing bacteria.

*Soil nitrogen fertilization and inoculation modifies tradeoffs between nitrogen and water use*

Photosynthetic least-cost theory predicts that plants should respond to increased nutrient availability by increasing leaf nitrogen allocation and decreasing stomatal conductance, leading to a reduction in photosynthetic nitrogen-use efficiency and stimulation in water-use efficiency (Prentice et al., 2014; Wright et al., 2003). Our results support these outcomes, indicating a stimulation in leaf nitrogen allocation and reduction in stomatal conductance that corresponded with a reduction in photosynthetic nitrogen-use efficiency and stimulation in intrinsic water-use efficiency. Further corroborating the theory, we also observed a stimulation in leaf nitrogen allocation and *V*cmax per stomatal conductance with increasing soil nitrogen fertilization (Figs. 4A-D). These findings support patterns observed in Perkowski et al. (in prep), who show a stimulation in leaf nitrogen allocation per stomatal conductance with increasing soil nitrogen fertilization. However, Perkowski et al. (in prep) show limited stomatal responses to soil nitrogen availability and inferred that this was due to the lack of water limitation in the study system. Our results show here a strong stomatal response to soil nitrogen fertilization, which could indicate that stomatal responses to fertilization may be dependent on growth form, growth habit, and life stage.

Interestingly, inoculation had limited effects on leaf water-nitrogen use tradeoffs. While a stimulation in leaf nitrogen allocation led to a reduction in photosynthetic nitrogen use efficiency for inoculated individuals growing under low soil nitrogen, there was no individual or interactive effect of inoculation on stomatal conductance or intrinsic water use efficiency. Additionally, there was no observable effect of inoculation on leaf nitrogen per stomatal conductance or *V*cmax per stomatal conductance. These patterns suggest that inoculation does not necessarily modify leaf nitrogen-water use tradeoffs, although could allow individuals to hedge bets against dry growing conditions. These results also indicate that a reduction in photosynthetic nitrogen-use efficiency may be driven by a lack of nutrient limitation, as nitrogen is less limited in the environment through nitrogen fixation than more finite pools of nitrogen in the soil. Thus, leaf nutrient demand to build and maintain photosynthetic machinery may have already been met, which might explain the increase in total leaf area with inoculation.

*Integrating leaf and whole plant processes into a single framework*

There is a need to integrate leaf and whole plant processes into least-cost frameworks.

*Study limitations*

This study does have 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 hardly equipped to address any of the nonlinearities of this response. 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 species and a single inoculation species. While this did allow us to isolate mechanisms that drive leaf water-nitrogen responses to soil nutrients and inoculation independent of phylogeny or genetic diversity, future work should consider conducting similar experiments using a suite of diverse, albeit phylogenetically related legumes, 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*

In summary, increasing soil nitrogen fertilization increased leaf nitrogen allocation per stomatal conductance through an increase in leaf nitrogen allocation with no change in stomatal conductance. These patterns corresponded with a decrease in photosynthetic nitrogen-use efficiency and increase in intrinsic water-use efficiency with increasing fertilization. Interestingly, these nitrogen-water use tradeoffs occurred alongside an increase in total leaf area and whole plant biomass, indicating no apparent tradeoff between leaf and whole plant allocation decisions. The stimulation in leaf nitrogen allocation per stomatal conductance occurred alongside a reduction in structural carbon costs to acquire nitrogen, which could infer that these tradeoffs were driven by a reduction in the cost of acquiring nitrogen relative to water. Inoculation with *B. japonicum* only increased leaf nitrogen allocation and decreased photosynthetic nitrogen-use efficiency under low soil nitrogen, which supports the common observation that advantages of nitrogen fixation may only be apparent under low soil nutrient environments due to high energetic costs of nitrogen fixation. Despite this, there was no inoculation effect on leaf nitrogen per stomatal conductance under low fertilization. These results deserve to be investigated again using a larger suite of soil fertilization treatments and more than a single species or inoculation type to determine whether these patterns are generalizable across species capable of forming associations with symbiotic nitrogen-fixing bacteria.

**Acknowledgements**

**Author contributions**

EAP coordinated all leaf physiological measurements, conducted data analysis, wrote the first draft of the manuscript, and

**Data Availability Statement**

All statistical analyses and plots were created in R version 4.1.1. All R code and data for this manuscript are available in a GitHub repository at <insert URL here> (<insert DOI from Zenodo here>).

**References**

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Duursma, R. (2015). Plantecophys - An R package for analyzing and modelling leaf gas exchange data. *PLos ONE*, *10*(11), e0143346. https://doi.org/10.1371/journal.pone.0143346>

Farquhar, G. D., von Caemmerer, S., & Berry, J. A. (1980). A biochemical model of photosynthetic CO*2* assimilation in leaves of C3 species. *Planta*, *149*(1), 78–90. https://doi.org/10.1007/BF00386231

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (Third edit). Sage. https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Gregory, L. M., McClain, A. M., Kramer, D. M., Pardo, J. D., Smith, K. E., Tessmer, O. L., Walker, B. J., Ziccardi, L. G., & Sharkey, T. D. (2021). The triose phosphate utilization limitation of photosynthetic rate: Out of global models but important for leaf models. *Plant, Cell & Environment*, *44*(10), 3223–3226. https://doi.org/10.1111/pce.14153

Heskel, M. A., O’Sullivan, O. S., Reich, P. B., Tjoelker, M. G., Weerasinghe, K. W. L. K., Penillard, A., Egerton, J. J. G., Creek, D., Bloomfield, K. J., Xiang, J., Sinca, F., Stangl, Z. R., Martinez-de la Torre, A., Griffin, K. L., Huntingford, C., Hurry, V., Meir, P., Turnbull, M. H., & Atkin, O. K. (2016). Convergence in the temperature response of leaf respiration across biomes and plant functional types. *Proceedings of the National Academy of Sciences*, *113*(14), 3832–3837. https://doi.org/10.1073/pnas.1520282113

Hoagland, D. R., & Arnon, D. I. (1950). The water-culture method for growing plants without soil. *California Agricultural Experiment Station: 347*, *347*(2), 1–32.

Katabuchi, M. (2015). LeafArea: An R package for rapid digital analysis of leaf area. *Ecological Research*, *30*(6), 1073–1077.

Kattge, J., & Knorr, W. (2007). Temperature acclimation in a biochemical model of photosynthesis: a reanalysis of data from 36 species. *Plant, Cell & Environment*, *30*(9), 1176–1190. https://doi.org/10.1111/j.1365-3040.2007.01690.x

Kenward, M. G., & Roger, J. H. (1997). Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics*, *53*(3), 983. https://doi.org/10.2307/2533558

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

Medlyn, B. E., Dreyer, E., Ellsworth, D. S., Forstreuter, M., Harley, P. C., Kirschbaum, M. U. F., Le Roux, X., Montpied, P., Strassemeyer, J., Walcroft, A., Wang, K., & Loustau, D. (2002). Temperature response of parameters of a biochemically based model of photosynthesis. II. A review of experimental data. *Plant, Cell & Environment*, *25*(9), 1167–1179. https://doi.org/10.1046/j.1365-3040.2002.00891.x

O’Sullivan, O. S., Weerasinghe, K. W. L. K., Evans, J. R., Egerton, J. J. G., Tjoelker, M. G., & Atkin, O. K. (2013). High-resolution temperature responses of leaf respiration in snow gum (*Eucalyptus pauciflora*) reveal high-temperature limits to respiratory function. *Plant, Cell & Environment*, *36*(7), 1268–1284. https://doi.org/10.1111/pce.12057

Perkowski, E. A., Waring, E. F., & Smith, N. G. (2021). Root mass carbon costs to acquire nitrogen are determined by nitrogen and light availability in two species with different nitrogen acquisition strategies. *Journal of Experimental Botany*, *72*(15), 5766–5776. https://doi.org/10.1093/jxb/erab253

Poorter, H., Bühler, J., Van Dusschoten, D., Climent, J., & Postma, J. A. (2012). Pot size matters: A meta-analysis of the effects of rooting volume on plant growth. *Functional Plant Biology*, *39*(11), 839–850. https://doi.org/10.1071/FP12049

Prentice, I. C., Dong, N., Gleason, S. M., Maire, V., & Wright, I. J. (2014). Balancing the costs of carbon gain and water transport: testing a new theoretical framework for plant functional ecology. *Ecology Letters*, *17*(1), 82–91. https://doi.org/10.1111/ele.12211

R Core Team. (2021). *R: A language and environment for statistical computing* (4.1.1). R Foundation for Statistical Computing. https://www.r-project.org/

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. https://doi.org/10.1038/nmeth.2089

Wright, I. J., Reich, P. B., & Westoby, M. (2003). Least-cost input mixtures of water and nitrogen for photosynthesis. *The American Naturalist*, *161*(1), 98–111. https://doi.org/0003-0147/2003/16101-010387