**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:**

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**Manuscript compilation details**

**Abstract:**

**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**

**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. We also observed 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) and the ratio of *R*d25 to *V*cmax25 (*R*d25: *V*cmax25).

*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 XXX. 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 *A*net, *V*cmax25, *J*max25, *J*max25:*V*cmax25, *R*d25, *R*d25:*V*cmax25, *N*area, *SLA*, *N*mass, *L*area, *L*mass, *g*s, χ, *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).

**Results**

*Leaf nitrogen allocation*

*N*area and *N*mass were both driven by an interaction between inoculation status and nitrogen fertilization (Table 1; Figs. 1A-B). This interaction indicated that inoculation with *B. japonicum* 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). Soil nitrogen fertilization generally increased *N*area and *N*mass when averaged across inoculation treatments (Table 1; Figs. 1A-B). Interestingly, nitrogen fertilization had no individual or interactive effect on specific leaf area, but there was a positive effect of inoculation with *B. japonicum* (Table 1; Fig. 1C).

**Table 1** 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**

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**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 2; Fig. 2A-C). In all cases, there was no effect of inoculation with *B. japonicum* or any interaction between fertilization or inoculation status (Table 2; Fig. 2A-C). However, *J*max25: *V*cmax25 was driven by a weak interaction between soil nitrogen fertilization and inoculation status. This interaction indicated that the general negative effect of increasing soil nitrogen fertilization on *J*max25: *V*cmax25 was stronger in non-inoculated than inoculated individuals. In fact, there was no effect of soil nitrogen fertilization on *J*max25: *V*cmax25 between inoculated individuals.

Interestingly, *R*d25 was determined through a weak interaction between nitrogen fertilization and inoculation with *B. japonicum* (Table 2; Fig. 2D). This interaction indicated that inoculation with *B. japonicum* generally increased the general positive effect of soil nitrogen fertilization on *R*d25 (Table 2; Fig. 2D). In fact, the positive effect of soil nitrogen fertilization on *R*d25 was almost entirely driven by individuals inoculated with *B. japonicum*, as there was no statistical difference between soil nitrogen fertilization treatments between 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 drove an increase in the positive effect of soil nitrogen fertilization on *R*d25:*V*cmax25 in inoculated individuals, but not non-inoculated individuals.

Increasing soil nitrogen fertilization generally increased *g*s (Table 2). There was no effect of inoculation with *B*. *japonicum* or interaction between fertilization and inoculation status on *g*s (Table 2).

**Table 2** 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 | 0.34 | 2.37 | 0.123 | 4.13 | **0.042** | 4.34 | **0.037** |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  | | ***R*d25:*V*cmax25** | | ***g*s** | |  | |  | |  | |
|  | df | χ2 | *p* | χ2 | *p* |  |  |  |  |  |  |
| N fertilization (N) | 1 | 7.56 | **0.006** | 23.72 | **<0.001** |  |  |  |  |  |  |
| Inoculation (I) | 1 | 0.07 | 0.797 | 0.41 | 0.522 |  |  |  |  |  |  |
| N\*I | 1 | 3.71 | *0.054* | 0.06 | 0.804 |  |  |  |  |  |  |

\*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; *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 2**

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**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 with *B. japonicum* (Table 3; Fig. 3A). This interaction indicated that inoculation with *B. japonicum* generally decreased the negative effect of increasing nitrogen fertilization on PNUE. Specifically, inoculated individuals grown under high soil nitrogen (marginal mean ± SE; 8.94±0.61) had 29.3% lower PNUE than inoculated individuals grown under high soil nitrogen (12.64±0.59; Tukey: p), while non-inoculated individuals grown under high soil nitrogen (7.94±0.59) had 45.9% lower PNUE than non-inoculated individuals grown under low soil nitrogen (14.68±0.61; Tukey: p).

Increasing nitrogen fertilization generally increased iWUE (Table 3; Fig. 3B). There was no effect of inoculation with *B. japonicum* or interactive effect of fertilization and inoculation status on iWUE.

Increasing nitrogen fertilization also generally increased *N*area:*g*s (Table 3; Fig 3C), a pattern driven by a stimulation in *N*area (Table 1; Fig 1A) and reduction in *g*s (Table 2) with increasing fertilization. Similarly, nitrogen fertilization also generally increased *V*cmax25:*g*s (Table 3; Fig 3D), a pattern driven by a stronger reduction in *g*s than *V*cmax25 (Table 2; Fig. 2C) with increasing soil fertilization. There was no effect of inoculation with *B. japonicum* or interactive effect of fertilization and inoculation status on *N*area:*g*s or *V*cmax25:*g*s (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 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**

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**Figure 2** 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*

*N*cost was driven by a strong interaction between nitrogen fertilization and inoculation with *B. japonicum*. This interaction indicated that, while nitrogen fertilization generally decreased *N*cost, inoculation with *B. japonicum* only decreased *N*cost in the low nitrogen fertilization treatment. There was no statistical difference in *N*cost between inoculation treatments in the high nitrogen fertilization treatment (Tukey: p=), and no *N*cost difference between inoculated individuals under the low soil nitrogen treatment and inoculated (Tukey: p=) or non-inoculated (Tukey: p=) individuals growing in the high soil nitrogen treatment.

Effects of soil nitrogen fertilization and inoculation with *B. japonicum* were driven by *N*wp – the denominator of the carbon cost to acquire nitrogen calculation. *N*wp was driven by a strong interaction between fertilization and inoculation. This interaction indicated that, while nitrogen fertilization generally increased *N*wp, inoculation with *B. japonicum* only increased *N*wp in the low nitrogen fertilization treatment. There was no statistical difference in *N*wp between inoculation treatments in the high nitrogen fertilization treatment (Tukey: p=).

There was no effect of soil nitrogen fertilization or an interactive effect of fertilization and inoculation on *C*bg (Table 2). However, there was a weak inoculation effect that indicated a positive effect of inoculation with *B. japonicum* on *C*bg.

*T*LA was similarly driven by a strong interaction between nitrogen fertilization and inoculation with *B. japonicum*. This interaction indicated that, while nitrogen fertilization generally increased *T*LA, inoculation with with *B. japonicum* only increased *T*LA in the low nitrogen fertilization treatment (Tukey: p). There was no difference between inoculation treatments under high soil nitrogen (Tukey: p).

Increasing nitrogen fertilization generally increased *T*bio, with no inoculation or interactive effect between fertilization and inoculation status.

**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 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 |

\*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

**Figure 4**

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**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**

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**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).

**Discussion**

**References**

**Bates D, Mächler M, Bolker B, Walker S**. **2015**. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**: 1–48.

**Duursma R**. **2015**. Plantecophys - An R package for analyzing and modelling leaf gas exchange data. *PLos ONE* **10**: e0143346.

**Farquhar GD, von Caemmerer S, Berry JA**. **1980**. A biochemical model of photosynthetic CO*2* assimilation in leaves of C3 species. *Planta* **149**: 78–90.

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

**Gregory LM, McClain AM, Kramer DM, Pardo JD, Smith KE, Tessmer OL, Walker BJ, Ziccardi LG, Sharkey TD**. **2021**. The triose phosphate utilization limitation of photosynthetic rate: Out of global models but important for leaf models. *Plant, Cell & Environment* **44**: 3223–3226.

**Heskel MA, O’Sullivan OS, Reich PB, Tjoelker MG, Weerasinghe KWLK, Penillard A, Egerton JJG, Creek D, Bloomfield KJ, Xiang J, *et al.*** **2016**. Convergence in the temperature response of leaf respiration across biomes and plant functional types. *Proceedings of the National Academy of Sciences* **113**: 3832–3837.

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

**Katabuchi M**. **2015**. LeafArea: An R package for rapid digital analysis of leaf area. *Ecological Research* **30**: 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**: 1176–1190.

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

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

**Medlyn BE, Dreyer E, Ellsworth D, Forstreuter M, Harley PC, Kirschbaum MUF, Le Roux X, Montpied P, Strassemeyer J, Walcroft A, *et al.*** **2002**. Temperature response of parameters of a biochemically based model of photosynthesis. II. A review of experimental data. *Plant, Cell & Environment* **25**: 1167–1179.

**O’Sullivan OS, Weerasinghe KWLK, Evans JR, Egerton JJG, Tjoelker MG, Atkin OK**. **2013**. High-resolution temperature responses of leaf respiration in snow gum (*Eucalyptus pauciflora*) reveal high-temperature limits to respiratory function. *Plant, Cell & Environment* **36**: 1268–1284.

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

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

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

**Schneider CA, Rasband WS, Eliceiri KW**. **2012**. NIH Image to ImageJ: 25 years of image analysis. *Nature methods* **9**: 671–675.