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**Abstract**

Plants acclimate to increasing CO2 by reducing leaf nutrient allocation and photosynthetic capacity at the leaf level, a response that often occurs alongside growth stimulation at the whole plant level. Nutrient limitation has been hypothesized to be the primary driver of plant acclimation to CO2, as nutrient availability commonly limits primary productivity and may decrease with increasing CO2 over time. However, recent work leveraging photosynthetic least-cost theory indicates that these acclimation responses may instead be the result of optimal resource investment toward photosynthetic capacity, which maximizes nutrient allocation to whole plant growth. To understand whether nutrient limitation or optimal leaf resource investment controls plant acclimation to CO2 and how nutrient acquisition strategy modifies these responses, we grew soybean under two atmospheric CO2 levels, two inoculation treatments, and nine soil nitrogen fertilization treatments in a full factorial growth chamber experiment.

We found that …

These results suggest that XX is the dominant control of plant acclimation responses to CO2, providing important empirical data needed to refine our understanding of mechanisms driving plant acclimation to CO2.

**Keywords**

photosynthetic acclimation, soil nutrient availability, nutrient acquisition, global change

**Introduction**

Photosynthesis in terrestrial systems is constrained by ecosystem carbon and nutrient biogeochemical cycle dynamics (Hungate et al., 2003). Specifically, plants fix carbon dioxide from the atmosphere into simple sugars using enzymes, such as Ribulose-1,5-bisphosphate carboxylase/oxygenase (“Rubisco”), that have large nitrogen requirements to build and maintain (Evans, 1989). Recent photosynthetically derived carbon (“photosynthate”) can be accumulated as biomass (cite), lost as a substrate of plant respiration (Glover, 1973), or allocated belowground to acquire nutrients (cite). Belowground photosynthate can be used by plants to acquire nutrients either directly from the soil (cite), indirectly through root exudates that prime soil microbial communities and organic matter decomposition (Bengtson et al., 2012), or indirectly through symbioses with mycorrhizal fungi and/or symbiotic nitrogen-fixing bacteria (S. E. Smith & Read, 2008).

Anthropogenic activities have been the proximal cause of increasing atmospheric CO2 concentrations since the start of the Industrial Revolution in the mid 1700s. The Intergovernmental Panel on Climate Change suggests that atmospheric CO2 concentrations will continue to increase under business-as-normal emissions scenarios, with some scenarios suggesting that CO2 concentrations will exceed 1,000 ppm by 2100 (IPCC, 2013). Plant ecologists and physiologists have been long interested in understanding long-term effects of elevated CO2 on plant photosynthetic processes, where large swaths of studies report that increasing CO2 concentrations generally results in reductions in leaf nutrient allocation and photosynthetic capacity, a pattern that often corresponds with a stimulation in whole plant growth and net primary productivity (Ainsworth et al., 2002; Ainsworth & Rogers, 2007; Curtis, 1996; Makino, 2003; Morgan et al., 2004; Poorter et al., 2022; N. G. Smith & Dukes, 2013).

There are two conflicting hypotheses that explain the inverse leaf and whole plant acclimation responses to increasing CO2. Some have hypothesized that nutrient limitation may be the primary control of plant acclimation to CO2, as nutrient availability commonly limits primary productivity and may decrease over time in elevated CO2 environments (Fay et al., 2015; LeBauer & Treseder, 2008; Liang et al., 2016; Luo et al., 2004) through chronic stimulations in whole plant nutrient demand. The nutrient limitation hypothesis predicts that plants decrease leaf nutrient allocation and photosynthetic capacity as a direct response to progressive reductions in soil nutrient availability due to elevated CO2. The nutrient limitation hypothesis also predicts an acute stimulation in whole plant growth due to elevated CO2 that dampens over time because of progressive nutrient limitation.

An alternative hypothesis to the leaf response, based on photosynthetic least-cost theory (Prentice et al., 2014; Wright et al., 2003) suggests that plants growing under elevated CO2 environments instead downregulate nutrient allocation to Rubisco to optimize resource use efficiencies at the leaf level, which maximizes resource allocation to whole plant growth. Importantly, the nutrient limitation and least-cost hypotheses predict similar leaf acclimation responses to CO2, but result in different outcomes at the whole plant level.

Nutrient acquisition strategy, or the method in which plants acquire nutrients, may also impact how plants acclimate to CO2 (N. G. Smith & Keenan, 2020; Terrer et al., 2018). Plants acquire nutrients via direct uptake from their rooting systems or through symbiotic associations with mycorrhizal fungi or symbiotic nitrogen-fixing bacteria (S. E. Smith & Read, 2008). In plants that form associations with microbial symbionts, plants allocate recent photosynthate belowground in exchange for nutrients acquired by microbial symbionts. However, not all microbial symbioses require the same belowground carbon investments to exchange nutrients. Carbon costs to acquire nitrogen, or the amount of carbon plants allocate belowground per nitrogen acquired, vary across nutrient acquisition strategies and soil nutrient availability thresholds (Perkowski et al., 2021). Interestingly, a recent global meta-analysis indicates that carbon costs to acquire nitrogen may modify plant acclimation responses to CO2 (Terrer et al., 2016, 2018), although manipulation experiments that directly test the mechanisms driving these responses are rare.

In this study, I will investigate the influence of inoculation with symbiotic nitrogen-fixing bacteria and direct soil nutrient manipulation on soybean (*Glycine max* L.) acclimation responses to CO2. This experiment will determine whether nutrient limitation or optimal leaf resource investment is the primary driver of plant acclimation to CO2 and how nutrient acquisition strategy modifies these responses. I hypothesize that leaf acclimation to CO2 will be driven by optimal leaf resource investment, not nutrient limitation. Specifically, I predict that increasing CO2 will decrease stomatal conductance, leaf nutrient allocation, and photosynthesis independent of nutrient acquisition strategy or soil nutrient availability, which will maximize resource allocation to whole plant growth. While I do not expect that soil nutrients or acquisition strategy will modify leaf acclimation responses to CO2, I do expect that soil nutrient availability will increase the positive effect of CO2 on whole plant growth. I also predict that inoculation with nitrogen-fixing bacteria will increase whole plant growth responses to CO2. However, I only expect an inoculation effect in low soil nutrient environments, as inoculated individuals should shift away from nitrogen fixation and toward direct uptake with increasing soil nutrient availability (Perkowski et al., 2021; Rastetter et al., 2001).

**Methods**

*Seed treatments and experimental design*

*Glycine max* L. (Merr) seeds were planted in 144 6-liter surface sterilized pots (NS-600, Nursery Supplies, Orange, CA, USA) containing a steam-sterilized 70:30 v:v mix of *Sphagnum* peat moss (Premier Horticulture, Quakertown, PA, USA) to sand (fill in manufacturer info here). Before planting, all *G. max* seeds were surface sterilized in 2% sodium hypochlorite for 3 minutes, followed by three separate 3-minute washes with ultrapure water (MilliQ 7000; MilliporeSigma, Burlington, MA USA). A subset of surface sterilized seeds were also inoculated with *Bradyrhizobium japonicum* (Verdesian N-Dure™ Soybean, Cary, NC, USA) in a slurry following manufacturer recommendations (3.12 g inoculant and 241 g deionized water per 1 kg seed).

Seventy-two pots were randomly planted with seeds inoculated with *B.* *japonicum*, and the remaining 72 pots were planted with uninoculated seeds. Within each inoculation treatment, 36 pots were randomly placed in one of two atmospheric CO2 treatments (ambient and 1000 μmol mol-1 CO2). Finally, pots within each unique inoculation-by- CO2 treatment combination randomly received one of nine soil nitrogen fertilization treatments equivalent to 0, 35, 70, 105, 140, 210, 280, 350, or 630 ppm N. Nitrogen fertilization treatments were created using a modified Hoagland solution (Hoagland & Arnon, 1950), and were designed to keep concentrations of other macronutrients and micronutrients equivalent across treatments (Table S1) such that the only change across treatments was the nitrogen concentration. Fertilization treatments were applied twice per week in 150mL doses as topical agents to the soil surface throughout the duration of the experiment. All individuals were well watered to minimize water stress potential.

*Growth conditions*

This experiment was conducted using six Percival LED-41L2 growth chambers (Percival Scientific Inc., Perry, IA, USA) over two iterations due to chamber space limitation. The two iterations were conducted such that one iteration included all elevated CO2 pots and the second iteration included all ambient CO2 pots. Daytime growing conditions were simulated using a 16-hour photoperiod, with incoming light radiation set to a 1240±32 μmol m-2 s-1 daytime maximum, chamber temperature set to 25°C, and relative humidity set to 50%. The remaining 8 hours simulated nighttime growing conditions, with incoming light radiation set to 0 μmol m-2 s-1 and relative humidity set to 50%. Chambers varied in their capacity to maintain nighttime temperatures, which were originally set to maintain 17°C, but instead averaged XX ± °C across chambers throughout the experiment. To account for climatic differences between chambers, we moved pots between chambers daily throughout the entire experiment. Transitions between daytime and nighttime chamber conditions were by ramping temperature and incoming light radiation in 45-minute increments over a three-hour period (see Table S2). Because of this, chambers averaged 1049±27 μmol m-2 s-1 and XX ± °C during the daytime. Across all machines, CO2 concentrations for the ambient CO2 treatment averaged XX ± μmol mol-1 CO2, while the CO2 concentrations for the elevated CO2 treatment averaged XX ± μmol mol-1 CO2. All individuals grew under these treatment combinations and growing conditions for a seven-week growth period.

*Leaf gas exchange measurements*

Gas exchange measurements were collected on the most recent fully expanded leaf for all experimental pots on the seventh week of development. Specifically, we measured net photosynthesis (*A*net; μmol m-2s-1), stomatal conductance (*g*s; mol m-2s-1), and intercellular CO2 (*C*i; μmol mol-1) concentrations across a range of atmospheric CO2 concentrations (i.e. an *A*net/*C*i curve) using the Dynamic Assimilation Technique™. The Dynamic Assimilation Technique™ has been previously shown in *G. max* to correspond well with traditional steady-state CO2 response curves (Saathoff & Welles, 2021). *A*net/*C*i curves were generated using the split method, which measured *A*net, *g*s, and *C*i along a reference CO2 ramp down from 420 µmol mol-1 CO2 to 20 µmol mol-1 CO2, followed by a ramp up from 420 µmol mol-1 CO2 to 1620 µmol mol-1 CO2 after a 90-second wait period at 420 µmol mol-1 CO2. The ramp rate for each curve was set to 200 μmol mol-1 min-1, logging every five seconds, which generated 96 data points per response curve. All *A*net/*C*i curves were generated with the cuvette flow rate stabilized at 500 mol s-1, fan set to 10,000rpm, vapor pressure deficit set to 1.5 kPa, leaf temperature set to 25°C, and incoming light radiation set to 2000 μmol m-2 s-1.

After *A*net/*C*i curves were generated, we subjected individuals to at least a 30-minute period of darkness. Dark respiration (*R*d; μmol m-2 s-1) measurements were then collected on the same focal leaf used to generate *A*net/*C*i curves. Measurements were collected on a 5-second log interval for 60 seconds after stabilizing in a LI-6800 cuvette where flow rate was stabilized at 500 mol s-1, reference CO2 was set to 420 μmol mol-1, vapor pressure deficit was set to 1.5 kPa, leaf temperature was set to 25°C, and incoming light radiation was set to 0 μmol m-2 s-1. A single dark respiration value was determined for each focal leaf by calculating the absolute assimilation average across the logging interval.

*Leaf trait measurements*

At the end of the seventh week of the experiment, leaf trait measurements were collected on the same focal leaf used to generate *A*net/*C*i response curves. 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. Leaf mass per area (*M*area; g cm-2) was calculated as the ratio of dry leaf biomass to fresh leaf area. 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), and sent samples to the University of California-Davis Stable Isotope Facility to determine leaf δ13C and δ15N. Leaf nitrogen content per unit leaf area (*N*area; gN m-2) was calculated by multiplying *N*mass and *M*area.

We used leaf δ13C values to estimate the ratio of intercellular (*C*i) to extracellular (*C*a) CO2 (χ; Pa Pa-1) following the approach of Farquhar *et al.* (1989) described in Cernusak *et al.* (2013). While intercellular and extracellular CO2 concentrations were directly measured during each CO2 response curve, deriving χ from δ13C provides a more integrative estimate of the *C*i:*C*a over an individual leaf’s lifespan . We derived χ as:

(Eqn. 1)

Δ13C represents the relative difference between leaf δ13C (‰) and air δ13C (‰), and is calculated from the following equation:

(Eqn. 2)

where δ13Cair is assumed to be -8‰ (Farquhar et al., 1989; Keeling et al., 1979), *a* represents the fractionation between 12C and 13C due to diffusion in air, assumed to be 4.4‰, and *b* represents the fractionation caused by Rubisco carboxylation, assumed to be 27‰ (Farquhar et al., 1989).

*A*/*C*i *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). Triose phosphate utilization (TPU) limitation and dark respiration were included in all curve fits. We determined Michaelis-Menten coefficients for Rubisco affinity to CO2 (*K*c; μmol mol-1) and O2 (*K*o; mmol mol-1), and the CO2 compensation point *(Γ*\*; μmol mol-1) using leaf temperature and equations described in Medlyn et al. (2002) and derived in Bernacchi et al. (2001). Specifically, *K*c and *K*o were calculated as:

(Eqn. 3a)

and

(Eqn. 3b)

while *Γ*\* was calculated as:

(Eqn. 3c)

In all three equations, *T*k is the leaf temperature (in Kelvin) during each CO2 response curve and R is the universal gas constant (8.314 J mol-1 K-1).

*V*cmax, *J*max, and *R*d were estimated using measurements that were collected at a common leaf temperature (25°C). Therefore, there was no need to temperature standardize rate estimates. For clarity, we reference rate estimates from this point forward as *V*cmax25, *J*max25, and *R*d25.

*Stomatal limitation*

We quantified the extent by which stomatal conductance limited photosynthesis (*l*; unitless) following equations originally described in Farquhar & Sharkey (1982). Stomatal limitation is calculated as:

(Eqn. 6)

*A*net represents the net photosynthesis rate where reference CO2 was set to 420 μmol mol-1 CO2, while *A*mod represents the photosynthetic rate where *C*i = *C*a. *A*mod was calculated as:

(Eqn. 7)

*V*cmax represents the temperature unstandardized maximum rate of Rubisco carboxylation. We used the temperature unstandardized *V*cmax value because *A*net values were not standardized to 25°C. *R*d represents dark respiration, which was the dark respiration value we temperature standardized to the CO2 response curve fit. Γ\* (Pa) is the CO2 compensation point in the absence of dark respiration, while *K*m is the Michaelis-Menten coefficient for Rubisco-limited photosynthesis. *K*m was calculated as:

(Eqn. 8)

*K*c refers to the Michaelis-Menten coefficient for Rubisco affinity to CO2, *K*o refers to the Michaelis-Menten coefficient for Rubisco affinity to O2, and *O*i refers to leaf intercellular O2 concentrations. Γ\* and *K*m were standardized to the average temperature of each *A*net/*C*i curve using equations and parameters described in Bernacchi et al. (2001).

*Tradeoffs between nitrogen and water use*

Photosynthetic nitrogen use efficiency (*PNUE*; µmol CO2 mol N-1 s-1) was calculated by dividing *A*net measured at 420 μmol mol-1 CO2 by *N*area, where the numerator (gN) was converted to mol N by dividing by 14 gN mol-1 N. We used χ, mentioned above, to estimate water use efficiency. Tradeoffs between nitrogen and water use were determined by calculating the ratio of *N*area toχ (*N*area: χ; gN m-2) and *V*cmax25 to χ(*V*cmax: χ; μmol m-2s-1).

*Whole plant traits*

Fifty days after experiment initiation, we harvested all experimental individuals and separated biomass of each experimental individual into major organ types (leaves, stems, roots, and root nodules when present). 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, and included the focal leaf measured during the dynamic 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. Leaves and nodules were manually ground with a mortar and pestly, while stems and roots were mechanically ground by first passing material through a Wiley mill, then passing material through a MiniG tissue grinder () using vials equipped with steel balls. 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).

*Nitrogen fixation*

We calculated plant investments in nitrogen fixation as the ratio of root nodule biomass to root biomass, where increasing values indicate an increase in plant investments to nitrogen fixation (Dovrat et al., 2018, 2020; Perkowski et al., 2021). We also calculated the percent of leaf nitrogen acquired from the atmosphere (%Ndfa) using leaf δ15N and the following equation from Andrews et al. (2011):

(Eqn. 9)

where δ15Nreference refers to a reference plant that exclusively acquires nutrients via direct uptake, δ15Nsample refers to an individual’s leaf δ15N, and B refers to individuals that are entirely reliant on nitrogen fixation. Within each nitrogen fertilization treatment x CO2 treatment combination (n=18), we calculated the mean leaf δ15N for individuals growing in the non-inoculated treatment for δ15Nreference. Any individuals with visual confirmation of root nodule formation or nodule initiation were omitted from the calculation of δ15Nreference. Following recommendations from Andrews et al. (2011) we calculated B within each CO2 treatment by calculating the mean leaf δ15N of inoculated individuals that formed nodules. We did not calculate B within each unique soil nitrogen x CO2 treatment combination, as previous studies suggest decreased reliance on nitrogen fixation with increasing soil nitrogen availability (Perkowski et al., 2021). This approach for estimating nitrogen fixation standardizes values such that approaching 1 indicates increasing reliance on nitrogen fixation, while values that approach 0 indicate decreasing reliance on nitrogen fixation.

*Statistical analyses*

We built a series of linear multiple regression models to investigate the impacts of atmospheric CO2, soil nitrogen fertilization, and inoculation with *B. japonicum* on *G. max* leaf photosynthesis, tradeoffs between nitrogen and water use, whole plant growth, and reliance on nitrogen fixation. All models included CO2 treatment as a categorical coefficient, inoculation as a categorical coefficient, nitrogen fertilization as a continuous coefficient. Models also included interaction terms between all three fixed effects. Models with this independent structure were created for each of the following dependent variables: *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, 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, root biomass, and %N from the atmosphere.

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 [add traits here] (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[add traits here] and square root transformed [add traits here].

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).

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