**Title**: Inoculation with *Bradyrhizobium japonicum* elicits stronger whole plant responses to soil nitrogen fertilization than leaf photosynthesis: the need to incorporate acquisition strategy and whole plant traits in photosynthetic least-cost frameworks

**Running Head:** Soil N and inoculation increases whole plant growth at expense of leaf physiology

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**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**

Relationships between soil nutrient availability, leaf nutrient allocation, and photosynthetic capacity are commonly observed. However, recent work suggests leaf nutrient allocation and photosynthetic capacity can also be predicted via aboveground climate or through interactions between soil nutrient availability and climate. Photosynthetic least cost theory provides a framework for understanding the effects of climate and soils on leaf nutrient allocation and photosynthetic capacity. The theory predicts that an increase in soil nutrient availability will decrease the cost of nutrient use relative to water, which should increase water-use efficiency, decrease nitrogen-use efficiency, and increase leaf nitrogen allocation per stomatal conductance. Empirical evidence for these patterns is sparse, and further experimentation is needed to test underlying assumptions of this theoretical framework. We measured leaf and whole plant traits of *G. max* under two soil nitrogen fertilization treatments both with and without inoculation with *Bradyrhizobium japonicum*. We found that soil nitrogen fertilization decreased photosynthetic nitrogen-use efficiency, increased intrinsic water-use efficiency, and increased leaf nitrogen allocation per stomatal conductance, supporting patterns expected from theory. We also found that soil nitrogen fertilization had a strong positive effect on total leaf area and whole plant biomass, which may have dampened the effect of these treatments on leaf nitrogen-water use tradeoffs. We found no effect of inoculation on leaf nitrogen-water use tradeoffs; however, did observe a strong effect of inoculation on structural carbon costs to acquire nitrogen, total leaf area, and leaf nitrogen allocation under low soil nitrogen environments. These findings indicate that soil nitrogen availability is more important in determining leaf nitrogen-water use tradeoffs. They also indicate that associations with symbiotic nitrogen-fixing bacteria do not modify nitrogen-water use tradeoffs but may instead contribute to a stimulation in whole plant photosynthesis through an increase in total leaf area.

**Keywords**

**Introduction**

Photosynthesis is a process in terrestrial systems where carbon and nutrient biogeochemical cycles are closely coupled. Terrestrial plants fix carbon dioxide from the atmosphere into simple sugars using enzymes that have high nitrogen requirements to build and maintain, such as Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Evans, 1989; Evans & Seemann, 1989; Walker et al., 2014). As terrestrial photosynthesis is also the largest carbon flux between the atmosphere and biosphere (IPCC, 2013), terrestrial biosphere models are sensitive to the formulation of photosynthetic processes and the underlying biogeochemical cycles that regulate photosynthesis (Bonan et al., 2011; Davies-Barnard et al., 2020; Friedlingstein et al., 2014; Hungate et al., 2003; Smith et al., 2016). It is therefore critical that these models include reliable, robust, and realistic formulations of photosynthesis and the biogeochemical cycles that regulate photosynthesis, especially under future environmental scenarios (Oreskes et al., 1994; Prentice et al., 2015).

Current generation terrestrial biosphere models predict leaf level photosynthesis using parameters assigned by plant functional type through relationships between soil nutrient availability, leaf nutrient allocation, and photosynthetic capacity (Rogers, 2014; Rogers et al., 2017; Smith & Dukes, 2013). These models predict leaf nutrient allocation from soil nutrient availability based on the assumption that increasing soil nutrient availability generally increases leaf nutrient allocation (Firn et al., 2019; Li et al., 2020; Liang et al., 2020), which often corresponds with increases in photosynthetic capacity (Li et al., 2020; Liang et al., 2020). However, recent work suggests that leaf nutrient allocation and photosynthetic capacity can also be predicted by aboveground climate, leaf traits, or other edaphic characteristics such as soil pH (Dong et al., 2017, 2020; Maire et al., 2015; Paillassa et al., 2020; Smith et al., 2019). These studies suggest that leaf nutrient allocation and photosynthetic capacity may instead be driven by leaf demand to build and maintain photosynthetic machinery or whole plant demand to build and maintain structures that support whole plant growth.

Photosynthetic least-cost theory provides a framework for understanding how climatic factors, edaphic factors, or other leaf traits might predict leaf nutrient allocation and photosynthetic capacity. First principles of the theory suggest that plants can acclimate to a given environment by maximizing photosynthetic carbon uptake at the lowest summed cost of water and nitrogen use (Prentice et al., 2014; Wright et al., 2003). The theory predicts that costs of water and nutrient use are substitutable, such that plants can maintain the lowest summed cost of water and nitrogen use, and therefore their maximal photosynthetic carbon assimilation rate, by sacrificing less efficient use of a relatively more abundant and less costly resource to use in exchange for more efficient use of a relatively less abundant and more costly resource to use (Wright et al., 2003). For example, all else equal, plants should respond to an increase in soil nitrogen availability by sacrificing less efficient nitrogen use for more efficient water use, a response that would be achieved by maintaining leaf net photosynthesis rates with greater leaf nitrogen allocation and decreased stomatal conductance (Prentice et al., 2014). A few recent global environmental gradient analyses support these nitrogen-water tradeoffs in response to shifts in soil nutrient availability (Maire et al., 2015; Paillassa et al., 2020); however, more work needs to be done to understand how generalizable these patterns are across different spatiotemporal scales, species, or ecosystem types.

Importantly, patterns expected from photosynthetic least-cost theory likely depend on whole plant responses to changing environments. Soil nutrient availability, particularly soil nitrogen availability, has been shown to exert stronger effects on whole plant growth and total leaf area than leaf photosynthesis, photosynthetic capacity, leaf nitrogen allocation, and water use efficiency (LeBauer & Treseder, 2008; Liang et al., 2020). The stronger whole plant response to soil nutrient availability may be explained through multiple nutrient limitation, which limits net primary productivity at global and regional scales (Fay et al., 2015; LeBauer & Treseder, 2008; Wieder et al., 2015). In such cases where net primary productivity is limited by soil nutrient availability, or if individuals have high resource requirements for growth (e.g., juvenile individuals or annual species), nutrient demand to build and maintain structures that support whole plant growth and primary productivity may be higher than nutrient demand to build and maintain photosynthetic enzymes in a single leaf. Thus, high whole plant nutrient demand may dampen or eliminate leaf responses to soil nutrient availability. No studies that investigate patterns expected from photosynthetic least-cost theory have considered whole plant responses to soil nutrient availability in their analyses, which could explain any patterns that deviate from those expected from theory.

Finally, leaf and whole plant responses to soil nutrient availability may depend on a species’ dominant mode of nutrient acquisition (or “nutrient acquisition strategy”). This is because the costs of nutrient use that are included in photosynthetic least-cost frameworks implicitly include costs of nutrient acquisition, which have been shown to vary between species with different nutrient acquisition strategies (Brzostek et al., 2014; Perkowski et al., 2021; Terrer et al., 2018). As a result, a species’ nutrient acquisition strategy should influence the cost of using nitrogen relative to water (Bialic‐Murphy et al., 2021; Paillassa et al., 2020), or could modify any tradeoffs between leaf and whole plant nutrient demand. In a given environment, species with low costs of nutrient acquisition should exhibit a larger difference in the cost of using nitrogen relative to water compared to species with higher costs of nutrient acquisition. These patterns should result in stronger nitrogen-water use tradeoffs in species that have lower costs of nutrient acquisition, and weaker nitrogen-water use tradeoffs in species that have higher costs of nutrient acquisition.

Effects of species acquisition strategy may be may be context dependent on soil nutrient availability, as costs of nutrient acquisition via direct uptake should decrease with increasing nutrient availability (Perkowski et al., 2021), and may cause a shift in the dominant mode of nutrient acquisition if the cost of nutrient acquisition via direct uptake becomes less costly than a previous dominant mode of nutrient acquisition. This may be especially true in species who form associations with facultative symbiotic nitrogen-fixing bacteria, where species should shift from acquiring nitrogen via nitrogen fixation to direct uptake once costs associated with nitrogen fixation exceed costs associated with direct uptake (Perkowski et al., 2021; Rastetter et al., 2001). Furthermore, as soil nutrient availability may also modify tradeoffs between leaf or whole plant nutrient allocation, species with low costs of nutrient acquisition may more easily satisfy whole plant nutrient demand, so we might again expect stronger nitrogen-water use tradeoffs in species with low costs of nutrient acquisition relative to species with higher costs of nutrient acquisition. To date, no study has directly investigated the effect of acquisition strategy on tradeoffs between nitrogen and water use while integrating whole plant traits across different soil nutrient thresholds.

In this study, we grew *G. max* grown under two soil nitrogen fertilization treatments and two inoculation treatments levels in a full factorial greenhouse experiment. We used this experiment to test the following hypotheses:

1. Soil nitrogen fertilization will increase whole plant growth through an increase in total leaf area, which will increase whole plant growth. We predict that inoculation will also increase total leaf area and whole plant growth, but only under the low soil nitrogen treatment due to a reduction in nodulation with increasing fertilization
2. Soil nitrogen fertilization will increase leaf nitrogen per stomatal conductance through an increase in leaf nitrogen allocation and reduction in stomatal conductance. We predict 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 also predict that inoculation will increase the magnitude of nitrogen-water tradeoffs but will only be observed in the low soil nitrogen treatment.
3. Effects of soil nitrogen fertilization and inoculation on leaf nitrogen-water use tradeoffs will depend on whole plant allocation decisions. We predict that weak growth 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 predicted 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. 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 prior to planting 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 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 no light 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 Medlyn *et al.* (2002). Dark respiration measurements were included in all curve fits. As leaf temperature was different between the dark respiration and *A*net/*C*i curves, we standardized all dark respiration measurements to the temperature of the *A*net/*C*i curves using a log-polynomial approach explained in Heskel *et al.* (2016), where:

(Eqn. 1)

*R*T is the temperature standardized respiration rate, *T* is the temperature in which a given respiration rate is being standardized, 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. We also set *T*ref to the air temperature of each response curve for all *A*net/*C*i curve fits. Finally, we standardized dark respiration to 25C (*R*d25; μmol m-2 s-1), using the same coefficients explained above with *T*ref set to 25C.

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. 2)

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. 3a)

and:

(Eqn. 3b)

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 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 through elemental combustion (Costech-4010, Costech, Inc., Valencia, CA, USA) of each respective organ type 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 that are 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 *J*max25:*V*cmax25, *R*d25, *iWUE*, *N*area:*g*s, *V*cmax:*g*s, *N*cost, *C*bg, and total leaf area, 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 total leaf area, 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 |
| *C*bg | g C | - |
| *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 |
| Nodule biomass: root biomass | unitless | - |
| *N*wp | g N | whole plant nitrogen biomass |
| *PNUE* | µmol CO2 g-1 N s-1 | photosynthetic nitrogen-use efficiency |
| *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 |
| Root biomass | g | - |
| Root nodule biomass | g | - |
| Specific leaf area | cm2 g-1 | - |
| 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 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 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*

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 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 *C*i: *C*a (Table 3). There was no effect of inoculation or any observable interaction between 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 | 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

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**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 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 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 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).

*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 increasing nitrogen fertilization generally decreased carbon costs to acquire nitrogen, inoculation only decreased carbon costs to acquire nitrogen in the low nitrogen fertilization treatment (Tukey: p<0.001). There was no difference in 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 (denominator of carbon cost to acquire nitrogen calculation; Table 5; Fig. 5C). This interaction indicated that, while increasing nitrogen fertilization generally increased whole plant nitrogen biomass, inoculation only increased whole plant nitrogen biomass 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 increasing 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 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

Description automatically generated**

**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*wp”; 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).

**Figure 5**

**Chart, box and whisker chart

Description automatically generated**

**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*

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 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 can acclimate to growing conditions by minimizing the 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 depend on whole plant nutrient demand (LeBauer & Treseder, 2008; Liang et al., 2020).

To understand how acquisition strategy and whole plant nutrient demand might modify these patterns, we grew *G. max* under two soil nitrogen fertilization treatments and two inoculation treatments levels in a full factorial greenhouse experiment. In support of our first hypothesis, increasing soil nitrogen fertilization increased total leaf area and whole plant growth. However, 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. 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, supporting our third and final hypothesis.

*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.

*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 slight 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.

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.

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

The land surface component of Earth system models commonly predict photosynthesis based on empirically observed relationships between soil nitrogen availability, leaf nitrogen allocation, and photosynthetic capacity (Rogers, 2014; Rogers et al., 2017). However, recent work suggests that leaf nitrogen allocation (Dong et al., 2017, 2020) and photosynthetic capacity (Peng et al., 2021; Smith et al., 2019) can each be predicted independent of soil nitrogen availability, indicating a possible decoupling of these relationships that require further inquiry. Our results show that soil nitrogen fertilization increased leaf nitrogen allocation, a pattern that did not coincide with an increase in leaf photosynthesis or photosynthetic capacity. Instead, soil nitrogen fertilization increased total leaf area, which effectively increased whole plant photosynthesis through a greater area of total light interception and stomata per plant.

*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 not equipped to address any of the 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 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 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 made revisions based on collaborator and reviewer feedback. JT designed and carried out the experiment, and contributed to manuscript revisions. HG assisted with post-experiment harvest and also contributed to manuscript revisions. NGS oversaw experiment progress, assisted with the post-experiment harvest, and contributed to manuscript revisions. All authors supported publication of this manuscript to XX.

**Data Availability Statement**

All statistical analyses and plots were created in R version 4.2.0. All R code and data for this manuscript are available in a GitHub repository at <insert URL here> (<insert DOI from Zenodo here>).

**References**

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

Bialic‐Murphy, L., Smith, N. G., Voothuluru, P., McElderry, R. M., Roche, M. D., Cassidy, S. T., Kivlin, S. N., & Kalisz, S. (2021). Invasion‐induced root–fungal disruptions alter plant water and nitrogen economies. *Ecology Letters*, *24*(6), 1145–1156. https://doi.org/10.1111/ele.13724

Bonan, G. B., Lawrence, P. J., Oleson, K. W., Levis, S., Jung, M., Reichstein, M., Lawrence, D. M., & Swenson, S. C. (2011). Improving canopy processes in the Community Land Model version 4 (CLM4) using global flux fields empirically inferred from FLUXNET data. *Journal of Geophysical Research*, *116*(G2), G02014. https://doi.org/10.1029/2010JG001593

Brzostek, E. R., Fisher, J. B., & Phillips, R. P. (2014). Modeling the carbon cost of plant nitrogen acquisition: Mycorrhizal trade-offs and multipath resistance uptake improve predictions of retranslocation. *Journal of Geophysical Research: Biogeosciences*, *119*, 1684–1697. https://doi.org/10.1002/2014JG002660.Received

Davies-Barnard, T., Meyerholt, J., Zaehle, S., Friedlingstein, P., Brovkin, V., Fan, Y., Fisher, R. A., Jones, C. D., Lee, H., Peano, D., Smith, B., Wårlind, D., & Wiltshire, A. J. (2020). Nitrogen cycling in CMIP6 land surface models: progress and limitations. *Biogeosciences*, *17*(20), 5129–5148. https://doi.org/10.5194/bg-17-5129-2020

Dong, N., Prentice, I. C., Evans, B. J., Caddy-Retalic, S., Lowe, A. J., & Wright, I. J. (2017). Leaf nitrogen from first principles: field evidence for adaptive variation with climate. *Biogeosciences*, *14*(2), 481–495. https://doi.org/10.5194/bg-14-481-2017

Dong, N., Prentice, I. C., Wright, I. J., Evans, B. J., Togashi, H. F., Caddy-Retalic, S., McInerney, F. A., Sparrow, B., Leitch, E., & Lowe, A. J. (2020). Components of leaf‐trait variation along environmental gradients. *New Phytologist*, *228*(1), 82–94. https://doi.org/10.1111/nph.16558

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>

Evans, J. R. (1989). Photosynthesis and nitrogen relationships in leaves of C3 plants. *Oecologia*, *78*(1), 9–19. https://doi.org/10.1007/BF00377192

Evans, J. R., & Seemann, J. R. (1989). The allocation of protein nitrogen in the photosynthetic apparatus: costs, consequences, and control. *Photosynthesis*, *8*, 183–205.

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

Fay, P. A., Prober, S. M., Harpole, W. S., Knops, J. M. H., Bakker, J. D., Borer, E. T., Lind, E. M., MacDougall, A. S., Seabloom, E. W., Wragg, P. D., Adler, P. B., Blumenthal, D. M., Buckley, Y. M., Chu, C., Cleland, E. E., Collins, S. L., Davies, K. F., Du, G., Feng, X., … Yang, L. H. (2015). Grassland productivity limited by multiple nutrients. *Nature Plants*, *1*(7), 15080. https://doi.org/10.1038/nplants.2015.80

Firn, J., McGree, J. M., Harvey, E., Flores-Moreno, H., Schütz, M., Buckley, Y. M., Borer, E. T., Seabloom, E. W., La Pierre, K. J., MacDougall, A. S., Prober, S. M., Stevens, C. J., Sullivan, L. L., Porter, E., Ladouceur, E., Allen, C., Moromizato, K. H., Morgan, J. W., Harpole, W. S., … Risch, A. C. (2019). Leaf nutrients, not specific leaf area, are consistent indicators of elevated nutrient inputs. *Nature Ecology & Evolution*, *3*(3), 400–406. https://doi.org/10.1038/s41559-018-0790-1

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

Friedlingstein, P., Meinshausen, M., Arora, V. K., Jones, C. D., Anav, A., Liddicoat, S. K., & Knutti, R. (2014). Uncertainties in CMIP5 climate projections due to carbon cycle feedbacks. *Journal of Climate*, *27*(2), 511–526. https://doi.org/10.1175/JCLI-D-12-00579.1

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.

Hungate, B. A., Dukes, J. S., Shaw, M. R., Luo, Y., & Field, C. B. (2003). Nitrogen and climate change. *Science*, *302*(5650), 1512–1513. https://doi.org/10.1126/science.1091390

IPCC. (2013). *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*.

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

LeBauer, D. S., & Treseder, K. (2008). Nitrogen limitation of net primary productivity. *Ecology*, *89*(2), 371–379. https://doi.org/10.1890/06-2057.1

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

Li, W., Zhang, H., Huang, G., Liu, R., Wu, H., Zhao, C., & McDowell, N. G. (2020). Effects of nitrogen enrichment on tree carbon allocation: A global synthesis. *Global Ecology and Biogeography*, *29*(3), 573–589. https://doi.org/10.1111/geb.13042

Liang, X., Zhang, T., Lu, X., Ellsworth, D. S., BassiriRad, H., You, C., Wang, D., He, P., Deng, Q., Liu, H., Mo, J., & Ye, Q. (2020). Global response patterns of plant photosynthesis to nitrogen addition: A meta‐analysis. *Global Change Biology*, *26*(6), 3585–3600. https://doi.org/10.1111/gcb.15071

Maire, V., Wright, I. J., Prentice, I. C., Batjes, N. H., Bhaskar, R., van Bodegom, P. M., Cornwell, W. K., Ellsworth, D. S., Niinemets, Ü., Ordonez, A., Reich, P. B., & Santiago, L. S. (2015). Global effects of soil and climate on leaf photosynthetic traits and rates. *Global Ecology and Biogeography*, *24*(6), 706–717. https://doi.org/10.1111/geb.12296

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

Oreskes, N., Shrader-Frechette, K., & Belitz, K. (1994). Verification , Validation , and Confirmation of Numerical Models in the Earth Sciences. *Science*, *263*(5147), 641–646. https://www.jstor.org/stable/2883078

Paillassa, J., Wright, I. J., Prentice, I. C., Pepin, S., Smith, N. G., Ethier, G., Westerband, A. C., Lamarque, L. J., Wang, H., Cornwell, W. K., & Maire, V. (2020). When and where soil is important to modify the carbon and water economy of leaves. *New Phytologist*, *228*(1), 121–135. https://doi.org/10.1111/nph.16702

Peng, Y., Bloomfield, K. J., Cernusak, L. A., Domingues, T. F., & Prentice, I. C. (2021). Global climate and nutrient controls of photosynthetic capacity. *Communications Biology*, *4*(1), 462. https://doi.org/10.1038/s42003-021-01985-7

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

Prentice, I. C., Liang, X., Medlyn, B. E., & Wang, Y.-P. (2015). Reliable, robust and realistic: The three R’s of next-generation land-surface modelling. *Atmospheric Chemistry and Physics*, *15*, 5987–6005. https://doi.org/10.5194/acp-15-5987-2015

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/

Rastetter, E. B., Vitousek, P. M., Field, C. B., Shaver, G. R., Herbert, D., & Ågren, G. I. (2001). Resource optimization and symbiotic nitrogen fixation. *Ecosystems*, *4*(4), 369–388. https://doi.org/10.1007/s10021-001-0018-z

Rogers, A. (2014). The use and misuse of Vc,max in Earth System Models. *Photosynthesis Research*, *119*(1–2), 15–29. https://doi.org/10.1007/s11120-013-9818-1

Rogers, A., Medlyn, B. E., Dukes, J. S., Bonan, G., von Caemmerer, S., Dietze, M. C., Kattge, J., Leakey, A. D. B., Mercado, L. M., Niinemets, Ü., Prentice, I. C., Serbin, S. P., Sitch, S., Way, D. A., & Zaehle, S. (2017). A roadmap for improving the representation of photosynthesis in Earth system models. *New Phytologist*, *213*(1), 22–42. https://doi.org/10.1111/nph.14283

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

Smith, N. G., & Dukes, J. S. (2013). Plant respiration and photosynthesis in global-scale models: Incorporating acclimation to temperature and CO2. *Global Change Biology*, *19*(1), 45–63. https://doi.org/10.1111/j.1365-2486.2012.02797.x

Smith, N. G., Keenan, T. F., Prentice, I. C., Wang, H., Wright, I. J., Niinemets, Ü., Crous, K. Y., Domingues, T. F., Guerrieri, R., Ishida, F. oko, Kattge, J., Kruger, E. L., Maire, V., Rogers, A., Serbin, S. P., Tarvainen, L., Togashi, H. F., Townsend, P. A., Wang, M., … Zhou, S.-X. (2019). Global photosynthetic capacity is optimized to the environment. *Ecology Letters*, *22*(3), 506–517. https://doi.org/10.1111/ele.13210

Smith, N. G., Malyshev, S. L., Shevliakova, E., Kattge, J., & Dukes, J. S. (2016). Foliar temperature acclimation reduces simulated carbon sensitivity to climate. *Nature Climate Change*, *6*(4), 407–411. https://doi.org/10.1038/nclimate2878

Terrer, C., Vicca, S., Stocker, B. D., Hungate, B. A., Phillips, R. P., Reich, P. B., Finzi, A. C., & Prentice, I. C. (2018). Ecosystem responses to elevated <scp>CO</scp> 2 governed by plant–soil interactions and the cost of nitrogen acquisition. *New Phytologist*, *217*(2), 507–522. https://doi.org/10.1111/nph.14872

Walker, A. P., Beckerman, A. P., Gu, L., Kattge, J., Cernusak, L. A., Domingues, T. F., Scales, J. C., Wohlfahrt, G., Wullschleger, S. D., & Woodward, F. I. (2014). The relationship of leaf photosynthetic traits - Vcmax and Jmax - to leaf nitrogen, leaf phosphorus, and specific leaf area: a meta-analysis and modeling study. *Ecology and Evolution*, *4*(16), 3218–3235. https://doi.org/10.1002/ece3.1173

Wieder, W. R., Cleveland, C. C., Smith, W. K., & Todd-Brown, K. (2015). Future productivity and carbon storage limited by terrestrial nutrient availability. *Nature Geoscience*, *8*(6), 441–444. https://doi.org/10.1038/ngeo2413

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