

Drivers of plant nutrient acquisition and allocation strategies and their influence
on plant responses to environmental change

by

Evan A. Perkowski, B.S.

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Approved

Nicholas G. Smith, Ph.D.
Chair of Committee

Aimée T. Classen, Ph.D.

Natasja van Gestel, Ph.D.

Lindsey C. Slaughter, Ph.D.

Dylan W. Schwilk, Ph.D.

Mark Sheridan, Ph.D.
Dean of the Graduate School

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Abstract

Photosynthesis is the largest carbon flux between the atmosphere and is constrained by ecosystem carbon and nutrient cycle dynamics, which causes terrestrial biosphere models to be sensitive to the formulation of photosynthetic processes. Terrestrial biosphere models exhibit high divergence in simulated carbon and nitrogen fluxes under future environmental conditions, which may be due to uncertainty in the acclimation response of photosynthetic processes to environmental change. Photosynthetic least-cost theory provides a promising framework for understanding effects of climatic and edaphic factors on photosynthetic acclimation responses to changing environments. Yet, empirical tests of the theory are rare, limiting our ability to assess whether the theory is suitable for implementation in next-generation terrestrial biosphere models.

Here, I present four experiments designed to test assumptions of photosynthetic least-cost theory. Experiment chapters are flanked by a general introduction chapter and conclusions chapter. The first experimental chapter quantifies structural carbon costs to acquire nitrogen in *Glycine max* and *Gossypium hirsutum* grown under four nitrogen fertilization levels and four light availability levels in a full factorial greenhouse experiment. I find that carbon costs to acquire nitrogen in both species increase with increasing light availability and decrease with increasing fertilization, though responses to fertilization in *G. max* were markedly less than *G. hirsutum*. The second experimental chapter quantifies leaf nitrogen and photosynthetic traits in upper canopy leaves of deciduous trees growing in a 9-year nitrogen-by-sulfur field manipulation experiment. I find strong evi-

dence for nitrogen-water use tradeoffs with increasing soil nitrogen availability, evidenced through a strong negative relationship between leaf nitrogen content and leaf $C_i:C_a$ and stronger increase in leaf nitrogen content with increasing soil nitrogen availability than leaf $C_i:C_a$. The third experiment investigates variance in leaf nitrogen content across a climate and soil resource availability gradient in Texan grasslands, showing that effects of soil resource availability and climate on leaf nitrogen content are driven by changes in leaf $C_i:C_a$. Finally, the fourth experiment quantifies leaf and whole plant acclimation responses in *G. max* grown under two atmospheric CO₂ levels, with and without inoculation with *Bradyrhizobium japonicum*, and across nine nitrogen fertilization treatments in a full factorial growth chamber experiment. I find that leaf acclimation responses to CO₂ were independent of soil nitrogen fertilization or inoculation treatment, though stimulations in whole plant growth under elevated CO₂ were enhanced with increasing soil nitrogen fertilization and in inoculated pots under low nitrogen fertilization.

Experiments included in this dissertation provide consistent support for patterns expected from photosynthetic least-cost theory. The use of multiple experimental approaches allowed me to examine mechanisms driving patterns expected from the theory and investigate whether these patterns occur in the field across environmental gradients. Findings from these chapters challenge common paradigms in plant ecophysiology, providing empirical evidence suggesting that including photosynthetic least-cost frameworks in terrestrial biosphere models may improve the longstanding observed divergence in simulated outcomes across terrestrial biosphere model products.

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1 Chapter 1

2 Introduction

3 Photosynthesis represents the largest carbon flux between the atmosphere
4 and biosphere, and is regulated by complex ecosystem carbon and nutrient cy-
5 cles (Hungate et al. 2003; IPCC 2021). As a result, the inclusion of robust,
6 empirically tested representations of photosynthetic processes is critical in order
7 for terrestrial biosphere models to accurately and reliably simulate carbon and
8 nutrient fluxes between the atmosphere and terrestrial biosphere (Oreskes et al.
9 1994; Smith and Dukes 2013; Prentice et al. 2015; Wieder et al. 2015). Despite
10 evidence that the inclusion of coupled carbon and nutrient cycles can improve
11 model uncertainty, widespread divergence in predicted carbon and nutrient fluxes
12 is still apparent across model products (Friedlingstein et al. 2014; Arora et al.
13 2020; Davies-Barnard et al. 2020). Divergence in predicted carbon and nutrient
14 fluxes across terrestrial biosphere models may be due to an incomplete under-
15 standing of how plants acclimate to changing environments (Smith and Dukes
16 2013; Davies-Barnard et al. 2020), as terrestrial biosphere models are sensitive to
17 the formulation of photosynthetic processes (Bonan et al. 2011; Ziehn et al. 2011;
18 Booth et al. 2012; Smith et al. 2016; Smith et al. 2017; Rogers et al. 2017).

Many terrestrial biosphere models predict leaf-level photosynthesis through linear relationships between area-based leaf nitrogen content and the maximum rate of Ribulose-1,5-bisphosphate carboxylase/oxygenase (“Rubisco”), following the idea that large fractions of leaf nitrogen content are allocated to the construction and maintenance of Rubisco and other photosynthetic enzymes (Evans

24 1989). The inclusion of coupled carbon and nutrient cycles in terrestrial bio-
25 sphere models (Shi et al. 2016; Braghieri et al. 2022) allows for the prediction
26 of leaf nitrogen content through soil nitrogen availability, which causes models to
27 indirectly predict photosynthetic processes through shifts in soil nitrogen avail-
28 ability (Smith et al. 2014; Lawrence et al. 2019). While these patterns are
29 commonly observed in ecosystems globally (Brix 1971; Evans 1989; Firn et al.
30 2019; Liang et al. 2020), this formulation does not allow for the prediction of
31 leaf and whole plant acclimation responses to changing environments (Smith and
32 Dukes 2013; Rogers et al. 2017; Harrison et al. 2021), and suggests that constant
33 leaf nitrogen-photosynthesis relationships are ubiquitous across ecosystems.

34 Photosynthetic least-cost theory (Prentice et al. 2014; Wang et al. 2017;
35 Smith et al. 2019; Paillassa et al. 2020; Scott and Smith 2022; Harrison et al.
36 2021) provides a contemporary framework for predicting leaf and whole plant ac-
37 climation responses to environmental change. The theory, which unifies optimal
38 coordination (Chen et al. 1993; Maire et al. 2012) and least-cost (Wright et al.
39 2003) theories, posits that plants optimize photosynthetic processes by minimizing
40 the summed cost of nutrient and water use (i.e., β). The summed cost of nutrient
41 and water use is predicted to be positively correlated with the ratio of intercellular
42 CO₂ to atmospheric CO₂ (leaf $C_i:C_a$). Leaf $C_i:C_a$ is determined by factors that
43 influence leaf nutrient demand, such as CO₂, temperature, vapor pressure deficit,
44 and light availability (Prentice et al. 2014; Wang et al. 2017; Smith et al. 2019;
45 Stocker et al. 2020), and may change in response to changing edaphic charac-
46 teristics through changes in β (Paillassa et al. 2020). Photosynthetic processes
47 are optimized such that nutrients and water are allocated to photosynthetic en-

48 zymes to allow net photosynthesis rates to be equally co-limited by the maximum
49 rate of Rubisco carboxylation and the maximum rate of Ribulose-1,5-bisphosphate
50 (RuBP) regeneration (Chen et al. 1993; Maire et al. 2012). The theory indicates
51 that costs of nutrient and water use are substitutable such that, in a given en-
52 vironment, optimal photosynthesis rates can be achieved by sacrificing inefficient
53 use of a relatively more abundant (and less costly to acquire) resource for more
54 efficient use of a relatively less abundant (and more costly to acquire) resource.

55 Optimality models leveraging patterns expected from photosynthetic least-
56 cost theory have been developed for both C₃ (Wang et al. 2017; Smith et al. 2019;
57 Stocker et al. 2020) and more recently for C₄ species (Scott and Smith 2022).
58 Such models show broad agreement with patterns observed across environmental
59 gradients (Smith et al. 2019; Stocker et al. 2020; Paillassa et al. 2020; Querejeta
60 et al. 2022; Westerband et al. 2023), and are capable of reconciling dynamic
61 leaf nitrogen-photosynthesis relationships and acclimation responses to elevated
62 CO₂, temperature, light availability, and vapor pressure deficit (Dong et al. 2017;
63 Dong et al. 2020; Smith and Keenan 2020; Luo et al. 2021; Peng et al. 2021;
64 Dong et al. 2022; Dong et al. 2022; Querejeta et al. 2022; Westerband et al.
65 2023). Current versions of optimality models that invoke patterns expected from
66 photosynthetic least-cost theory hold β constant across growing environments.
67 As growing evidence suggests that costs of nutrient use change across resource
68 availability and climatic gradients in species with different nutrient acquisition
69 strategies (Fisher et al. 2010; Brzostek et al. 2014; Terrer et al. 2018; Allen et al.
70 2020), one might expect that β should dynamically change across environments
71 and in species with different nutrient acquisition strategies.

72 Despite recent recognition that patterns expected from photosynthetic
73 least-cost theory occur across broad environmental gradients, a limited number
74 of studies have investigated how β varies across edaphic and climatic gradients
75 and how variance in β might scale to influence leaf nutrient-water use tradeoffs
76 (Lavergne et al. 2020; Paillassa et al. 2020). Furthermore, no previous study has
77 investigated whether β varies in species with different nutrient acquisition strate-
78 gies, or if changes in β due to changes in edaphic characteristics scale to influence
79 leaf or whole plant acclimation responses to changing environments. The lack of
80 such studies provided motivation for the experimental chapters included in this
81 dissertation.

82 In this dissertation, I use a combination of greenhouse, field manipulation,
83 environmental gradient, and growth chamber experiments to quantify leaf and
84 whole plant acclimation responses across various climatic and edaphic conditions
85 and different nutrient acquisition strategies. Together, these experiments eval-
86 uated patterns expected from photosynthetic least-cost theory and test mechanisms
87 predicted to drive responses expected from theory. The empirical data collected
88 in these experiments provide important information needed to refine existing opti-
89 mality models that include photosynthetic least-cost frameworks, and could help
90 determine whether such models are suitable for implementing in next-generation
91 terrestrial biosphere models. While theory suggests that plants acclimate across
92 environments by minimizing the summed cost of nutrients relative to water, I chose
93 to isolate effects of soil nitrogen availability on costs of nitrogen acquisition rela-
94 tive to water for the sake of brevity. I acknowledge that patterns expected from
95 theory may be modified by other nutrients (e.g., phosphorus) or other edaphic

96 characteristics (Smith et al. 2019; Paillassa et al. 2020; Westerband et al. 2023),
97 and, though not included here, should also be investigated.

98 In the first experimental chapter, I re-analyze data from a greenhouse ex-
99 periment that grew *Glycine max* and *Gossypium hirsutum* seedlings under full-
100 factorial combinations of four light treatments and four fertilization treatments
101 to examine effects of nitrogen and light availability on structural carbon costs to
102 acquire nitrogen. In the second experimental chapter, I measure leaf physiological
103 traits in the upper canopy of mature trees growing in a 9-year nitrogen-by-pH
104 field manipulation experiment to assess whether changes in soil nitrogen availabil-
105 ity or soil pH modify nitrogen-water use trade-offs expected from photosynthetic
106 least-cost theory. The third experimental chapter leverages a broad precipitation
107 and soil nitrogen availability gradient in Texan grasslands to investigate primary
108 drivers of leaf nitrogen content. In the fourth experimental chapter, I use growth
109 chambers to quantify leaf and whole plant acclimation responses to CO₂ across
110 a soil nitrogen fertilization gradient, while also manipulating nutrient acquisition
111 strategy by controlling whether seedlings were able to form associations with sym-
112 biotic nitrogen-fixing bacteria.

113 Across experiments, I find strong and consistent support for patterns ex-
114 pected from photosynthetic least-cost theory, showing that shifts in edaphic char-
115 acteristics predictably alter β , and that shifts in β facilitate changes in leaf
116 nitrogen-water use tradeoffs and leaf nitrogen-photosynthesis relationships. I also
117 show that costs of nitrogen acquisition vary in species with different nitrogen
118 acquisition strategies. Finally, I show strong evidence suggesting that leaf accli-
119 mation responses to elevated CO₂ are decoupled from soil nitrogen availability and

120 inoculation with symbiotic nitrogen-fixing bacteria. It is my hope that these ex-
121 periments will encourage future iterations of optimality models that adopt photo-
122 synthetic least-cost frameworks to consider frameworks for implementing dynamic
123 β values across soil resource availability gradients and in species with different nu-
124 trient acquisition strategies.

125 The four experimental chapters included in this dissertation are presented
126 either as previously published journal articles or as manuscript drafts currently
127 in preparation for journal submission. Specifically, the first experimental chapter
128 was published in *Journal of Experimental Botany* in 2021 and the second chapter
129 is currently in review, while the third and fourth chapters are each in preparation
130 for journal submission. The dissertation concludes with a sixth chapter that sum-
131 marizes experiment findings, briefly synthesizes common themes observed across
132 experiments, and provides some suggestions for future experimentation.

133

Chapter 2

134

Structural carbon costs to acquire nitrogen are determined by
135 nitrogen and light availability in two species with different nitrogen
136 acquisition strategies

137 Perkowski EA, EF Waring, NG Smith, "Root mass carbon costs to acquire nitro-
138 gen are determined by nitrogen and light availability in two species with different
139 nitrogen acquisition strategies", *Journal of Experimental Botany*, 2021, Volume
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141 2.1 Introduction

142 Carbon and nitrogen cycles are tightly coupled in terrestrial ecosystems. This
143 tight coupling influences photosynthesis (Walker et al. 2014; Rogers et al. 2017),
144 net primary productivity (LeBauer and Treseder 2008; Thomas et al. 2013), de-
145 composition (Cornwell et al. 2008; Bonan et al. 2013; Sulman et al. 2019), and
146 plant resource competition (Gill and Finzi 2016; Xu-Ri and Prentice 2017). Ter-
147 restrial biosphere models are beginning to include connected carbon and nitrogen
148 cycles to improve the realism of their simulations (Fisher et al. 2010; Brzostek
149 et al. 2014; Wieder et al. 2015; Shi et al. 2016; Zhu et al. 2019). Simula-
150 tions from these models indicate that coupling carbon and nitrogen cycles can
151 drastically influence future biosphere-atmosphere feedbacks under global change,
152 such as elevated carbon dioxide or nitrogen deposition (Thornton et al. 2007;
153 Goll et al. 2012; Wieder et al. 2015; Wieder et al. 2019). Nonetheless, there
154 are still limitations in our quantitative understanding of connected carbon and
155 nitrogen dynamics (Thomas et al. 2015; Meyerholt et al. 2016; Rogers et al.
156 2017; Exbrayat et al. 2018; Shi et al. 2019), forcing models to make potentially
157 unreliable assumptions.

158 Plant nitrogen acquisition is a process in terrestrial ecosystems by which
159 carbon and nitrogen are tightly coupled (Vitousek and Howarth 1991; Delaire
160 et al. 2005; Brzostek et al. 2014). Plants must allocate photosynthetically de-
161 rived carbon belowground to produce and maintain root systems or exchange with
162 symbiotic soil microbes in order to acquire nitrogen (Högberg et al. 2008; Hög-
163 berg et al. 2010). Thus, plants have an inherent carbon cost associated with
164 acquiring nitrogen, which can include both direct energetic costs associated with
165 nitrogen acquisition and indirect costs associated with building structures that
166 support nitrogen acquisition (Gutschick 1981; Rastetter et al. 2001; Vitousek
167 et al. 2002; Menge et al. 2008). Model simulations (Fisher et al. 2010; Brzostek
168 et al. 2014; Shi et al. 2016; Allen et al. 2020) and meta-analyses (Terrer et al.
169 2018) suggest that these carbon costs vary between species, particularly those
170 with different nitrogen acquisition strategies. For example, simulations using iter-
171 ations of the Fixation and Uptake of Nitrogen (FUN) model indicate that species
172 that acquire nitrogen from non-symbiotic active uptake pathways (e.g. mass flow)
173 generally have larger carbon costs to acquire nitrogen than species that acquire
174 nitrogen through symbiotic associations with nitrogen-fixing bacteria (Brzostek
175 et al. 2014; Allen et al. 2020).

176 Carbon costs to acquire nitrogen likely vary in response to changes in soil
177 nitrogen availability. For example, if the primary mode of nitrogen acquisition
178 is through non-symbiotic active uptake, then nitrogen availability could decrease
179 carbon costs to acquire nitrogen as a result of increased per-root nitrogen up-
180 take (Franklin et al. 2009; Wang et al. 2018). However, if the primary mode of
181 nitrogen acquisition is through symbiotic active uptake, then nitrogen availabil-

182 ity may incur additional carbon costs to acquire nitrogen if it causes microbial
183 symbionts to shift toward parasitism along the parasitism–mutualism continuum
184 (Johnson et al. 1997; Hoek et al. 2016; Friel and Friesen 2019) or if it reduces
185 the nitrogen acquisition capacity of a microbial symbiont (van Diepen et al. 2007;
186 Soudzilovskaia et al. 2015; Muñoz et al. 2016). Species may respond to shifts in
187 soil nitrogen availability by switching their primary mode of nitrogen acquisition
188 to a strategy with lower carbon costs to acquire nitrogen in order to maximize
189 the magnitude of nitrogen acquired from a belowground carbon investment and
190 outcompete other individuals for soil resources (Rastetter et al. 2001; Menge et al.
191 2008).

192 Environmental conditions that affect demand to acquire nitrogen to sup-
193 port new and existing tissues could also be a source of variance in plant carbon
194 costs to acquire nitrogen. For example, an increase in plant nitrogen demand could
195 increase carbon costs to acquire nitrogen if this increases the carbon that must be
196 allocated belowground to acquire a proportional amount of nitrogen (Kulmatiski
197 et al. 2017; Noyce et al. 2019). This could be driven by a temporary state of
198 diminishing return associated with investing carbon toward building and main-
199 taining structures that are necessary to support enhanced nitrogen uptake, such
200 as fine roots (Matamala and Schlesinger 2000; Norby et al. 2004; Arndal et al.
201 2018), mycorrhizal hyphae (Saleh et al. 2020), or root nodules (Parvin et al.
202 2020). Alternatively, if the environmental factor that increases plant nitrogen de-
203 mand causes nitrogen to become more limiting in the system (e.g. atmospheric
204 CO₂) (Luo et al. 2004; LeBauer and Treseder 2008; Vitousek et al. 2010; Liang
205 et al. 2016), species might switch their primary mode of nitrogen acquisition to

206 a strategy with lower relative carbon costs to acquire nitrogen in order to gain a
207 competitive advantage over species with either different or more limited modes of
208 nitrogen acquisition (Ainsworth and Long 2005; Taylor and Menge 2018).

209 Using a plant economics approach, I examined the influence of plant ni-
210 trogen demand and soil nitrogen availability on plant carbon costs to acquire
211 nitrogen. This was done by growing a species capable of forming associations
212 with nitrogen-fixing bacteria (*Glycine max* L. (Merr)) and a species not capable
213 of forming these associations (*Gossypium hirsutum* L.) under four levels of light
214 availability (plant nitrogen demand proxy) and four levels of soil nitrogen fertil-
215 ization (soil nitrogen availability proxy) in a full-factorial, controlled greenhouse
216 experiment. I used this experimental set-up to test the following hypotheses:

- 217 1. An increase in plant nitrogen demand due to increasing light availability will
218 increase carbon costs to acquire nitrogen through a proportionally larger
219 increase in belowground carbon than whole-plant nitrogen acquisition. This
220 will be the result of an increased investment of carbon toward belowground
221 structures that support enhanced nitrogen uptake, but at a lower nitrogen
222 return.
- 223 2. An increase in soil nitrogen availability will decrease carbon costs to acquire
224 nitrogen as a result of increased per root nitrogen uptake in *G. hirsutum*.
225 However, soil nitrogen availability will not affect carbon costs to acquire
226 nitrogen in *G. max* because of the already high return of nitrogen supplied
227 through nitrogen fixation.

228 2.2 Methods

229 2.2.1 *Experiment setup*

230 *Gossypium hirsutum* and *G. max* were planted in individual 3 liter pots (NS-300; **231** Nursery Supplies, Orange, CA, USA) containing a 3:1 mix of unfertilized potting **232** mix (Sungro Sunshine Mix #2, Agawam, MA, USA) to native soil extracted from **233** an agricultural field most recently planted with *G. max* at the USDA-ARS Lab-**234** oratory in Lubbock, TX, USA (33.59°N, -101.90°W). The field soil was classified **235** as Amarillo fine sandy loam (75% sand, 10% silt, 15% clay). Upon planting, **236** all *G. max* pots were inoculated with *Bradyrhizobium japonicum* (Verdesian N-**237** Dure™ Soybean, Cary, NC, USA) to stimulate root nodulation. Individuals of **238** both species were grown under similar, unshaded, ambient greenhouse conditions **239** for 2 weeks to germinate and begin vegetative growth.

240 Three blocks were set up in the greenhouse, each containing four light **241** treatments created using shade cloth that reduced incoming radiation by either 0 **242** (full sun), 30, 50, or 80%. Two weeks post-germination, individuals were randomly **243** placed in the four light treatments in each block. Individuals received one of four **244** nitrogen fertilization doses as 100mL of a modified Hoagland solution (Hoagland **245** and Arnon 1950) equivalent to either 0, 70, 210, or 630 ppm N twice per week **246** within each light treatment. Nitrogen fertilization doses were received as topical **247** agents to the soil surface. Each Hoagland solution was modified to keep concen-**248** trations of other macro- and micronutrients equivalent (Table A1). Plants were **249** routinely well watered to eliminate water stress.

250 2.2.2 *Plant measurements and calculations*

251 Each individual was harvested after 5 weeks of treatment, and biomass was sepa-
252 rated by organ type (leaves, stems, and roots). Nodules on *G. max* roots were also
253 harvested. Except for the 0% shade cover and 630 ppm N treatment combination,
254 all treatment combinations in both species had lower average dry biomass:pot vol-
255 ume ratios than the 1:1 ratio recommended by Poorter et al. (2012) to minimize
256 the likelihood of pot volume-induced growth limitation (Table A2, A3; Fig. A1).

257 All harvested material was dried, weighed, and ground by organ type.
258 Carbon and nitrogen content (g g^{-1}) was determined by subsampling from ground
259 and homogenized biomass of each organ type using an elemental analyzer (Costech
260 4010; Costech, Inc., Valencia, CA, USA). I scaled these values to total leaf, stem,
261 and root carbon and nitrogen biomass (g) by multiplying dry biomass of each
262 organ type by carbon or nitrogen content of each corresponding organ type. Whole
263 plant nitrogen biomass (g) was calculated as the sum of total leaf (g), stem (g),
264 and root (g) nitrogen biomass. Root nodule carbon biomass was not included in
265 the calculation of root carbon biomass; however, relative plant investment toward
266 root or root nodule standing stock was estimated as the ratio of root biomass to
267 root nodule biomass (g g^{-1}), following similar metrics to those adopted by Dovrat
268 et al. (2018) and Dovrat et al. (2020).

269 Carbon costs to acquire nitrogen (N_{cost} ; gC gN^{-1}) were estimated as the
270 ratio of total root carbon biomass (C_{bg} ; gC) to whole-plant nitrogen biomass
271 (N_{wp} ; gN). This calculation quantifies the relationship between carbon spent on
272 nitrogen acquisition and whole plant nitrogen acquisition by using root carbon
273 biomass as a proxy for estimating the magnitude of carbon allocated toward ni-

274 trogen acquisition. This calculation therefore assumes that the magnitude of root
275 carbon standing stock is proportional to carbon transferred to root nodules or my-
276 corrhizae, or lost through root exudation or turnover. The assumption has been
277 supported in species that associate with ectomycorrhizal fungi (Hobbie 2006; Hob-
278 bie and Hobbie 2008), but is less clear in species that acquire nitrogen through
279 non-symbiotic active uptake or symbiotic nitrogen fixation. It is also unclear
280 whether relationships between root carbon standing stock and carbon transfer to
281 root nodules are similar in magnitude to carbon lost through exudation or when
282 allocated toward other active uptake pathways. Thus, because of the way mea-
283 surements were calculated, proximal values of carbon costs to acquire nitrogen are
284 underestimates.

285 2.2.3 *Statistical analyses*

286 I explored the effects of light and nitrogen availability on carbon costs to acquire
287 nitrogen using separate linear mixed-effects models for each species. Models in-
288 cluded shade cover, nitrogen fertilization, and interactions between shade cover
289 and nitrogen fertilization as continuous fixed effects, and also included block as a
290 random intercept term. Three separate models for each species were built with
291 this independent variable structure for three different dependent variables: (i)
292 carbon costs to acquire nitrogen (gC gN^{-1}); (ii) whole plant nitrogen biomass
293 (denominator of carbon cost to acquire nitrogen; gN); and (iii) belowground car-
294 bon biomass (numerator of carbon cost to acquire nitrogen; gC). I constructed two
295 additional models for *G. max* with the same model structure described above to
296 investigate the effects of light availability and nitrogen fertilization on root nodule

297 biomass (g) and the ratio of root nodule biomass to root biomass (unitless).

298 I used Shapiro–Wilk tests of normality to determine whether species spe-
299 cific linear mixed-effects model residuals followed a normal distribution. Zero
300 models satisfied residual normality assumptions when models were fit using un-
301 transformed data (Shapiro–Wilk: $p < 0.05$ in all cases). I attempted to satisfy
302 residual normality assumptions by first fitting models using dependent variables
303 that were natural-log transformed. If residual normality assumptions were still
304 not met (Shapiro–Wilk: $p > 0.05$), then models were fit using dependent variables
305 that were square root transformed. All residual normality assumptions were satis-
306 fied when models were fit with either a natural-log or square root transformation
307 (Shapiro–Wilk: $p > 0.05$ in all cases). Specifically, I natural-log transformed *G.*
308 *hirsutum* carbon costs to acquire nitrogen and *G. hirsutum* whole-plant nitrogen
309 biomass. I also square root transformed *G. max* carbon costs to acquire nitrogen,
310 *G. max* whole-plant nitrogen biomass, root carbon biomass in both species, *G.*
311 *max* root nodule biomass, and the *G. max* ratio of root nodule biomass to root
312 biomass. I used the ‘lmer’ function in the ‘lme4’ R package (Bates et al. 2015) to
313 fit each model and the ‘Anova’ function in the ‘car’ R package (Fox and Weisberg
314 2019) to calculate Wald’s χ^2 to determine the significance ($\alpha = 0.05$) of each fixed
315 effect coefficient. Finally, I used the ‘emmeans’ R package (Lenth 2019) to conduct
316 post-hoc comparisons of our treatment combinations using Tukey’s tests. Degrees
317 of freedom for all Tukey’s tests were approximated using the Kenward–Roger ap-
318 proach (Kenward and Roger 1997). All analyses and plots were conducted in R
319 version 4.0.1 (R Core Team 2021).

320 2.3 Results

321 2.3.1 *Carbon costs to acquire nitrogen*

322 Carbon costs to acquire nitrogen in *G. hirsutum* increased with increasing light
323 availability ($p<0.001$; Table 2.1; Fig. 2.1) and decreased with increasing nitrogen
324 fertilization ($p<0.001$; Table 2.1; Fig. 2.1). There was no interaction between
325 light availability and nitrogen fertilization ($p=0.486$, Table 2.1; Fig. 2.1).

326 Carbon costs to acquire nitrogen in *G. max* also increased with increasing
327 light availability ($p<0.001$, Table 2.1; Fig. 2.1) and decreased with increasing
328 nitrogen fertilization ($p<0.001$; Table 2.1; Fig. 2.1). There was no interaction
329 between light availability and nitrogen fertilization ($p=0.261$, Table 2.1; Fig. 2.1).

Table 2.1. Analysis of variance results exploring species-specific effects of light availability, nitrogen fertilization, and their interactions on carbon costs to acquire nitrogen (N_{cost} ; gC gN $^{-1}$), whole plant nitrogen biomass (N_{wp} ; gN), and root carbon biomass (C_{bg} ; gC)

	N_{cost}			N_{wp}			C_{bg}			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
<i>G. hirsutum</i>										
Intercept		1.594	-	-	-3.232	-	-	0.432	-	-
Light (L)	1	-1.09E-02	56.494	<0.001	-6.41E-03	91.275	<0.001	-2.62E-03	169.608	<0.001
Nitrogen (N)	1	-1.34E-03	54.925	<0.001	1.83E-03	118.784	<0.001	1.15E-04	2.901	0.089
L*N	1	3.88E-06	0.485	0.486	-1.34E-05	10.721	0.001	-1.67E-06	3.140	0.076
<i>G. max</i>										
Intercept		1.877	-	-	0.239	-	-	0.438	-	-
Light (L)	1	-7.67E-03	174.156	<0.001	-6.72E-04	39.799	<0.001	-2.55E-03	194.548	<0.001
Nitrogen (N)	1	-2.35E-04	21.948	<0.001	1.55E-04	70.771	<0.001	2.52E-04	19.458	<0.001
L*N	1	-2.89E-06	1.262	0.261	-6.32E-07	1.435	0.231	-3.16E-06	10.803	0.001

16

330 *Significance determined using Wald's χ^2 tests ($p=0.05$). P -values less than 0.05 are in bold and p -values between
 331 0.05 and 0.1 are italicized. Negative coefficients for light treatments indicate a positive effect of increasing light
 332 availability on all response variables, as light availability is treated as percent shade cover in all linear mixed-effects
 333 models.

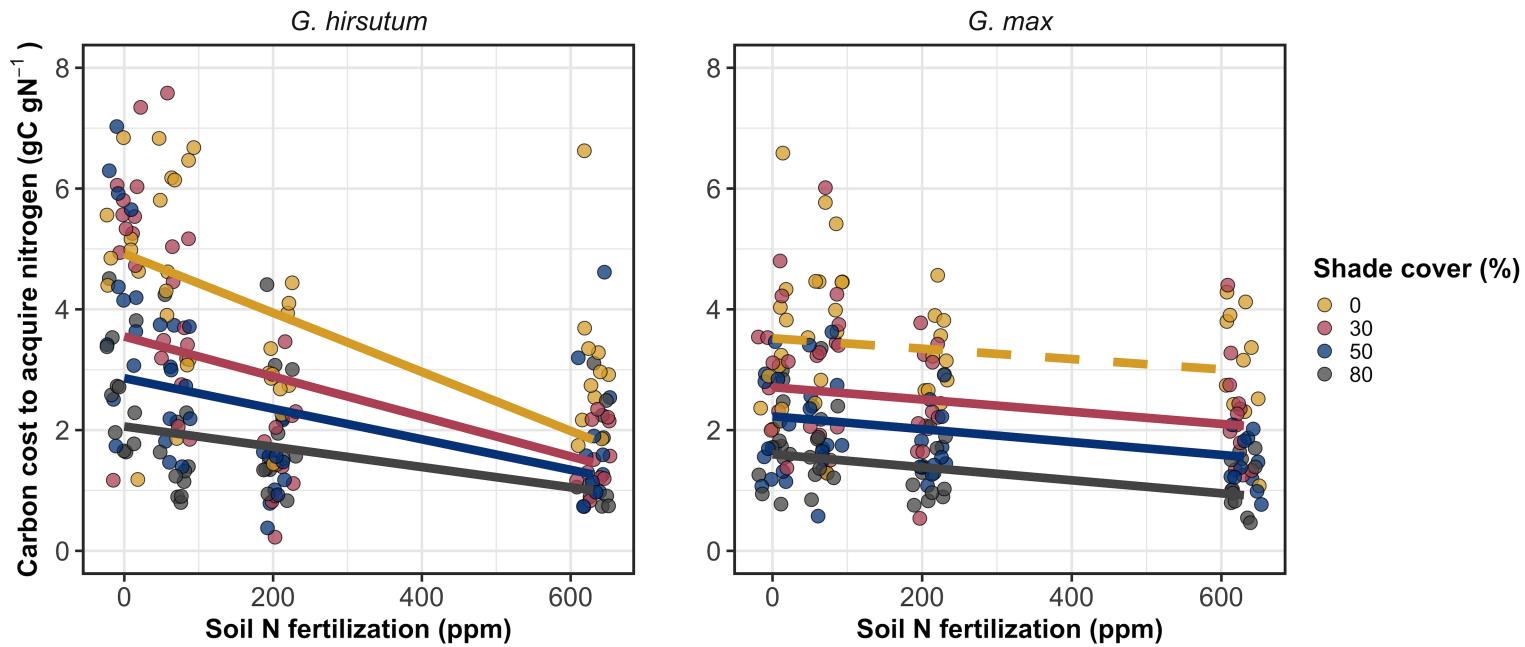


Figure 2.1. Relationships between soil nutrient fertilization and light availability on carbon costs to acquire nitrogen in *G. hirsutum* and *G. max*. Nitrogen fertilization treatments are represented on the x-axis. Shade cover treatments are represented through colored points and trendlines. Trendlines were created by back-transforming marginal mean slopes and intercepts from species-specific linear mixed-effects models. These values were calculated using the ‘emtrends’ and ‘emmeans’ functions in the ‘emmeans’ R package (Lenth, 2019). Yellow points and trendlines represent the 0% shade cover treatment, red points and trendlines represent the 30% shade cover treatment, blue points and trendlines represent the 50% shade cover treatment, and gray points and trendlines represent the 80% shade cover treatment. Solid trendlines indicate slopes that are significantly different from zero (Tukey: $p < 0.05$), while dashed trendlines indicate slopes that are not statistically different from zero.

334 2.3.2 *Whole plant nitrogen biomass*

335 Whole plant nitrogen biomass in *G. hirsutum* was driven by an interaction between
336 light availability and nitrogen fertilization ($p=0.001$; Table 2.1; Fig. 2.2). This
337 interaction indicated a greater stimulation of whole-plant nitrogen biomass by
338 nitrogen fertilization as light levels increased (Table 2.1; Fig. 2.2).

339 Whole plant nitrogen biomass in *G. max* increased with increasing light
340 availability ($p<0.001$) and nitrogen fertilization ($p<0.001$), with no interaction
341 between light availability and nitrogen fertilization ($p=0.231$; Table 2.1; Fig. 2.2).

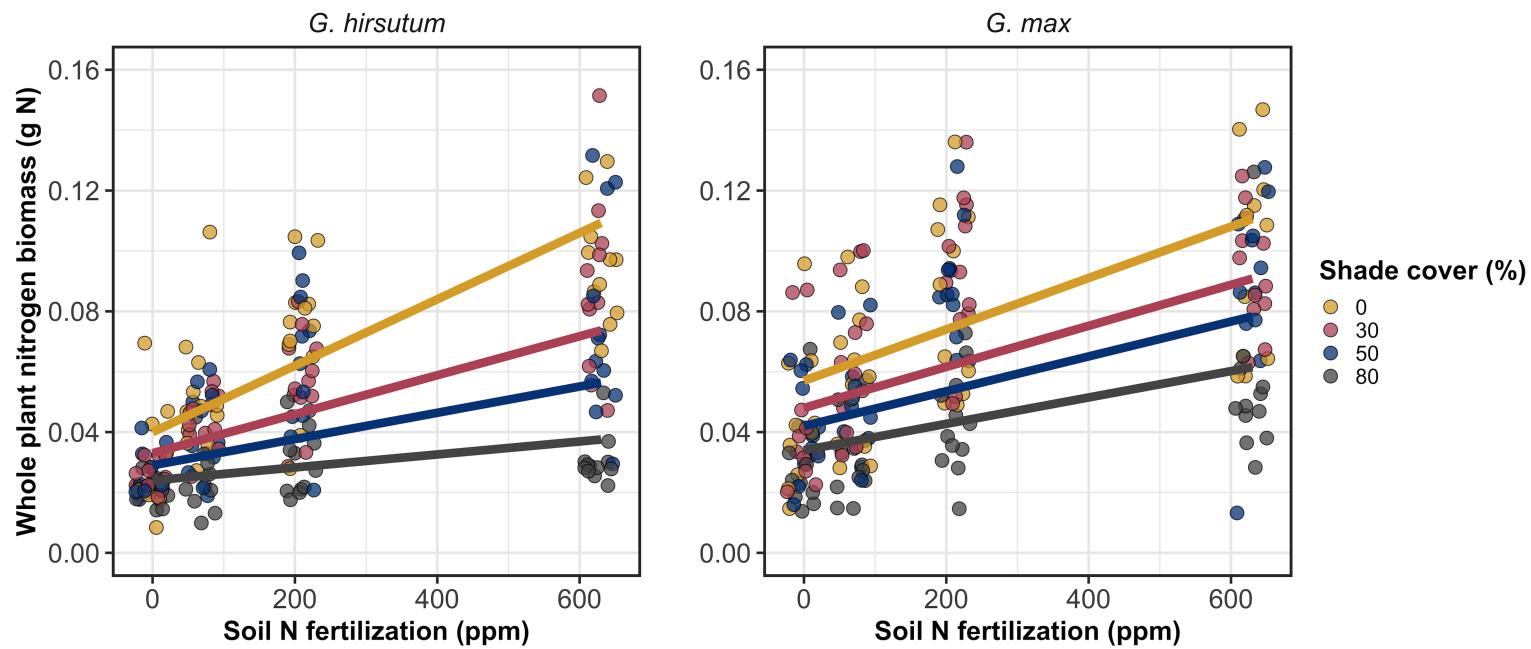


Figure 2.2. Relationships between soil nutrient fertilization and light availability on whole-plant nitrogen biomass in *G. hirsutum* and *G. max*. Whole-plant nitrogen biomass is the denominator of the carbon cost to acquire nitrogen calculation. Nitrogen fertilization treatments are represented on the x-axis. Shade cover treatments are represented through colored points and trendlines. Trendlines were created by back-transforming marginal mean slopes and intercepts from species-specific linear mixed-effects models. These values were calculated using the ‘emtrends’ and ‘emmeans’ functions in the ‘emmeans’ R package (Lenth 2019). Points are jittered for visibility. Colored points and trendlines are as explained in Fig. 2.1. Solid trendlines indicate slopes that are significantly different from zero (Tukey: $p < 0.05$), while dashed trendlines indicate slopes that are not statistically different from zero.

342 2.3.3 *Root carbon biomass*

343 Root carbon biomass in *G. hirsutum* significantly increased with increasing light availability ($p<0.001$; Table 2.1; Fig. 2.3) and marginally increased with nitrogen fertilization ($p=0.089$; Table 2.1; Fig. 2.3). There was also a marginal interaction between light availability and nitrogen fertilization ($p=0.076$; Table 2.1), driven by an increase in the positive response of root carbon biomass to increasing nitrogen fertilization as light availability increased (Table 2.3). This resulted in significantly positive trends between root carbon biomass and nitrogen fertilization in the two highest light treatments (Tukey: $p<0.05$ in both cases; Table 2.3; Fig. 2.3) and no effect of nitrogen fertilization in the two lowest light treatments (Tukey: $p>0.05$ in both cases; Table 2.3; Fig. 2.3).

353 There was an interaction between light availability and nitrogen fertilization on root carbon biomass in *G. max* ($p=0.001$; Table 2.1; Fig. 2.3). Post-hoc analyses indicated that the positive effects of nitrogen fertilization on *G. max* root carbon biomass increased with increasing light availability (Table 2.3; Fig. 2.3). There were also positive individual effects of increasing nitrogen fertilization ($p<0.001$; Table 2.3) and light availability ($p<0.001$; Table 2.3) on *G. max* root carbon biomass (Table 2.1; Fig. 2.3).

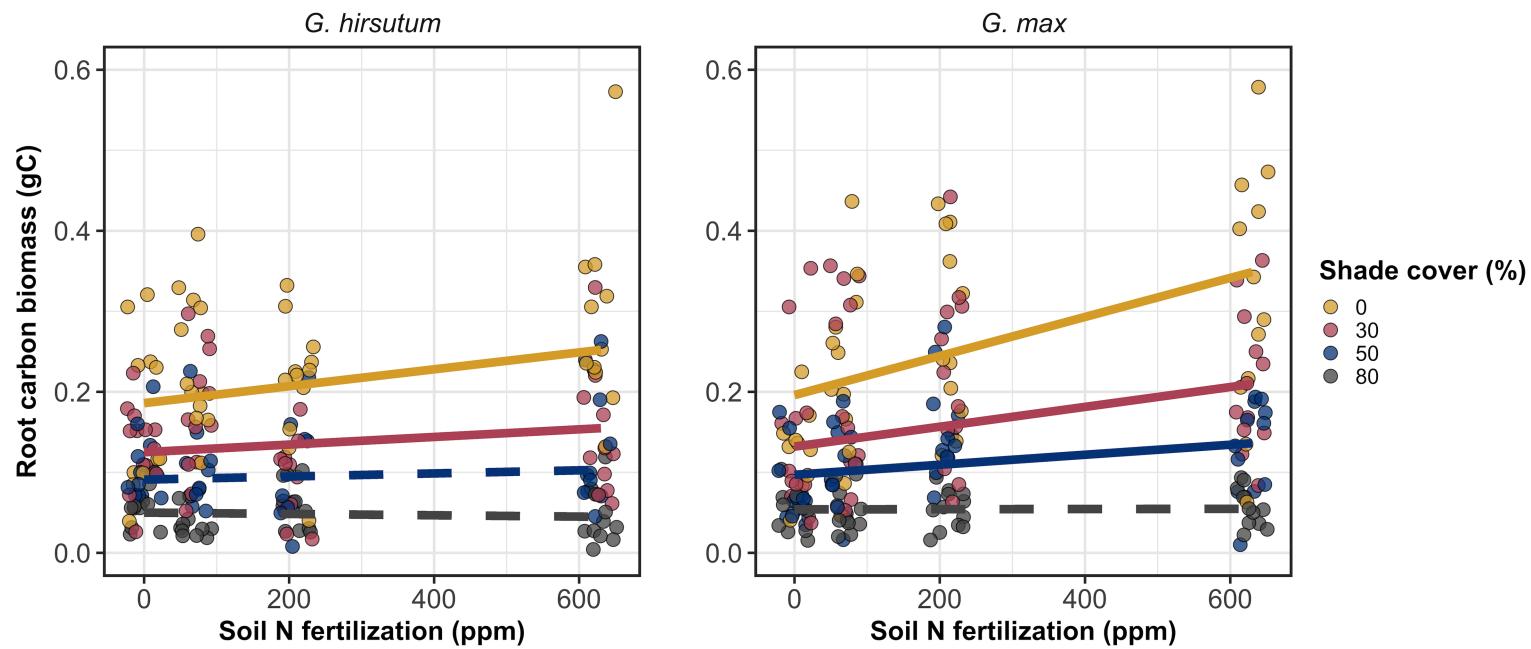


Figure 2.3. Relationships between soil nutrient fertilization and light availability on root carbon biomass in *G. hirsutum* and *G. max*. Root carbon biomass is the numerator of the carbon cost to acquire nitrogen calculation. Nitrogen fertilization treatments are represented on the x-axis. Colored points and trendlines are as explained in Fig. 2.1. Trendlines were created by back-transforming marginal mean slopes and intercepts from species-specific linear mixed-effects models. These values were calculated using the ‘emtrends’ and ‘emmeans’ functions in the ‘emmeans’ R package (Lenth 2019). Points are jittered for visibility. Colored points and trendlines are as explained in Fig. 2.1.

360 2.3.4 *Root nodule biomass*

361 Root nodule biomass in *G. max* increased with increasing light availability ($p <$
362 0.001; Table 2.2; Fig. 2.4a) and decreased with increasing nitrogen fertilization
363 ($p < 0.001$; Table 2.2; Fig. 2.4a). There was no interaction between nitrogen
364 fertilization and light availability ($p = 0.133$; Table 2.2; Fig. 2.4a). The ratio of
365 root nodule biomass to root biomass did not change in response to light availability
366 ($p = 0.481$; Table 2.2; Fig. 2.4b) but decreased with increasing nitrogen fertilization
367 ($p < 0.001$; Table 2.2; Fig. 2.4b). There was no interaction between nitrogen
368 fertilization and light availability on the ratio of root nodule biomass to root
369 biomass ($p = 0.621$; Table 2.2; Fig. 2.4b).

Table 2.2. Analysis of variance results exploring effects of light availability, nitrogen fertilization, and their interactions on *G. max* root nodule biomass (g) and the ratio of root nodule biomass to root biomass (g g⁻¹)*

	Nodule biomass			Nodule biomass: root biomass			<i>p</i>
	df	Coefficient	χ^2	<i>p</i>	Coefficient	χ^2	
(Intercept)		0.302	-	-	0.448	-	-
Light (L)	1	-1.81E-03	72.964	<0.001	-8.76E-05	0.496	0.481
Nitrogen (N)	1	-2.83E-04	115.377	<0.001	-5.09E-04	156.476	<0.001
L*N	1	1.14E-06	2.226	0.133	-7.30E-07	0.244	0.621

370 *Significance determined using Wald's χ^2 tests ($\alpha=0.05$). *P*-values less than 0.05 are in bold. Negative coefficients for
 371 light treatments indicate a positive effect of increasing light availability on all response variables, as light availability
 372 is treated as percent shade cover in all linear mixed-effects models. Root nodule biomass and nodule biomass: root
 373 biomass models were only constructed for *G. max* because *G. hirsutum* was not inoculated with *B. japonicum* and
 374 is not capable of forming root nodules.

Table 2.3. Slopes of the regression line describing the relationship between each dependent variable and nitrogen fertilization at each light level*

Shade cover	Carbon cost to acquire nitrogen	Whole plant nitrogen biomass	Belowground carbon biomass	Root nodule biomass	Nodule biomass: root biomass
<i>G. hirsutum</i>					
0%	-1.34E-03^a	1.83E-03^a	1.15E-04^b	-	-
30%	-1.22E-03^a	1.43E-03^a	1.17E-04^b	-	-
50%	-1.14E-03^a	1.17E-03^a	3.12E-05 ^b	-	-
80%	-1.02E-03^a	7.66E-04^a	-1.89E-06 ^b	-	-
<i>G. max</i>					
0%	-2.35E-04 ^b	1.55E-05^b	2.51E-04^b	-2.83E-04^b	-5.09E-04^b
30%	-3.22E-04^b	1.35E-05^b	1.57E-04^b	-2.49E-04^b	-5.31E-04^b
50%	-3.80E-04^b	1.23E-05^b	9.37E-05^b	-2.26E-04^b	-5.45E-04^b
80%	-4.66E-04^b	1.04E-05^b	-9.95E-07 ^b	-1.92E-04^b	-5.67E-04^b

24

375 * Slopes represent estimated marginal mean slopes from linear mixed-effects models described in the Methods. Slopes
 376 were calculated using the ‘emmeans’ R package (Lenth 2019). Superscripts indicate slopes fit to natural-log (^a) or
 377 square root (^b) transformed data. Slopes statistically different from zero (Tukey: $p<0.05$) are indicated in bold.
 378 Marginally significant slopes (Tukey: $0.05< p<0.1$) are italicized.

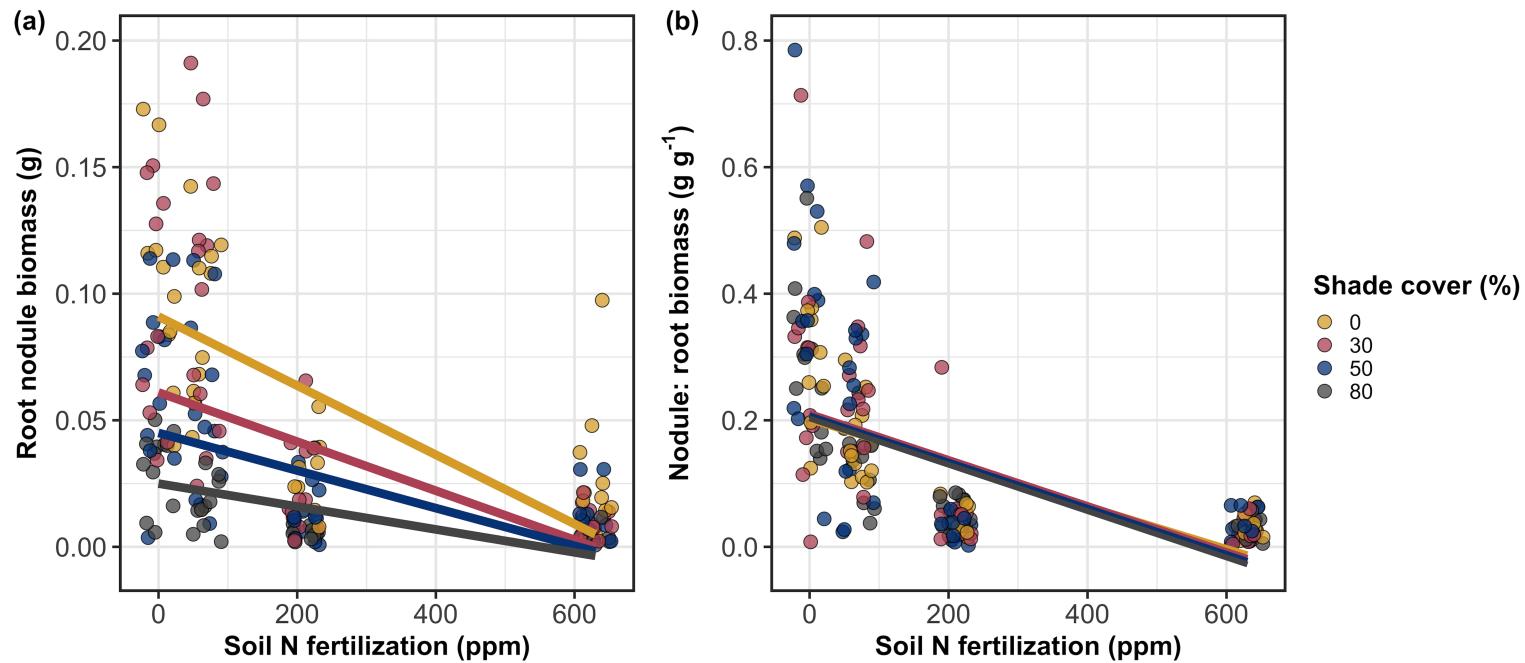


Figure 2.4. Effects of shade cover and nitrogen fertilization on root nodule biomass (a) and the ratio of root nodule biomass to root biomass (b) in *G. max*. Nitrogen fertilization treatments are represented on the x-axis. Shade cover treatments are represented through colored points and trendlines. Trendlines were created by back-transforming marginal mean slopes and intercepts from species-specific linear mixed-effects models. These values were calculated using the ‘emtrends’ and ‘emmeans’ functions in the ‘emmeans’ R package (Lenth 2019). Points are jittered for visibility. Yellow points and trendlines represent the 0% shade cover treatment, blue points and trendlines represent the 30% shade cover treatment, green points and trendlines represent the 50% shade cover treatment, and purple points and trendlines represent the 80% shade cover treatment. Solid trendlines indicate slopes that are significantly different from zero (Tukey: $p < 0.05$), while dashed trendlines indicate slopes that are not statistically different from zero.

379 2.4 Discussion

380 In this chapter, I determined the effects of light availability and soil nitrogen
381 fertilization on root mass carbon costs to acquire nitrogen in *G. hirsutum* and *G.*
382 *max*. In support of my hypotheses, I found that carbon costs to acquire nitrogen
383 generally increased with increasing light availability and decreased with increasing
384 soil nitrogen fertilization in both species. These findings suggest that carbon costs
385 to acquire nitrogen are determined by factors that influence plant nitrogen demand
386 and soil nitrogen availability. In contrast to my second hypothesis, root nodulation
387 data suggested that *G. max* and *G. hirsutum* achieved similar directional carbon
388 cost responses to nitrogen fertilization despite a likely shift in *G. max* allocation
389 from nodulation to root biomass along the nitrogen fertilization gradient.

390 2.4.1 *Carbon costs to acquire nitrogen increase with light availability and*
391 *decrease with fertilization*

392 Both *G. max* and *G. hirsutum* experienced an increase in carbon costs to ac-
393 quire nitrogen due to increasing light availability. These patterns were driven by
394 a larger increase in root carbon biomass than whole-plant nitrogen biomass. In-
395 creases in root carbon biomass due to factors that increase plant nitrogen demand
396 are a commonly observed pattern, as carbon allocated belowground provides sub-
397 strate needed to produce and maintain structures that satisfy aboveground plant
398 nitrogen demand (Nadelhoffer and Raich 1992; Giardina et al. 2005; Raich et al.
399 2014). Findings suggest that plants allocate relatively more carbon for acquiring
400 nitrogen when demand increases over short temporal scales, which may cause a
401 temporary state of diminishing return due to asynchrony between belowground

402 carbon and whole-plant nitrogen responses to plant nitrogen demand (Kulmatiski
403 et al. 2017; Noyce et al. 2019). These responses might be attributed to a temporal
404 lag associated with producing structures that enhance nitrogen acquisition. For
405 example, fine roots (Matamala and Schlesinger 2000; Norby et al. 2004; Arndal
406 et al. 2018) and root nodules (Parvin et al. 2020) take time to build and first
407 require the construction of coarse roots. Thus, full nitrogen returns from these
408 investments may not occur immediately (Kayler et al. 2010; Kayler et al. 2017),
409 and may vary by species acquisition strategy. I speculate that increases in ni-
410 trogen acquisition from a given carbon investment may occur beyond the 5-week
411 scope of this experiment. A similar study conducted over a longer temporal scale
412 would address this.

413 Increasing soil nitrogen fertilization generally decreased carbon costs to
414 acquire nitrogen in both species. These patterns were driven by a larger increase
415 in whole-plant nitrogen biomass than root carbon biomass. In *G. hirsutum*, re-
416 ductions in carbon costs to acquire nitrogen may have been due to an increase in
417 per-root nitrogen uptake, allowing individuals to maximize the amount of nitro-
418 gen acquired from a belowground carbon investment. Interestingly, increased soil
419 nitrogen fertilization increased whole-plant nitrogen biomass in *G. max* despite
420 reductions in root nodule biomass that likely reduced the nitrogen-fixing capac-
421 ity of *G. max* (Andersen et al. 2005; Muñoz et al. 2016). While reductions in
422 root nodulation due to increased soil nitrogen availability are commonly observed
423 (Gibson and Harper 1985; Fujikake et al. 2003), root nodulation responses were
424 observed in tandem with increased root carbon biomass, implying that *G. max*
425 shifted relative carbon allocation from nitrogen fixation to soil nitrogen acquisition

426 (Markham and Zekveld 2007; Dovrat et al. 2020). This was likely because there
427 was a reduction in the carbon cost advantage of acquiring fixed nitrogen relative
428 to soil nitrogen, and suggests that species capable of associating with symbiotic
429 nitrogen-fixing bacteria shift their relative nitrogen acquisition pathway to opti-
430 mize nitrogen uptake (Rastetter et al. 2001). Future studies should investigate
431 these patterns with a larger quantity of phylogenetically related species, or differ-
432 ent varieties of a single species that differ in their ability to form associations with
433 symbiotic nitrogen-fixing bacteria to more directly test the impact of nitrogen
434 fixation on the patterns observed in this study.

435 2.4.2 *Modeling implications*

436 Carbon costs to acquire nitrogen are subsumed in the general discussion of eco-
437 nomic analogies to plant resource uptake (Bloom et al. 1985; Rastetter et al.
438 2001; Vitousek et al. 2002; Phillips et al. 2013; Terrer et al. 2018; Henneron
439 et al. 2020). Despite this, terrestrial biosphere models rarely include costs of
440 nitrogen acquisition within their framework for predicting plant nitrogen uptake.
441 There is currently one plant resource uptake model, FUN, that quantitatively
442 predicts carbon costs to acquire nitrogen within a framework for predicting plant
443 nitrogen uptake for different nitrogen acquisition strategies (Fisher et al. 2010;
444 Brzostek et al. 2014). Iterations of FUN are currently coupled to two terrestrial
445 biosphere models: the Community Land Model 5.0 and the Joint UK Land En-
446 vironment Simulator (Clark et al. 2011; Shi et al. 2016; Lawrence et al. 2019).
447 Recent work suggests that coupling FUN to CLM 5.0 caused a large overpredic-
448 tion of plant nitrogen uptake associated with nitrogen fixation (Davies-Barnard

449 et al. 2020) compared to other terrestrial biosphere model products. Thus, em-
450 pirical data from manipulative experiments that explicitly quantify carbon costs
451 to acquire nitrogen in species capable of associating with nitrogen-fixing bacteria
452 across different environmental contexts is an important step toward identifying
453 potential biases in models such as FUN.

454 These findings broadly support the FUN formulation of carbon costs to
455 acquire nitrogen in response to soil nitrogen availability. FUN calculates carbon
456 costs to acquire nitrogen based on the sum of carbon costs to acquire nitrogen
457 via nitrogen fixation, mycorrhizal active uptake, non-mycorrhizal active uptake,
458 and retranslocation (Fisher et al. 2010; Brzostek et al. 2014). Carbon costs to
459 acquire nitrogen via mycorrhizal or non-mycorrhizal active uptake pathways are
460 derived as a function of nitrogen availability, root biomass, and two parameterized
461 values based on nitrogen acquisition strategy (Brzostek et al. 2014). Due to this,
462 FUN simulates a net decrease in carbon costs to acquire nitrogen with increasing
463 nitrogen availability for mycorrhizal and non-mycorrhizal active uptake pathways,
464 assuming constant root biomass. This was a pattern I observed in *G. hirsutum* re-
465 gardless of light availability. In contrast, FUN would not simulate a net change in
466 carbon costs to acquire nitrogen via nitrogen fixation due to nitrogen availability.
467 This is because carbon costs to acquire nitrogen via nitrogen fixation are derived
468 from a well established function of soil temperature, which is independent of soil
469 nitrogen availability (Houlton et al. 2008; Fisher et al. 2010). I observed a net
470 reduction in carbon costs to acquire nitrogen in *G. max*, except when individu-
471 als were grown under 0% shade cover. While a net reduction of carbon costs in
472 response to nitrogen fertilization runs counter to nitrogen fixation carbon costs

473 simulated by FUN, these patterns were likely because *G. max* individuals switched
474 their primary mode of nitrogen acquisition from symbiotic nitrogen fixation to a
475 non-symbiotic active uptake pathway.

476 2.4.3 *Study limitations*

477 It should be noted that the metric used in this study to determine carbon costs
478 to acquire nitrogen has several limitations. Most notably, this metric uses root
479 carbon biomass as a proxy for estimating the amount of carbon spent on nitrogen
480 acquisition. While it is true that most carbon allocated belowground has at least
481 an indirect structural role in acquiring soil resources, it remains unclear whether
482 this assumption holds true for species that acquire nitrogen via symbiotic nitro-
483 gen fixation. I also cannot quantify carbon lost through root exudates or root
484 turnover, which may increase due to factors that increase plant nitrogen demand
485 (Tingey et al. 2000; Phillips et al. 2011), and can increase the magnitude of
486 available nitrogen from soil organic matter through priming effects on soil micro-
487 bial communities (Uselman et al. 2000; Bengtson et al. 2012). It is also not
488 clear whether these assumptions hold under all environmental conditions, such
489 as those that shift belowground carbon allocation toward a different mode of ni-
490 trogen acquisition (Taylor and Menge 2018; Friel and Friesen 2019) or between
491 species with different acquisition strategies. In this study, increasing soil nitrogen
492 fertilization increased carbon investment to roots relative to carbon transferred to
493 root nodules. By assuming that carbon allocated to root carbon was proportional
494 to carbon allocated to root nodules across all treatment combinations, these ob-
495 served responses to soil nitrogen fertilization were likely to be overestimated in *G.*

496 *max*. I encourage future research to quantify these carbon fates independently.

497 Researchers conducting pot experiments must carefully choose pot volume
498 to minimize the likelihood of growth limitations induced by pot volume (Poorter
499 et al. 2012). Poorter et al. (2012) indicate that researchers are likely to avoid
500 growth limitations associated with pot volume if measurements are collected when
501 the plant biomass:pot volume ratio is less than 1 g L^{-1} . In this experiment, all
502 treatment combinations in both species had biomass:pot volume ratios less than
503 1 g L^{-1} except for *G. max* and *G. hirsutum* that were grown under 0% shade
504 cover and had received 630 ppm N. Specifically, *G. max* and *G. hirsutum* had
505 average respective biomass:pot volume ratios of $1.24 \pm 0.07 \text{ g L}^{-1}$ and 1.34 ± 0.13
506 g L^{-1} , when grown under 0% shade cover and received 630 ppm N (Table A2,
507 A3; Fig. A1). If growth in this treatment combination was limited by pot vol-
508 ume, then individuals may have had larger carbon costs to acquire nitrogen than
509 would be expected if they were grown in larger pots. This pot volume induced
510 growth limitation could cause a reduction in per-root nitrogen uptake associated
511 with more densely packed roots, which could reduce the positive effect of nitro-
512 gen fertilization on whole-plant nitrogen biomass relative to root carbon biomass
513 (Poorter et al. 2012).

514 Growth limitation associated with pot volume provides a possible expla-
515 nation for the marginally insignificant effect of increasing nitrogen fertilization on
516 *G. max* carbon costs to acquire nitrogen when grown under 0% shade cover. This
517 is because the regression line describing the relationship between carbon costs to
518 acquire nitrogen and nitrogen fertilization in *G. max* grown under 0% shade cover
519 would have flattened if growth limitation had caused larger than expected carbon

520 costs to acquire nitrogen in the 0% shade cover, 630 ppm N treatment combi-
521 nation. This may have been exacerbated by the fact that *G. max* likely shifted
522 relative carbon allocation from nitrogen fixation to soil nitrogen acquisition, which
523 could have increased the negative effect of more densely packed roots on nitrogen
524 uptake. These patterns could have also occurred in *G. hirsutum* grown under 0%
525 shade cover; however, there was no change in the effect of nitrogen fertilization on
526 *G. hirsutum* carbon costs to acquire nitrogen grown under 0% shade cover relative
527 to other shade cover treatments. Regardless, the possibility of growth limitation
528 due to pot volume suggests that effects of increasing nitrogen fertilization on car-
529 bon costs to acquire nitrogen in both species grown under 0% shade cover could
530 have been underestimated. Follow-up studies using a similar experimental design
531 with a larger pot volume would be necessary in order to determine whether these
532 patterns were impacted by pot volume-induced growth limitation.

533 2.4.4 *Conclusions*

534 In conclusion, this chapter provides empirical evidence that carbon costs to ac-
535 quire nitrogen are influenced by light availability and soil nitrogen fertilization
536 in a species capable of acquiring nitrogen via symbiotic nitrogen fixation and a
537 species not capable of forming such associations. We show that carbon costs to
538 acquire nitrogen generally increase with increasing light availability and decrease
539 with increasing nitrogen fertilization. This chapter provides important empirical
540 data needed to evaluate the formulation of carbon costs to acquire nitrogen in
541 terrestrial biosphere models, particularly carbon costs to acquire nitrogen that
542 are associated with symbiotic nitrogen fixation. Findings broadly support the

543 general formulation of these carbon costs in the FUN biogeochemical model in
544 response to shifts in nitrogen availability. However, there is a need for future
545 studies to explicitly quantify carbon costs to acquire nitrogen under different en-
546 vironmental contexts, over longer temporal scales, and using larger selections of
547 phylogenetically related species. In addition, I suggest that future studies mini-
548 mize the limitations associated with the metric used here by explicitly measuring
549 belowground carbon fates independently.

550 Chapter 3

551 Soil nitrogen availability modifies leaf nitrogen economies in mature 552 temperate deciduous forests: a direct test of photosynthetic least-cost 553 theory

554 3.1 Introduction

Photosynthesis represents the largest carbon flux between the atmosphere and land surface (IPCC 2021), and plays a central role in biogeochemical cycling at multiple spatial and temporal scales (Vitousek and Howarth 1991; LeBauer and Treseder 2008; Kaiser et al. 2015; Wieder et al. 2015). Therefore, carbon and energy fluxes simulated by terrestrial biosphere models are sensitive to the formulation of photosynthetic processes (Ziehn et al. 2011; Bonan et al. 2011; Booth et al. 2012; Smith et al. 2016; Smith et al. 2017) and must be represented using robust, empirically tested processes (Prentice et al. 2015; Wieder et al. 2019). Current formulations of photosynthesis vary across terrestrial biosphere models (Smith and Dukes 2013; Rogers et al. 2017), which causes variation in modeled ecosystem processes (Knorr 2000; Knorr and Heimann 2001; Bonan et al. 2011; Friedlingstein et al. 2014) and casts uncertainty on the ability of these models to accurately predict terrestrial ecosystem responses and feedbacks to global change (Zaehle et al. 2005; Schaefer et al. 2012; Davies-Barnard et al. 2020).

Terrestrial biosphere models commonly represent C₃ photosynthesis through rough variants of the Farquhar et al. (1980) biochemical model (Smith and Dukes 2013; Rogers 2014; Rogers et al. 2017). This well-tested photosynthesis model estimates leaf-level carbon assimilation, or photosynthetic capacity, as a function of the maximum rate of Ribulose-1,5-bisphosphate carboxylase-oxygenase (Ru-

574 bisco) carboxylation (V_{cmax}) and the maximum rate of Ribulose-1,5-bisphosphate
575 (RuBP) regeneration (J_{max}) (Farquhar et al. 1980). Many terrestrial biosphere
576 models predict these model inputs based on plant functional group specific lin-
577 ear relationships between leaf nutrient content and V_{cmax} (Smith and Dukes 2013;
578 Rogers 2014; Rogers et al. 2017) under the tenet that a large fraction of leaf nutri-
579 ents, and nitrogen in particular, are partitioned toward building and maintaining
580 enzymes that support photosynthetic capacity, such as Rubisco (Brix 1971; Gul-
581 mon and Chu 1981; Evans 1989; Kattge et al. 2009; Walker et al. 2014). Terres-
582 trial biosphere models predict leaf nutrient content from soil nutrient availability
583 based on the assumption that increasing soil nutrients generally increases leaf nu-
584 trients (Firn et al. 2019; Li et al. 2020; Liang et al. 2020) which, in the case of
585 nitrogen, generally corresponds with an increase in photosynthetic processes (Li
586 et al. 2020; Liang et al. 2020).

587 Recent work calls the generality of relationships between soil nutrient avail-
588 ability, leaf nutrient content, and photosynthetic capacity into question, suggest-
589 ing instead that leaf nutrients and photosynthetic capacity are better predicted as
590 an integrated product of aboveground climate, leaf traits, and soil nutrient avail-
591 ability, rather than soil nutrient availability alone (Dong et al. 2017; Dong et al.
592 2020; Dong et al. 2022; Firn et al. 2019; Smith et al. 2019; Peng et al. 2021).
593 It has been reasoned that this result is because plants allocate added nutrients to
594 growth and storage rather than alterations in leaf chemistry (Smith et al. 2019),
595 perhaps as a result of nutrient limitation of primary productivity (LeBauer and
596 Treseder 2008; Fay et al. 2015). Additionally, recent work suggests that relation-
597 ships between leaf nutrient content and photosynthesis vary across environments,

598 and that the proportion of leaf nutrient content allocated to photosynthetic tis-
599 sue varies over space and time with plant acclimation and adaptation responses
600 to light availability, vapor pressure deficit, soil pH, soil nutrient availability, and
601 environmental factors that influence leaf mass per area (Pons and Pearcy 1994;
602 Niinemets and Tenhunen 1997; Evans and Poorter 2001; Hikosaka and Shigeno
603 2009; Ghimire et al. 2017; Onoda et al. 2017; Luo et al. 2021). The use of linear
604 relationships between leaf nutrient content and V_{cmax} to predict photosynthetic
605 capacity, as commonly used in terrestrial biosphere models (Rogers 2014), is not
606 capable of detecting such responses.

607 Photosynthetic least-cost theory provides an alternative framework for un-
608 derstanding relationships between soil nutrient availability, leaf nutrient content,
609 and photosynthetic capacity (Harrison et al. 2021). Leveraging a two-input mi-
610 croeconomics approach (Wright et al. 2003), the theory posits that plants accli-
611 mate to a given environment by optimizing leaf photosynthesis rates at the lowest
612 summed cost of using nutrients and water (Prentice et al. 2014; Wang et al. 2017;
613 Smith et al. 2019; Paillassa et al. 2020). Across resource availability gradients,
614 the theory predicts that optimal photosynthetic rates can be achieved by trading
615 less efficient use of a resource that is less costly to acquire (or more abundant)
616 for more efficient use of a resource more costly to acquire (or less abundant). For
617 example, an increase in soil nutrient availability should reduce the cost of acquir-
618 ing and using nutrients (Bae et al. 2015; Eastman et al. 2021; Perkowski et al.
619 2021), which could increase leaf nutrient investments in photosynthetic proteins to
620 allow similar photosynthetic rates to be achieved with higher nutrient use (lower
621 nutrient use efficiency) but lower water use (greater water use efficiency). The

622 theory suggests similar tradeoffs in response to increasing soil pH (Paillassa et al.
623 2020), specifically, that increasing soil pH should reduce the cost of acquiring soil
624 nutrients due to an increase in plant-available nutrient concentration (Paillassa
625 et al. 2020; Dong et al. 2022). The theory is also capable of reconciling dynamic
626 leaf nutrient-photosynthesis relationships at global scales (Luo et al. 2021).

627 Patterns expected from photosynthetic least-cost theory have recently re-
628 ceived empirical support both in global environmental gradient (Smith et al.
629 2019; Paillassa et al. 2020; Luo et al. 2021; Querejeta et al. 2022; Wester-
630 band et al. 2023) and local manipulative invasion (Bialic-Murphy et al. 2021)
631 studies. However, nutrient addition experiments that directly examine nutrient-
632 water use tradeoffs expected from the theory are rare (but see Guerrieri et al.
633 2011), and only global gradient studies testing the theory have considered soil pH
634 in their analyses. As a result, there is a need to use nutrient addition and soil pH
635 manipulation experiments to test mechanisms driving responses predicted by the
636 theory.

637 In this study, I measured leaf responses to soil nitrogen availability in five
638 deciduous tree species growing in the upper canopy of mature closed canopy tem-
639 perate forests in the northeastern United States. Soil nitrogen availability and pH
640 were manipulated through a nitrogen-by-pH field manipulation experiment with
641 treatments applied since 2011, eight years prior to measurement. Two different soil
642 nitrogen treatments were applied to increase nitrogen availability with opposing
643 effects on soil pH. An additional nitrogen-free acidifying treatment was expected
644 to decrease soil pH. I hypothesized that increased soil nitrogen availability would
645 enable plants to increase nutrient uptake and create more photosynthetic enzymes

646 per leaf, allowing similar photosynthetic rates achieved with lower leaf C_i:C_a and
647 increased leaf nitrogen content allocated to photosynthetic leaf tissue. I expected
648 that this response would be driven by a reduction in the cost of acquiring nitrogen,
649 which would cause trees to sacrifice efficient nitrogen use to enable more efficient
650 use of other limiting resources (i.e., water). Finally, I hypothesized similar leaf
651 responses to increasing soil pH.

652 3.2 Methods

653 3.2.1 *Study site description*

654 I conducted this study in summer 2019 at three stands located within a 20-km ra-
655 dius of Ithaca, NY, USA (42.444 °N, 76.502 °W). All stands contain mature,
656 closed-canopy forests dominated by deciduous tree species. Stands contained
657 abundant sugar maple (*Acer saccharum* Marshall), American beech (*Fagus gran-*
658 *difolia* Ehrh.), and white ash (*Fraxinus americana* L.), accounting for 43%, 15%,
659 and 17% of the total aboveground biomass across the three stands, respectively,
660 with less frequent red maple (*Acer rubrum* L.; 9% of total aboveground biomass)
661 and red oak occurrences (*Quercus rubra* L.; 10% of total aboveground biomass).
662 Soils at each site were broadly classified as a channery silt loam Inceptisols using
663 the USDA NRCS Web Soil Survey data product (Soil Survey Staff 2022). Between
664 2006 and 2020, study sites averaged 972 mm of precipitation per year and had an
665 average temperature of 7.9 °C per a weather station located near the Cornell Uni-
666 versity campus (42.449 °N, 76.449 °W) part of the NOAA NCEI Global Historical
667 Climatology Network (Menne et al. 2012).

668 3.2.2 *Experimental design*

669 Four 40 m x 40 m plots were set up at each site in 2009, each with an additional
670 10 m buffer along plot perimeters (60 m x 60 m total). The plots were set up as a
671 nitrogen-by-pH field manipulation experiment, with one each of four treatments
672 at each site. Two nitrogen treatments were applied, both at $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, as
673 either sodium nitrate (NaNO_3) to raise soil pH, or ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$)
674 to acidify; an elemental sulfur treatment was selected to acidify without nitrogen,
675 applied at the same rate of S addition ($57 \text{ kg S ha}^{-1} \text{ yr}^{-1}$); and control plots
676 received no additions. All amendments were added in pelletized form using hand-
677 held fertilizer spreaders to both the main plots and buffers. Amendments were
678 divided into three equal doses distributed across the growing season from 2011-
679 2017 and added as a single dose from 2018 onward. During 2019, plots were
680 fertilized during the week of May 20.

681 3.2.3 *Leaf gas exchange and trait measurements*

682 I sampled one leaf each from 6 to 10 individuals per plot between June 25 and July
683 12, 2019 for gas exchange measurements (Table B1). Leaves were collected from
684 deciduous broadleaf trees represented across all sites and plots and were replicated
685 in efforts to mimic the species abundance of each plot at each site. I attempted
686 to collect leaves from the upper canopy to reduce differential shading effects on
687 leaf physiology. Leaves were accessed by pulling down small branches using an
688 arborist's slingshot and weighted beanbag attached to a throw line. Branches
689 were immediately recut under deionized water and remained submerged to reduce
690 stomatal closure and avoid xylem embolism, as done in Smith and Dukes (2018),

691 until gas exchange data were collected.

692 Randomly selected leaves with little to no visible external damage were
693 attached to a Li-COR LI-6800 (Li-COR Bioscience, Lincoln, Nebraska, USA)
694 portable photosynthesis machine to measure net photosynthesis (A_{net} ; $\mu\text{mol m}^{-2}$
695 s^{-1}), stomatal conductance (g_{sw} ; $\text{mol m}^{-2} \text{s}^{-1}$), and intercellular CO_2 concentra-
696 tion (C_i ; $\mu\text{mol mol}^{-1}$) at different reference CO_2 concentrations (C_a ; $\mu\text{mol mol}^{-1}$)
697 concentrations (i.e., an A_{net}/C_i curve) under saturating light conditions (2,000
698 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Reference CO_2 concentrations followed the sequence: 400, 300,
699 200, 100, 50, 400, 400, 600, 800, 1000, 1200, 1500, and 2000 $\mu\text{mol mol}^{-1} \text{CO}_2$. Leaf
700 temperatures were not controlled in the cuvette and ranged from 21.8 °C to 31.7
701 °C (mean±SD: 27.2±2.2 °C). A linear and second order log-polynomial nonlinear
702 regression suggested no effect of temperature on stomatal conductance measured
703 at 400 $\mu\text{mol mol}^{-1} \text{CO}_2$ or net photosynthesis measured at 400 $\mu\text{mol mol}^{-1} \text{CO}_2$
704 (Table B2, B3; Fig. B1). All A_{net}/C_i curves were generated within one hour of
705 branch severance.

706 Leaf morphological and chemical traits were collected on the same leaf used
707 to generate each A_{net}/C_i curve. Images of each leaf were taken using a flat-bed
708 scanner to determine fresh leaf area using the ‘LeafArea’ R package (Katabuchi
709 2015), which automates leaf area calculations using ImageJ software (Schneider
710 et al. 2012). Each leaf was dried at 65°C for at least 48 hours, weighed, and
711 ground using a Retsch MM200 ball mill grinder (Verder Scientific, Inc., Newtown,
712 PA, USA) until homogenized. Leaf mass per unit leaf area (M_{area} , g m^{-2}) was
713 calculated as the ratio of dry leaf biomass to fresh leaf area. Using a subsample
714 of ground and homogenized leaf biomass, leaf nitrogen content (N_{mass} ; gN g^{-1})

715 and leaf $\delta^{13}\text{C}$ (‰, relative to Vienna Pee Dee Belemnite international reference
 716 standard) were measured at the Cornell Stable Isotope Lab with an elemental
 717 analyzer (NC 2500, CE Instruments, Wigan, UK) interfaced to an isotope ratio
 718 mass spectrometer (Delta V Isotope Ratio Mass Spectrometer, ThermoFisher Sci-
 719 entific, Waltham, MA, USA). Leaf nitrogen content per unit leaf area (N_{area} ; g N
 720 m^{-2}) was calculated by multiplying N_{mass} by M_{area} .

721 I used leaf $\delta^{13}\text{C}$ values to estimate χ (unitless), which is an isotope-derived
 722 estimate of the leaf $C_i:C_a$ ratio. While intercellular and atmospheric CO_2 concen-
 723 trations were directly measured during each A_{net}/C_i curve, deriving χ from $\delta^{13}\text{C}$
 724 provides a more integrative estimate of the leaf $C_i:C_a$ over an individual leaf's
 725 lifespan. I derived χ following the approach of Farquhar et al. (1989) described
 726 in Cernusak et al. (2013):

$$\chi = \frac{\Delta^{13}\text{C} - a}{b - a} \quad (3.1)$$

727 where $\Delta^{13}\text{C}$ represents the relative difference between leaf $\delta^{13}\text{C}$ (‰) and air $\delta^{13}\text{C}$
 728 (‰), and is calculated from the following equation:

$$\Delta^{13}\text{C} = \frac{\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{leaf}}}{1 + \delta^{13}\text{C}_{\text{leaf}}} \quad (3.2)$$

729 where $\delta^{13}\text{C}_{\text{air}}$ is assumed to be -8‰ (Keeling et al. 1979; Farquhar et al. 1989), a
 730 represents the fractionation between ^{12}C and ^{13}C due to diffusion in air, assumed
 731 to be 4.4‰, and b represents the fractionation caused by Rubisco carboxylation,
 732 assumed to be 27‰ (Farquhar et al. 1989).

733 3.2.4 A_{net}/C_i curve-fitting and parameter estimation

734 I fit A_{net}/C_i curves of each individual using the ‘fitaci’ function in the ‘plante-
735 cophys’ R package (Duursma 2015). This function estimates the maximum rate
736 of Rubisco carboxylation (V_{cmax} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) and maximum rate of electron
737 transport for RuBP regeneration (J_{max} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) based on the Farquhar,
738 von Caemmerer, and Berry biochemical model of C₃ photosynthesis (Farquhar
739 et al. 1980). For each curve fit, I included triose phosphate utilization (TPU)
740 limitation to avoid underestimating J_{max} (Gregory et al. 2021). Curves were
741 visually examined to confirm the likely presence of TPU limitation.

742 I determined Michaelis-Menten coefficients for Rubisco affinity to CO₂ (K_c ;
743 $\mu\text{mol mol}^{-1}$) and O₂ (K_o ; $\mu\text{mol mol}^{-1}$), and the CO₂ compensation point (Γ^* ;
744 $\mu\text{mol mol}^{-1}$) using leaf temperature and equations described in Medlyn et al.
745 (2002) and derived in Bernacchi et al. (2001). Specifically, K_c and K_o were
746 calculated as:

$$K_c = 404.9 * \exp^{\frac{79430(T_k - 298)}{298RT_k}} \quad (3.3)$$

747 and

$$K_o = 278.4 * \exp^{\frac{36380(T_k - 298)}{298RT_k}} \quad (3.4)$$

748 while Γ^* was calculated as:

$$\Gamma^* = 42.75 * \exp^{\frac{37830(T_k - 298)}{298RT_k}} \quad (3.5)$$

749 In all three equations, T_k is the leaf temperature (in Kelvin) during each A_{net}/C_i

750 curve and R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$).

751 I standardized V_{cmax} and J_{max} estimates to 25°C using a modified Arrhe-

752 nius equation (Kattge and Knorr 2007):

$$k_{25} = \frac{k_{\text{obs}}}{e^{\frac{H_a(T_{\text{obs}} - T_{\text{ref}})}{T_{\text{ref}}RT_{\text{obs}}}} * \frac{1+e^{\frac{T_{\text{ref}}\Delta S - H_d}{T_{\text{obs}}\Delta S - H_d}}}{1+e^{\frac{T_{\text{obs}}\Delta S - H_d}{T_{\text{obs}}\Delta S - H_d}}}} \quad (3.6)$$

753 k_{25} represents the standardized V_{cmax} or J_{max} rate at 25°C , k_{obs} represents the

754 V_{cmax} or J_{max} estimate at the average leaf temperature measured inside the cuvette

755 during the A_{net}/C_i curve. H_a is the activation energy of V_{cmax} ($71,513 \text{ J mol}^{-1}$)

756 Kattge and Knorr (2007) or J_{max} ($49,884 \text{ J mol}^{-1}$) (Kattge and Knorr 2007).

757 H_d represents the deactivation energy of both V_{cmax} and J_{max} ($200,000 \text{ J mol}^{-1}$)

758 (Medlyn et al. 2002), and R represents the universal gas constant (8.314 J mol^{-1}

759 K^{-1}). T_{ref} represents the standardized temperature of 298.15 K (25°C) and T_{obs}

760 represents the mean leaf temperature (in K) during each A_{net}/C_i curve. ΔS is an

761 entropy term that (Kattge and Knorr 2007) derived as a linear relationship with

762 average growing season temperature (T_g ; $^\circ\text{C}$), where:

$$\Delta S_{v_{\text{cmax}}} = -1.07 T_g + 668.39 \quad (3.7)$$

763 and

$$\Delta S_{j_{\text{max}}} = -0.75 T_g + 659.70 \quad (3.8)$$

764 I estimated T_g in Equations 3.7 and 3.8 based on mean daily (24-hour) air tem-
765 perature of the 30 days leading up to the day of each sample collection using the
766 same weather station reported in the site description. I used V_{cmax25} and J_{max25}
767 estimates to calculate the ratio of J_{max25} to V_{cmax25} ($J_{max25}:V_{cmax25}$; unitless).

768 3.2.5 *Proportion of leaf nitrogen allocated to photosynthesis and structure*

769 I used equations from Niinemets and Tenhunen (1997) to estimate the proportion
770 of leaf nitrogen content allocated to Rubisco and bioenergetics. The proportion of
771 leaf nitrogen allocated to Rubisco ($\rho_{rubisco}$; gN gN⁻¹) was calculated as a function
772 of V_{cmax25} and N_{area} :

$$\rho_{rubisco} = \frac{V_{cmax25} N_r}{V_{cr} N_{area}} \quad (3.9)$$

773 where N_r is the amount of nitrogen in Rubisco, set to 0.16 gN (gN in Rubisco)⁻¹
774 and V_{cr} is the maximum rate of RuBP carboxylation per unit Rubisco protein,
775 set to 20.5 μmol CO₂ (g Rubisco)⁻¹. The proportion of leaf nitrogen allocated to
776 bioenergetics (ρ_{bioe} ; gN gN⁻¹) was similarly calculated as a function of J_{max25} and
777 N_{area} :

$$\rho_{bioe} = \frac{J_{max25} N_b}{J_{mc} N_{area}} \quad (3.10)$$

778 where N_b is the amount of nitrogen in cytochrome f, set to 0.12407 gN (μmol
779 cytochrome f)⁻¹ assuming a constant 1: 1: 1.2 cytochrome f: ferredoxin NADP
780 reductase: coupling factor molar ratio (Evans and Seemann 1989; Niinemets and
781 Tenhunen 1997), and J_{mc} is the capacity of electron transport per cytochrome f,

782 set to 156 μmol electron (μmol cytochrome f) $^{-1}\text{s}^{-1}$.

783 I estimated the proportion of leaf nitrogen content allocated to photosynthetic tissue (ρ_{photo} ; gN gN^{-1}) as the sum of ρ_{rubisco} and ρ_{bioe} . This calculation
784 is an underestimate of the proportion of leaf nitrogen allocated to photosynthetic
785 tissue because it does not include nitrogen allocated to light harvesting proteins.
786 This leaf nitrogen pool was not included because I did not perform chlorophyll
787 extractions on focal leaves. However, the proportion of leaf nitrogen content al-
788 located to light harvesting proteins tends to be small relative to ρ_{rubisco} and ρ_{bioe} ,
789 and may scale with changes in ρ_{rubisco} and ρ_{bioe} (Niinemets and Tenhunen 1997).

791 Finally, the proportion of leaf nitrogen content allocated to structural tissue
792 ($\rho_{\text{structure}}$; gN gN^{-1}) was estimated as:

$$\rho_{\text{structure}} = \frac{N_{\text{cw}}}{N_{\text{area}}} \quad (3.11)$$

793 where N_{cw} is the leaf nitrogen content allocated to cell walls (gN m^{-2}), calculated
794 as a function of M_{area} using an empirical equation from Onoda et al. (2017):

$$N_{\text{cw}} = 0.000355 * M_{\text{area}}^{1.39} \quad (3.12)$$

795 3.2.6 *Tradeoffs between nitrogen and water use*

796 Photosynthetic nitrogen use efficiency (PNUE; $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ N s}^{-1}$) was cal-
797 culated by dividing A_{net} by N_{area} , first converting N_{area} to mol N m^{-2} using the
798 molar mass of nitrogen (14 g mol^{-1}). I used χ as an indicator of water use effi-
799 ciency, which exploratory analyses suggest had similar responses to soil nitrogen

800 availability and pH as intrinsic water use efficiency measured from gas exchange
801 ($A_{\text{net}}/g_{\text{sw}}$). Tradeoffs between nitrogen and water use were determined by cal-
802 culating the ratio of N_{area} to χ ($N_{\text{area}}:\chi$; gN m⁻²) and V_{cmax25} to χ ($V_{\text{cmax25}}:\chi$;
803 $\mu\text{mol m}^{-2} \text{ s}^{-1}$). This approach is similar to tradeoff calculations in which nitrogen-
804 water use tradeoffs are measured as the ratio of N_{area} or V_{cmax25} to g_{sw} (Paillassa
805 et al. 2020; Bialic-Murphy et al. 2021). In this chapter, I quantify these rela-
806 tionships using χ in lieu of g_{sw} because g_{sw} rapidly changes with environmental
807 conditions and therefore may have been altered by recent tree branch severance
808 and/or placement in the cuvette.

809 3.2.7 *Soil nitrogen availability and pH*

810 To characterize soil nitrogen availability at the time of our leaf gas exchange
811 measurements, I used mixed bed resin bags to quantify mobile ammonium-N and
812 nitrate-N concentrations in each plot. Lycra mesh bags were filled with 5 g of
813 Dowex® Marathon MR-3 hydrogen and hydroxide form resin (MilliporeSigma,
814 Burlington, MA USA) and sealed with a zip tie. Each bag was activated by
815 soaking in 0.5 M HCl for 20 minutes, then in 2 M NaCl until pH of the saline
816 solution stabilized, as described in Allison et al. (2008). Five resin bags were
817 inserted about 10 cm below the soil surface at each plot on June 25, 2019: one
818 near each of the four plot corners and one near the plot center. All resin bags
819 were collected 24 days later on July 19, 2019 and were frozen until extracted.

820 Prior to anion and cation extraction, each resin bag was rinsed with ul-
821 trapure water (MilliQ IQ 7000; Millipore Sigma, Burlington, MA) to remove any
822 surface soil residues. Anions and cations were extracted from surface-cleaned

823 resin bags by individually soaking and shaking each bag in 100 mL of a 0.1 M
824 HCl/2.0 M NaCl matrix for one hour. Using a microplate reader (Biotek Synergy
825 H1; Biotek Instruments, Winooski, VT USA), I quantified nitrate-N concentra-
826 tions spectrophotometrically at 540 nm with the end product of a single reagent
827 vanadium (III) chloride reaction (Doane and Horwáth 2003), and ammonium-N
828 concentrations quantified at 650 nm with the end product of a modified phenol-
829 hypochlorite reaction (Weatherburn 1967; Rhine et al. 1998). Both the single
830 reagent vanadium (III) chloride and modified phenol-hypochlorite methodologies
831 are well established for determining nitrate-N and ammonium-N concentrations
832 in resin bag extracts (Arnone 1997; Allison et al. 2008). I used a series of nega-
833 tive and positive controls throughout each well plate to verify the accuracy and
834 precision of our measurements, assaying each resin bag extract and control in
835 triplicate. Soil nitrogen availability was estimated as the sum of the nitrate-N
836 and ammonium-N concentration in each resin bag, normalized per g of resin and
837 duration in the field ($\mu\text{g N g}^{-1} \text{ resin d}^{-1}$), then subsequently averaged across all
838 resin bags in a plot for a plot-level mean.

839 Soil pH was measured on 0-10 cm mineral soil samples collected prior to
840 fertilization in 2019. Near each of the four plot corners, three 5.5 cm diameter soil
841 cores were collected after first removing the forest floor where present. Each set
842 of three cores was placed in a plastic bag, and later composited by hand mixing
843 and sieved to 4mm. Soil pH was determined for a 1:2 soil:water slurry (10 g field-
844 moist soil to 20 mL DI water) of each sample using an Accumet AB15 pH meter
845 with flushable junction probe (Fisher Scientific; Hampton, NH, USA), and was
846 estimated at the plot level as the mean soil pH within each plot.

847 3.2.8 *Statistical analyses*

848 I built two separate series of linear mixed-effects models to explore effects of soil
849 nitrogen availability, soil pH, species, and leaf nitrogen content on leaf physiolog-
850 ical traits. In the first series of linear mixed-effects models, I explored the effect
851 of soil nitrogen availability, soil pH, and species on leaf nitrogen content, leaf
852 photosynthesis, stomatal conductance, and nitrogen-water use tradeoffs. Models
853 included plot-level soil nitrogen availability and plot-level soil pH as continuous
854 fixed effects, species as a categorical fixed effect, and site as a categorical ran-
855 dom intercept term. Interaction terms between fixed effects were not included
856 due to the small number of experimental plots. I built a series of separate mod-
857 els with this independent variable structure to quantify individual effects of soil
858 nitrogen availability, soil pH, and species on N_{area} , M_{area} , N_{mass} , A_{net} , V_{cmax25} ,
859 J_{max25} , $J_{\text{max25}}:V_{\text{cmax25}}$, ρ_{rubisco} , $\rho_{\text{bioenergetics}}$, ρ_{photo} , $\rho_{\text{structure}}$, χ , PNUE, $N_{\text{area}}:\chi$, and
860 $V_{\text{cmax25}}:\chi$.

861 A second series of linear mixed-effects models were built to investigate
862 relationships between leaf nitrogen content and photosynthetic parameters. Sta-
863 tistical models included N_{area} as a single continuous fixed effect with species and
864 site designated as individual random intercept terms. I used this independent
865 variable structure to quantify individual effects of leaf nitrogen content on A_{net} ,
866 V_{cmax25} , J_{max25} , $J_{\text{max25}}:V_{\text{cmax25}}$, and χ .

867 For all linear mixed-effects models, I used Shapiro-Wilk tests of normality
868 to determine whether linear mixed-effects models satisfied residual normality as-
869 sumptions. If residual normality assumptions were not met, then models were fit
870 using dependent variables that were natural log transformed. If residual normal-

871 ity assumptions were still not met (Shapiro-Wilk: $p<0.05$), then models were fit
872 using dependent variables that were square root transformed. All residual nor-
873 mality assumptions for both sets of models that did not originally satisfy residual
874 normality assumptions were met with either a natural log or square root data
875 transformation (Shapiro-Wilk: $p>0.05$ in all cases).

876 In the first series of models, models for N_{area} , M_{area} , N_{mass} , V_{cmax25} , J_{max25} ,
877 χ , $N_{\text{area}}:\chi$, and $V_{\text{cmax25}}:\chi$, ρ_{rubisco} , $\rho_{\text{bioenergetics}}$, ρ_{photo} , $\rho_{\text{structure}}$ satisfied residual
878 normality assumptions without data transformations (Shapiro-Wilk: $p>0.05$ in
879 all cases). The model for $J_{\text{max25}}:V_{\text{cmax25}}$ satisfied residual normality assumptions
880 with a natural log data transformation, while models for A_{net} and PNUE each
881 satisfied residual normality assumptions with square root data transformations.
882 In the second series of models, models for V_{cmax25} , J_{max25} , χ , and $V_{\text{cmax25}}:\chi$ satis-
883 fied residual normality assumptions without data transformations (Shapiro-Wilk:
884 $p>0.05$ in all cases). The model for $J_{\text{max25}}:V_{\text{cmax25}}$ required a natural log data
885 transformation and the model for A_{net} required a square root data transformation
886 (Shapiro-Wilk: $p>0.05$ in both cases).

887 In all models, I used the ‘lmer’ function in the ‘lme4’ R package (Bates
888 et al. 2015) to fit each model and the ‘Anova’ function in the ‘car’ R package
889 (Fox and Weisberg 2019) to calculate Type II Wald’s χ^2 and determine the signif-
890 icance level ($\alpha=0.05$) of each fixed effect coefficient. Finally, I used the ‘emmeans’
891 R package (Lenth, 2019) to conduct post-hoc comparisons using Tukey’s tests,
892 where degrees of freedom were approximated using the Kenward-Roger approach
893 (Kenward and Roger 1997). All analyses and plots were conducted in R version
894 4.1.1 (R Core Team 2021). All figure regression lines and associated 95% confi-

895 dence interval error bars were plotted using predictions generated across the soil
896 nitrogen availability gradient using the ‘emmeans’ R package (Lenth 2019).

897 3.3 Results

898 3.3.1 *Leaf nitrogen content*

899 Increasing soil nitrogen availability generally increased N_{area} (Table 3.1; Fig. 3.1a).
900 This pattern was driven by an increase in N_{mass} (Table 3.1; Fig. 3.1c) and a
901 marginal increase in M_{area} (Table 3.1; Fig. 3.1e) with increasing soil nitrogen
902 availability. There was no effect of soil pH on N_{area} , N_{mass} , or M_{area} (Table 3.1);
903 however, I also observed strong differences in N_{area} (Fig. 3.1b), N_{mass} (Fig. 3.1d),
904 and M_{area} (Fig. 3.1e) between species (Table 3.1).

Table 3.1. Effects of soil nitrogen availability, soil pH, and species on leaf nitrogen content per unit leaf area (N_{area} ; gN m⁻²), leaf nitrogen content per unit leaf mass (N_{mass} ; gN g⁻¹), and leaf mass per unit leaf area (M_{area} ; g m⁻²)*

	N_{area}			N_{mass}			M_{area}			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
Intercept	-	9.03E-01	-	-	1.68E+00	-	-	4.60E+01	-	-
Soil N	1	1.68E-02	11.990	0.001	1.25E-02	6.902	0.009	4.87E-01	4.143	0.042
Soil pH	1	9.28E-02	0.836	0.361	8.08E-02	0.663	0.415	4.05E+00	0.653	0.419
Species	4	-	72.128	<0.001	-	35.074	<0.001	-	29.869	<0.001

905 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values<0.05 are in bold.

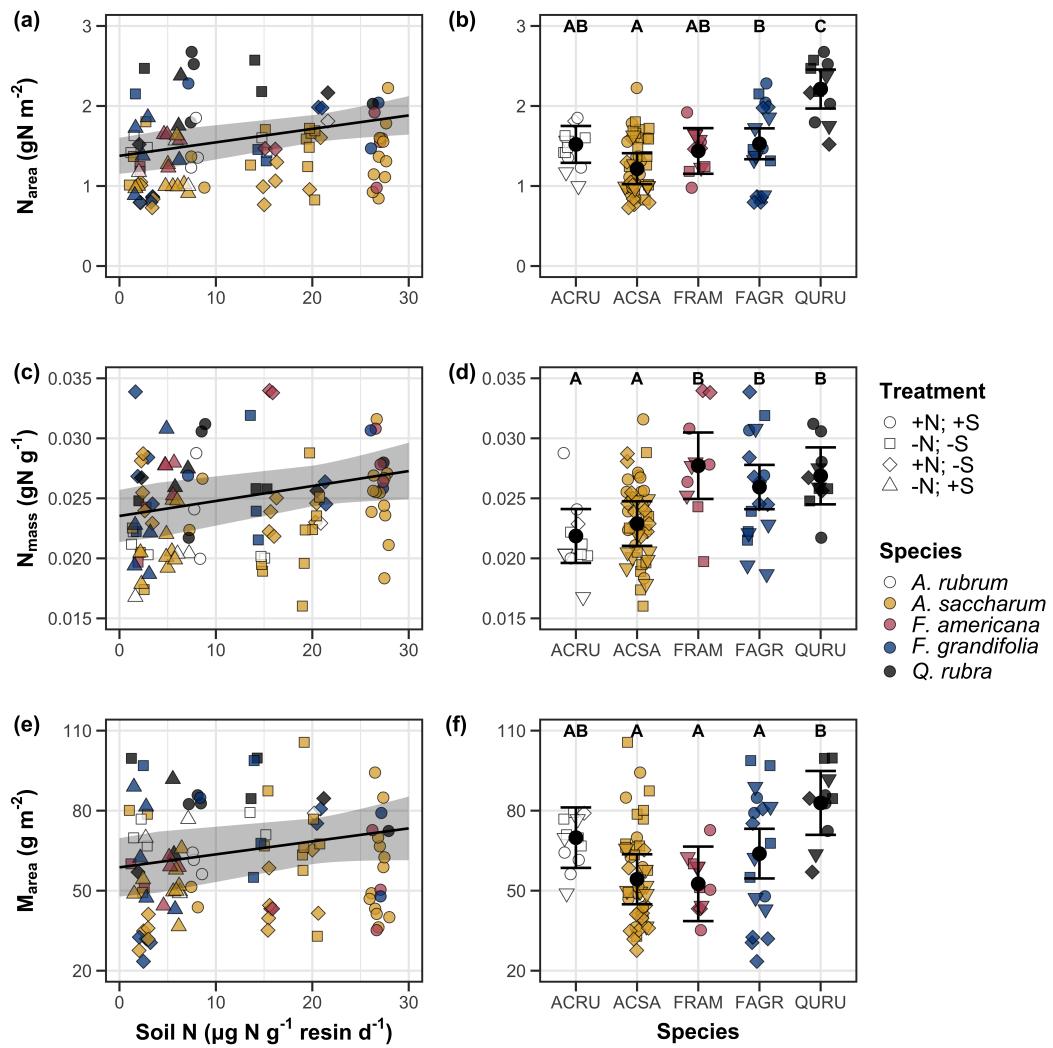


Figure 3.1. Effects of soil N availability and species on leaf nitrogen content per unit leaf area (a-b), leaf nitrogen content per unit leaf biomass (c-d), and leaf mass per unit leaf area (e-f). Soil nitrogen availability is represented on the x-axis in the left column of panels, while species is represented on the x-axis in the right column of panels. Tree species are represented as colored points and treatment plots are represented as shaped points, jittered for visibility. Species are abbreviated in the right column of panels through their assigned NRCS PLANTS Database symbol (USDA NRCS 2022), grouped along the x-axis per common mycorrhizal association, where the first three species commonly associate with arbuscular mycorrhizae (ACRU, ACSA, FAGR) and the second two species with ectomycorrhizae (FAGR, QURU). Trendlines are only included when the regression slope is statistically different from zero ($p < 0.05$).

906 3.3.2 *Net photosynthesis and leaf biochemistry*

907 Increasing soil nitrogen availability generally had no effect on A_{net} , V_{cmax25} , J_{max25} ,
908 or $J_{\text{max25}}:V_{\text{cmax25}}$ (Table 3.2, Figs. 3.2a, 3.2d, 3.2g). I also observed strong species
909 effects on all measured leaf photosynthetic traits (Table 3.2; Figs. 3.2b, 3.2e, 3.2h).
910 Increasing soil pH had a marginal negative effect on A_{net} , but had no effect on
911 V_{cmax25} , J_{max25} , or $J_{\text{max25}}:V_{\text{cmax25}}$ (Table 3.2). There was a weak positive effect of
912 increasing N_{area} on A_{net} (Fig. 3.2c), but quite strong positive effects of increasing
913 N_{area} on V_{cmax25} and J_{max25} (Table 3.2; Fig. 3.2f and 3.2i).

Table 3.2. Effects of soil nitrogen availability, soil pH, species, and N_{area} on net photosynthesis (A_{net} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$), the maximum rate of Rubisco carboxylation (V_{cmax25} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$), the maximum rate of RuBP regeneration (J_{max25} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$), and the ratio of the maximum rate of RuBP regeneration to the maximum rate of Rubisco carboxylation ($J_{\text{max25}}:V_{\text{cmax25}}$; unitless)*

	A_{net}			V_{cmax25}			J_{max25}			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	3.29E+00 ^b	-	-	6.38E+01	-	-	1.12E+02	-	-
Soil N	1	-1.23E-03 ^b	1.798	0.180	-3.84E-01	1.745	0.187	-6.70E-01	2.172	0.141
Soil pH	1	-3.09E-01 ^b	3.312	0.069	-4.91E+00	0.655	0.418	-8.18E+00	0.742	0.389
Species	4	-	11.838	0.019	-	31.748	<0.001	-	27.291	<0.001
(N_{area} int.)	-	6.59E-01 ^b	-	-	1.45E-01	-	-	2.86E+01	-	-
N_{area}	4	3.13E-01 ^b	4.790	0.029	2.43E+01	22.616	<0.001	4.04E+01	28.259	<0.001

	$J_{\text{max25}}:V_{\text{cmax25}}$			
	df	Coefficient	χ^2	p
(Intercept)	-	6.59E-01 ^a	-	-
Soil N	1	7.04E-04 ^a	0.088	0.767
Soil pH	1	-7.84E-03 ^a	0.025	0.874
Species	4	-	12.745	0.013
(N_{area} int.)	-	6.69E-01 ^a	-	-
N_{area}	4	-4.69E-02 ^a	1.142	0.285

54

914 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 915 between 0.05 and 0.1 are italicized. Superscript letters indicate model coefficients fit to natural-log (^a) or square-root
 916 (^b) transformed data. Relationships between N_{area} and each response variable were fit using the second series of
 917 bivariate mixed-effects models, so model coefficients and results are independent of model coefficients and results
 918 reported for relationships between soil nitrogen, soil pH, and species for each response variable.

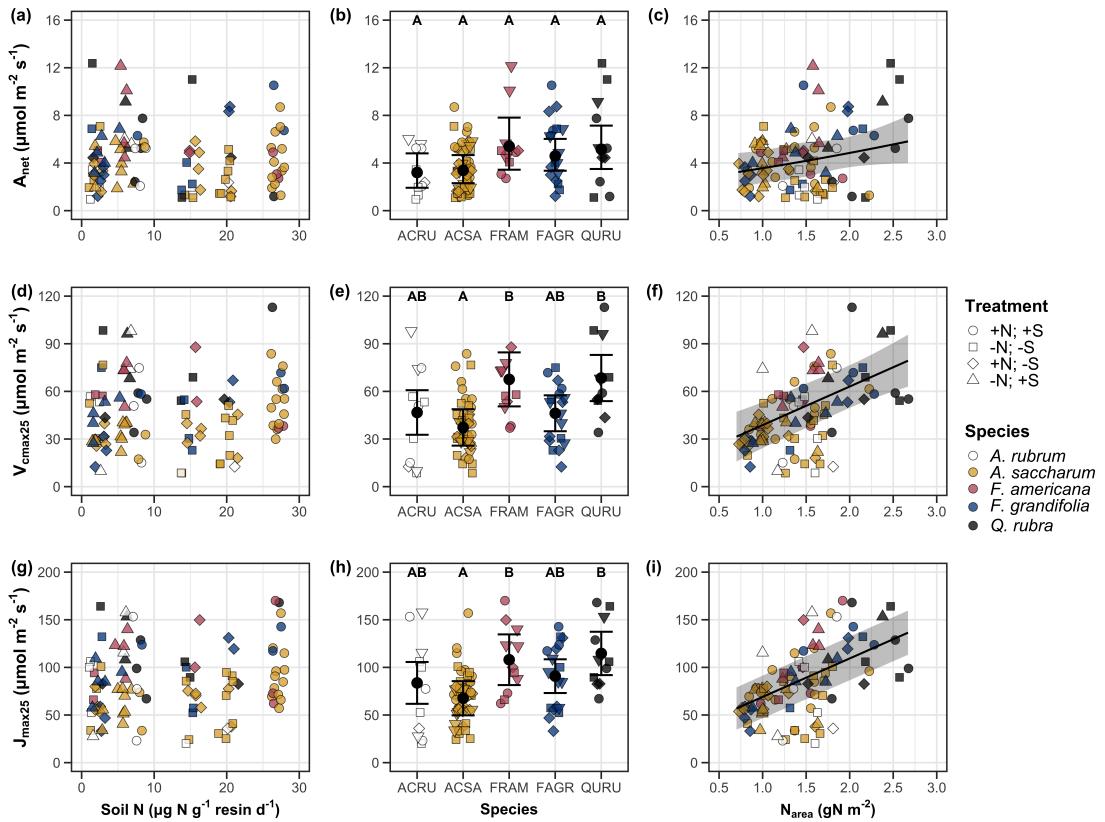


Figure 3.2. Effects of soil nitrogen availability (left column of panels), species (middle column of panels), and leaf nitrogen content per unit leaf area (right column of panels) on net photosynthesis (a-c), maximum Rubisco carboxylation rate (d-f), and maximum RuBP regeneration rate (g-i). Soil nitrogen availability is represented on the x-axis in the left column of panels, species is represented on the x-axis in the middle column of panels, and leaf nitrogen content per unit leaf area is represented continuously on the x-axis in the right column of panels. Species abbreviations and position along the x-axis in the middle column of panels, colored points, shapes, and trendlines are as explained in Figure 3.1.

919 3.3.3 *Leaf nitrogen allocation*

920 Neither soil nitrogen availability nor soil pH affected the proportion of leaf nitrogen
921 allocated to Rubisco or bioenergetics (Table 3.3; Fig. 3.3a, Fig. 3.3c). There was
922 also no effect of soil nitrogen availability or soil pH on the proportion of leaf
923 nitrogen allocated to photosynthesis (Table 3.3; Fig. 3.3f). I found no effect of
924 soil nitrogen availability or soil pH on the proportion of leaf nitrogen allocated to
925 structure (Table 3.3; Fig 3.3g). Species varied in the proportion of leaf nitrogen
926 allocated to Rubisco, photosynthesis, and structure (Fig 3.3b, Fig. 3.3f, Fig 3.3h),
927 with no detectable species effect on the proportion of leaf nitrogen allocated to
928 bioenergetics (Table 3.3, Fig. 3.3d).

Table 3.3. Effects of soil nitrogen availability, soil pH, and species on the proportion of leaf nitrogen content allocated to photosynthesis (ρ_{photo} ; gN gN⁻¹), Rubisco (ρ_{rubisco} ; gN gN⁻¹), bioenergetics (ρ_{bioe} ; gN gN⁻¹), and structure ($\rho_{\text{structure}}$; gN gN⁻¹)*

	ρ_{photo}			ρ_{rubisco}			ρ_{bioe}			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
Intercept	-	4.93E-01	-	-	4.17E-01	-	-	7.64E-02	-	-
Soil N	1	-1.23E-03	0.521	0.470	-1.04E-03	0.501	0.479	-1.77E-04	0.557	0.455
Soil pH	1	-4.37E-02	1.581	0.209	-3.70E-02	1.511	0.219	-6.84E-03	1.941	0.164
Species	4	-	13.106	0.011	-	14.152	0.007	-	7.300	0.121

	$\rho_{\text{structure}}$			
	df	Coefficient	χ^2	p
Intercept	-	9.77E-02	-	-
Soil N	1	-2.29E-04	1.165	0.280
Soil pH	1	-1.87E-03	0.179	0.672
Species	4	-	16.428	0.002

929 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold.

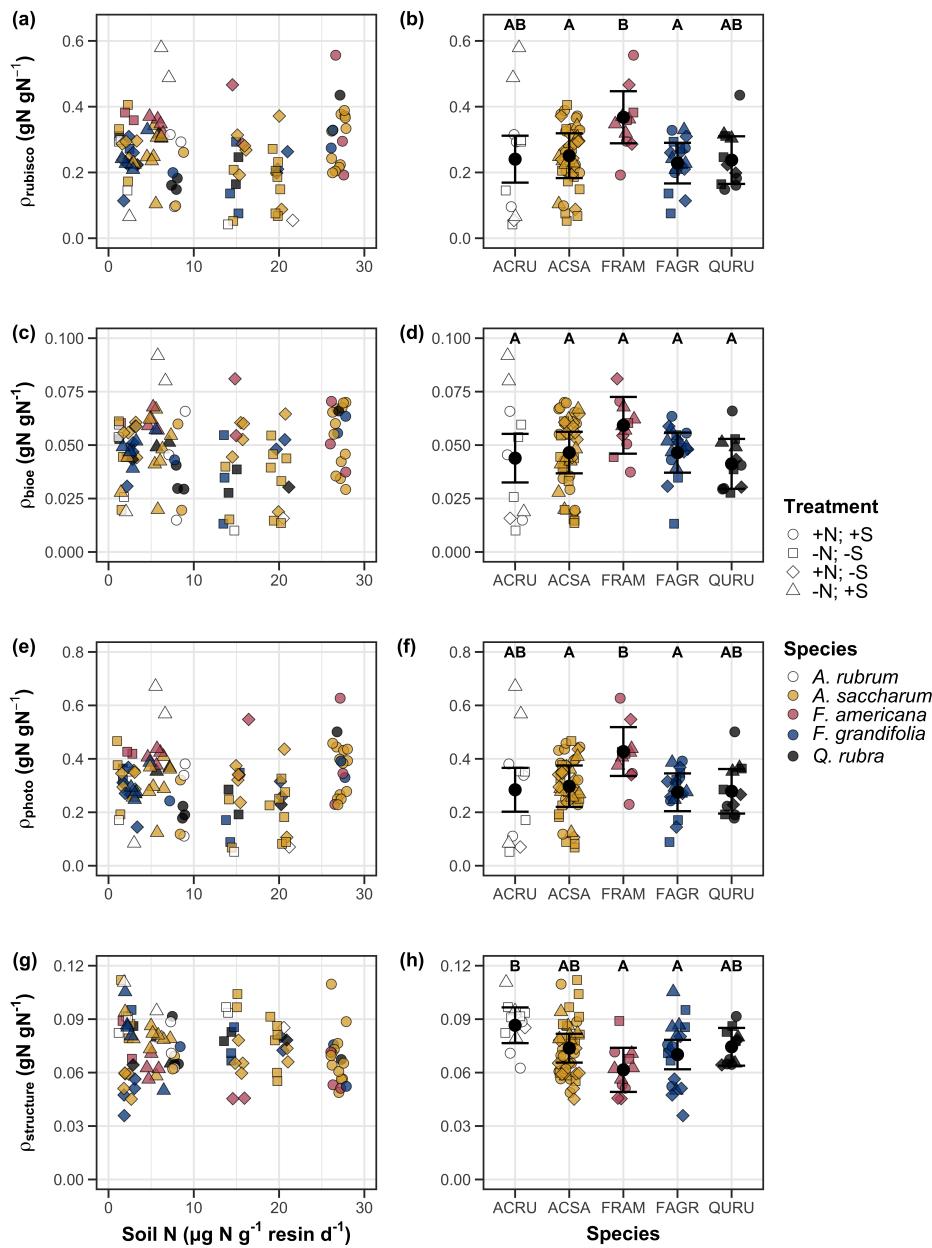


Figure 3.3. Effects of soil nitrogen availability and species on the proportion of leaf nitrogen content allocated to Rubisco (a-b), bioenergetics (c-d), photosynthesis (e-f), and structure (g-h). Soil nitrogen availability is represented on the x-axis in the left column of panels and species are represented on the x-axis in the right column of panels. Species abbreviations and position along the x-axis in the middle column of panels, colored points, shapes, trendlines, error bars, and compact lettering are as explained in Figure 3.1.

930 3.3.4 *Tradeoffs between nitrogen and water use*

931 Although soil nitrogen availability did not affect χ (Table 3.4; Fig. 3.4a), increasing
932 soil nitrogen availability decreased PNUE (Table 3.4; Fig. 3.4d) and increased
933 the ratio of $N_{\text{area}}:\chi$ (Table 3.4; Fig. 3.4f). Specifically, this response yielded a
934 26% reduction in PNUE and 37% stimulation in $N_{\text{area}}:\chi$ across the soil nitrogen
935 availability gradient. There was no apparent effect of soil nitrogen availability on
936 $V_{\text{cmax25}}:\chi$ (Table 3.4; Fig. 3.4h). Increasing soil pH had a weak marginal nega-
937 tive effect on PNUE, but did not influence χ , $N_{\text{area}}:\chi$, or $V_{\text{cmax25}}:\chi$ (Table 3.4). I
938 observed differences in χ (Fig. 3.4b), PNUE (Fig. 3.4e), $N_{\text{area}}:\chi$ (Fig. 3.4g), and
939 $V_{\text{cmax25}}:\chi$ (Fig. 3.4i) between species (Table 3.4). Finally, increasing N_{area} had a
940 strong negative effect on χ (Table 3.4; Fig. 3.4c) and a strong positive effect on
941 $V_{\text{cmax25}}:\chi$ (Table 3.4; Fig. 3.4j).

Table 3.4. Effects of soil nitrogen availability, soil pH, species, and N_{area} on χ (unitless), photosynthetic nitrogen use efficiency (PNUE; $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ N s}^{-1}$), leaf nitrogen content per unit χ ($N_{\text{area}}:\chi$; gN m^{-2}), and maximum Rubisco carboxylation rate per unit χ ($V_{\text{cmax25}}:\chi$; $\mu\text{mol m}^{-2} \text{ s}^{-1}$)^{*}

	χ			PNUE			$N_{\text{area}}:\chi$			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	8.12E-01	-	-	9.57E+00 ^b	-	-	9.19E-01	-	-
Soil N	1	-1.14E-03	1.698	0.193	-6.63E-02 ^b	6.396	0.011	2.60E-02	9.533	0.002
Soil pH	1	-1.91E-02	1.087	0.297	-9.25E-01 ^b	2.843	<i>0.092</i>	2.03E-01	1.321	0.250
Species	4	-	18.843	0.001	-	13.454	0.009	-	52.983	<0.001
(N_{area} int.)	-	8.93E-01	-	-	-	-	-	-	-	-
N_{area}	1	-1.11E-01	80.606	<0.001	-	-	-	-	-	-

	$V_{\text{cmax25}}:\chi$			
	df	Coefficient	χ^2	p
(Intercept)	-	7.20E+01	-	-
Soil N	1	3.99E-01	0.963	0.326
Soil pH	1	-3.12E+00	0.138	0.711
Species	4	-	31.450	<0.001
(N_{area} int.)	-	1.18E+01	-	-
N_{area}	4	3.87E+01	32.797	<0.001

60

942 *Significance determined using Type II Wald χ^2 tests ($\alpha = 0.05$). P-values less than 0.05 are in bold, while p-values
 943 between 0.05 and 0.1 are italicized. Superscript letters indicate model coefficients fit to natural-log (^a) or square-root
 944 (^b) transformed data. Relationships between N_{area} and each response variable were fit using the second series of
 945 bivariate mixed-effects models, so model coefficients and results are independent of model coefficients and results
 946 reported for relationships between soil nitrogen, soil pH, and species for each response variable.

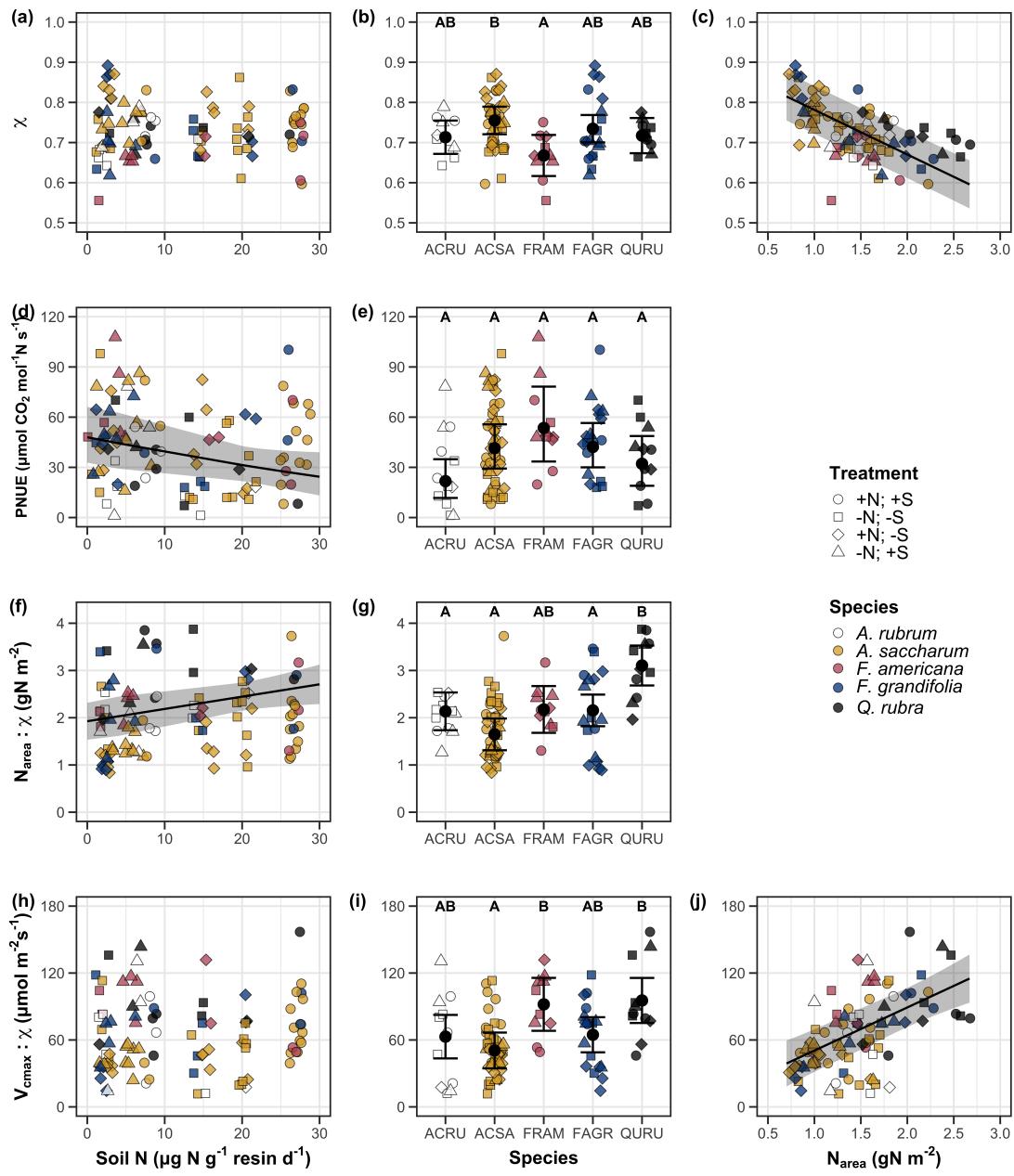


Figure 3.4. Effects of soil nitrogen availability and species on the proportion of leaf nitrogen content allocated to Rubisco (a-b), bioenergetics (c-d), photosynthesis (Rubisco + bioenergetics; e-f), and structure (g-h). Soil nitrogen availability is represented on the x-axis in the left column of panels and species are represented on the x-axis in the right column of panels. Species abbreviations and position along the x-axis in the middle column of panels, colored points, shapes, trendlines, error bars, and compact lettering are as explained in Figure 3.1.

947 3.4 Discussion

948 Photosynthetic least-cost theory provides an explanation for understanding rela-
949 tionships between soil nutrient availability, leaf nutrient allocation, and photosyn-
950 thetic capacity. The theory suggests that plants acclimate to a given environment
951 by optimizing leaf photosynthesis rates at the lowest summed cost of using nu-
952 trients and water (Prentice et al. 2014; Wang et al. 2017; Smith et al. 2019;
953 Paillassa et al. 2020). The theory predicts that an increase in soil nutrient avail-
954 ability should allow similar photosynthesis rates to be achieved with increased leaf
955 nutrient content and photosynthetic capacity (i.e., V_{cmax25} and J_{max25}) at lower
956 leaf $C_i:C_a$ (χ), resulting in an increase in water use efficiency, decrease in nutri-
957 ent use efficiency, and increase in both leaf nutrient content and photosynthetic
958 capacity per unit χ . The theory predicts similar leaf responses to increasing soil
959 pH under acidic conditions, presumably due to generally faster nutrient cycle dy-
960 namics and consequent reductions in the cost of acquiring nutrients relative to
961 water with increasing soil pH (Wang et al. 2017; Paillassa et al. 2020; Dong et al.
962 2020).

963 Supporting the theory, increasing soil nitrogen availability was associated
964 with increased leaf nitrogen content, a pattern that reduced photosynthetic nitro-
965 gen use efficiency and increased leaf nitrogen content per unit χ . Increasing soil
966 nitrogen coincided with slight, but non-significant decreases in χ and increases
967 in V_{cmax25} and J_{max25} ($p<0.2$, Table 3.2). The positive trend between soil ni-
968 trogen availability and photosynthetic capacity was supported by the concurrent
969 strong increase in leaf nitrogen content with increasing soil nitrogen availability,
970 which resulted in no change in the proportion of leaf nitrogen content allocated to

971 photosynthesis across the soil nitrogen availability gradient. Additionally, leaf ni-
972 trogen content exhibited a strong negative correlation with χ , indicative of strong
973 nitrogen-water use tradeoffs at the leaf level. Responses tended to vary more due
974 to soil nitrogen availability than soil pH. Overall, these findings are consistent
975 with the nutrient-water use tradeoffs predicted from theory.

976 3.4.1 *Soil nitrogen availability modifies tradeoffs between nitrogen and water use*
977 In support of expected least-cost outcomes and past environmental gradient stud-
978 ies (Dong et al. 2017; Paillassa et al. 2020), increasing soil nitrogen availability
979 was associated with increased leaf nitrogen content. Soil nitrogen availability had
980 smaller impacts on measures of net photosynthesis and χ , which led to reductions
981 in PNUE and increases in leaf nitrogen content per unit χ , as expected from the-
982 ory. Photosynthetic least-cost theory suggests that reductions in PNUE should
983 be driven by an increase in the proportion of leaf nitrogen allocated to photosyn-
984 thetic tissue, a pattern that should allow plants to achieve optimal photosynthetic
985 rates with greater photosynthetic capacity to make better use of available light.
986 Contrasting theory predictions, I found no effect of soil nitrogen availability on
987 photosynthetic capacity. However, photosynthetic capacity did tend to increase
988 with increasing soil nitrogen availability ($p<0.20$; Table 3.2) resulting in no effect
989 of soil nitrogen availability on the relative fraction of leaf nitrogen allocated to
990 photosynthesis, Rubisco, or bioenergetics. These lines of evidence support the
991 idea that trees use additional nitrogen to support increased leaf nitrogen alloca-
992 tion toward photosynthetic tissue and enhance photosynthetic capacity (Wright
993 et al. 2003).

994 Soil nitrogen availability had a stronger effect on leaf nitrogen than photo-
995 synthetic capacity. This pattern suggests that additional plant nitrogen up-
996 take due to increased soil nitrogen availability was also being used to support
997 non-photosynthetic nitrogen pools, possibly to structural tissue or stress-induced
998 amino acid and polyamine synthesis (Minocha et al. 2000; Onoda et al. 2004;
999 Bubier et al. 2011). While I found no change in the proportion of leaf nitrogen
1000 allocated to leaf structural tissue, the overall stimulation in leaf nitrogen content
1001 with increasing soil nitrogen availability suggests an increase in the net amount of
1002 nitrogen invested in leaf structural tissue along the nitrogen availability gradient.
1003 Importantly, leaf nitrogen allocated to structure was calculated using an empiri-
1004 cal relationship between M_{area} and the amount of leaf nitrogen allocated to cell
1005 walls (Onoda et al. 2017). As the generality of relationships between M_{area} and
1006 the amount of leaf nitrogen allocated to cell walls has been called into question
1007 (Harrison et al. 2009), future work should consider explicitly measuring nitrogen
1008 allocation to cell wall tissue and stress-induced amino acid synthesis to confirm
1009 these patterns.

1010 In opposition to patterns expected from least-cost theory, increasing soil
1011 nitrogen availability had no apparent effect on χ . Interestingly, despite the null
1012 effect of soil nitrogen availability on χ , I observed a strong negative effect of in-
1013 creasing N_{area} on χ , consistent with the nitrogen-water use tradeoffs expected from
1014 theory. The null response of χ to increasing soil nitrogen availability may have
1015 been due to a lack of water limitation in the system, given that the area received
1016 approximately 20% more precipitation (1167 mm) during the 12-month period
1017 leading up to our measurement period than normally expected (972 mm). How-

1018 ever, droughts can and do occur in temperate forests of the northeastern United
1019 States (Sweet et al. 2017), so the observed increase in leaf nitrogen content with
1020 increasing soil nitrogen availability could be a strategy that allows trees to hedge
1021 bets against drier than normal growing seasons (Onoda et al. 2004; Onoda et al.
1022 2017; Hallik et al. 2009). As was suggested in Paillassa et al. (2020), and more
1023 recently by Querejeta et al. (2022), negative effects of soil nitrogen availabil-
1024 ity on χ may increase with increasing aridity. This strategy would be especially
1025 advantageous if it allows individuals growing in arid regions to maintain carbon
1026 assimilation rates with reduced water loss. Future work should attempt to quan-
1027 tify interactive roles of climate and soil nitrogen availability on nitrogen-water use
1028 tradeoffs, which could be done by leveraging coordinated and multifactor nutrient
1029 (Borer et al. 2014) and water (Knapp et al. 2017) manipulation experiments
1030 across broad climatic gradients.

1031 3.4.2 *Soil pH did not modify tradeoffs between nitrogen and water usage*

1032 While the primary purpose of this study was to examine the role of soil nitrogen
1033 availability on nitrogen-water use tradeoffs, this experimental design manipulated
1034 both soil nitrogen and pH, providing an opportunity to isolate the roles of these
1035 variables. Previous correlational studies along environmental gradients have iden-
1036 tified soil pH as a particularly important factor that can modify tradeoffs between
1037 nutrient and water use (Smith et al. 2019; Paillassa et al. 2020; Westerband et al.
1038 2023) and the proportion of leaf nitrogen allocated to photosynthesis (Luo et al.
1039 2021). Such studies implied that these patterns may be driven by reductions in
1040 the cost of acquiring nutrients relative to water with increasing pH, which may

1041 be exacerbated in acidic soils.

1042 Consistent with theory (Wright et al. 2003; Prentice et al. 2014), results
1043 indicate that increasing soil pH was negatively associated with PNUE. However,
1044 there was no effect of soil pH on leaf nitrogen content, χ , or leaf nitrogen content
1045 per unit χ , most likely because the experimental nitrogen additions increased soil
1046 nitrogen supply while both increasing (sodium nitrate) and decreasing (ammo-
1047 nium sulfate) soil pH. These results suggest that soil pH did not play a major
1048 role in modifying expected photosynthetic least-cost theory patterns, contrasting
1049 findings from Paillassa et al. (2020) and other gradient studies that note positive
1050 effects of increasing soil pH on leaf nitrogen content, Rubisco carboxylation, and
1051 χ (Viet et al. 2013; Cornwell et al. 2018; Luo et al. 2021). Instead, null responses
1052 to soil pH show that leaf photosynthetic parameters depend more on soil nitrogen
1053 availability than pH per se, and that inferences from gradient studies might be
1054 confounding covariation between nitrogen availability and soil acidity.

1055 3.4.3 *Species identity explains a large amount of variation in leaf and whole*
1056 *plant traits*

1057 Species generally explained a larger amount of variation in measured leaf traits
1058 than soil nitrogen availability or soil pH. Interspecies variation is an important
1059 factor to consider when deducing mechanisms that drive photosynthetic least-
1060 cost theory, particularly for species that form distinct mycorrhizal associations or
1061 have different photosynthetic pathways, growth forms, or leaf habit (Espelta et al.
1062 2005; Adams et al. 2016; Bialic-Murphy et al. 2021; Scott and Smith 2022). The
1063 need to consider species may also be important when comparing nutrient-water

1064 use tradeoffs in early and late successional species, or in species with different
1065 resource economic strategies (Abrams and Mostoller 1995; Ellsworth and Reich
1066 1996; Wright et al. 2004; Reich 2014; Onoda et al. 2017; Ziegler et al. 2020).

1067 A strength of the study design and sampling effort is that it controls for
1068 many species differences that should modify nitrogen-water use tradeoffs expected
1069 from theory. All tree species measured in this study shared the leaf habit of de-
1070 ciduous broadleaves, were growing in forests of similar successional stage, but
1071 differed in mycorrhizal association and consequent resource economic strategies.
1072 As stands tended to be dominated by trees that associate with arbuscular myc-
1073 orrhizae (*Fraxinus* and both *Acer* species made up roughly 70% of total above-
1074 ground biomass across stands), ecosystem biogeochemical cycle dynamics may be
1075 more closely aligned to the inorganic nutrient economy proposed in Phillips et al.
1076 (2013), which may promote stronger nitrogen-water use tradeoffs in tree species
1077 that associate with arbuscular mycorrhizae. This result was not observed here,
1078 as photosynthetic properties varied as much within as across the two mycorrhizal
1079 associations represented. Given the high variability in measured photosynthetic
1080 traits within and across species, effects of mycorrhizal association likely require
1081 more intensive sampling efforts to detect than were possible here.

1082 3.4.4 *Implications for photosynthetic least-cost theory model development*

1083 In the field, soil nutrient availability is heterogeneous across time and space (Ta-
1084 ble B4). Unaccounted within-plot heterogeneity may have contributed to the low
1085 amount of variation explained by soil nitrogen availability in statistical models,
1086 as resin bags are a coarse surrogate for soil nitrogen availability. Despite this, I

1087 still observed evidence for nutrient-water use tradeoffs, suggesting that observed
1088 responses reported here may be an underestimate toward the net effect of soil ni-
1089 trogen availability on these tradeoffs. While I urge caution in the interpretation of
1090 these results, they do provide a promising baseline for future studies investigating
1091 patterns expected from photosynthetic least-cost theory at finer spatiotemporal
1092 resolutions.

1093 The general stronger relationship between leaf nitrogen content and photo-
1094 synthetic parameters versus between leaf nitrogen content and soil nitrogen avail-
1095 ability suggests that leaf nitrogen content is more directly tied to photosynthesis
1096 than soil nitrogen availability. While this could be due to the high spatiotemporal
1097 heterogeneity of soil nitrogen availability, principles from photosynthetic least-
1098 cost theory suggest that leaf nitrogen content is the downstream product of leaf
1099 nutrient demand to build and maintain photosynthetic machinery, which is set by
1100 aboveground environmental conditions such as light availability, CO₂, tempera-
1101 ture, or vapor pressure deficit (Smith et al. 2019; Paillassa et al. 2020; Peng et al.
1102 2021; Westerband et al. 2023). The stronger relationship between leaf nitrogen
1103 and photosynthetic parameters, paired with the strong negative relationship be-
1104 tween leaf nitrogen and χ , could indicate a relatively stronger effect of climate on
1105 leaf nitrogen-photosynthesis relationships than soil resource availability. However,
1106 the short distance between plots and across sites limited my ability to test this
1107 mechanism.

1108 Variation in soil pH affected least cost responses less than variations in soil
1109 nitrogen availability, in part because experimental treatments directly increased
1110 soil nitrogen and affected soil pH in opposite directions. While soil pH has been

1111 shown to drive nitrogen-water tradeoffs in global gradient analyses (Viet et al.
1112 2013; Paillassa et al. 2020), these responses may be due to covariations between
1113 soil pH and nutrient cycling rather than a role of pH per se. The direct manipula-
1114 tions of soil pH and soil nitrogen availability in this study partly disentangle these
1115 factors and show that variation in nitrogen availability matters more for least-cost
1116 tradeoffs than pH alone.

1117 3.4.5 *Conclusions*

1118 Increasing soil nitrogen availability generally increased leaf nitrogen content (both
1119 area- and mass-based), but did not significantly influence χ . This shift in leaf ni-
1120 trogen led to a reduction in PNUE, and an increase in leaf nitrogen per unit
1121 χ with increasing soil nitrogen availability. Despite null effects of soil nitrogen
1122 availability on χ , I observed a strong negative relationship between leaf nitrogen
1123 content and χ . These results provide empirical support for the nutrient-water use
1124 tradeoffs expected from photosynthetic least-cost theory in response to increas-
1125 ing soil nutrient availability, but suggest that all tenets of the theory may not
1126 hold in every environment. These results experimentally test previous work sug-
1127 gesting that leaf nitrogen-water economies vary across gradients of soil nutrient
1128 availability and pH, and show that variations in nutrient availability matter more
1129 for determining variation in leaf photosynthetic traits than soil pH.

1130

Chapter 4

1131 The relative cost of resource use for photosynthesis drives variance in
1132 leaf nitrogen content across a climate and soil resource availability
1133 gradient

1134 4.1 Introduction

1135 Terrestrial biosphere models, which comprise the land surface component of Earth
1136 system models, are sensitive to the formulation of photosynthetic processes (Knorr
1137 and Heimann 2001; Ziehn et al. 2011; Booth et al. 2012; Walker et al. 2021).
1138 This is because photosynthesis is the largest carbon flux between the atmosphere
1139 and terrestrial biosphere (IPCC 2021), and is constrained by ecosystem carbon
1140 and nutrient cycles (Hungate et al. 2003; LeBauer and Treseder 2008; Fay et al.
1141 2015). Many terrestrial biosphere models formulate photosynthesis by parame-
1142 terizing photosynthetic capacity within plant functional groups through empiri-
1143 cal linear relationships between area-based leaf nitrogen content (N_{area}) and the
1144 maximum carboxylation rate of Ribulose-1,5-bisphosphate carboxylase/oxygenase
1145 (V_{cmax}) (Kattge et al. 2009; Rogers 2014; Rogers et al. 2017). Models are also
1146 beginning to include connected carbon-nitrogen cycles (Wieder et al. 2015; Shi
1147 et al. 2016; Davies-Barnard et al. 2020; Braghieri et al. 2022), which allows leaf
1148 photosynthesis to be predicted directly through changes in N_{area} and indirectly
1149 through changes in soil nitrogen availability (e.g., LPJ-GUESS, CLM5.0) (Smith
1150 et al. 2014; Lawrence et al. 2019). Despite recent model developments, open
1151 questions remain regarding the generality of ecological relationships between soil
1152 nitrogen availability, leaf nitrogen content, and leaf photosynthesis across edaphic
1153 and climatic gradients.

1154 Empirical support for positive relationships between soil nitrogen availabil-
1155 ity and N_{area} is abundant (Firn et al. 2019; Liang et al. 2020), and is a result
1156 often attributed to the high nitrogen cost of building and maintaining Rubisco
1157 (Evans 1989; Evans and Seemann 1989; Onoda et al. 2004; Walker et al. 2014;
1158 Onoda et al. 2017; Dong et al. 2020). Such patterns imply that positive relation-
1159 ships between soil nitrogen availability and N_{area} should increase leaf photosyn-
1160 thesis and photosynthetic capacity by increasing the maximum rate of Rubisco
1161 carboxylation through increased investments to Rubisco construction and mainte-
1162 nance. This integrated N_{area} -photosynthesis response to soil nitrogen availability
1163 has been observed both in manipulative experiments and across environmental
1164 gradients (Field and Mooney 1986; Evans 1989; Walker et al. 2014; Li et al.
1165 2020), and is thought to be driven by ecosystem nitrogen limitation, which lim-
1166 its primary productivity globally (LeBauer and Treseder 2008; Fay et al. 2015).
1167 However, this response is not consistently observed, as recent studies note variable
1168 N_{area} -photosynthesis relationships across edaphic and climatic gradients (Liang
1169 et al. 2020; Luo et al. 2021) and that aboveground growing conditions (e.g., light
1170 availability, temperature, vapor pressure deficit) or species identity traits (e.g.,
1171 photosynthetic pathway, nitrogen acquisition strategy) may be more important
1172 for explaining variance in N_{area} and photosynthetic capacity across environmental
1173 gradients (Adams et al. 2016; Dong et al. 2017; Smith et al. 2019; Dong et al.
1174 2020; Peng et al. 2021; Dong et al. 2022; Westerband et al. 2023).

1175 One hypothesized mechanism to explain variance in N_{area} across environ-
1176 mental gradients has been proposed via photosynthetic least-cost theory (Wright
1177 et al. 2003; Prentice et al. 2014; Paillassa et al. 2020; Harrison et al. 2021).

1178 The theory predicts that plants acclimate to environments by optimizing photo-
1179 synthetic assimilation rates at the lowest summed cost of nitrogen and water use
1180 (Wright et al. 2003; Prentice et al. 2014). In a given environment, the theory
1181 suggests that nitrogen and water use can be substituted for each other to maintain
1182 the lowest summed cost of resource use, such that optimal photosynthetic rates
1183 are achieved with less efficient use of the more abundant and less costly resource
1184 to acquire in exchange for more efficient use of the less abundant and more costly
1185 resource to acquire.

1186 Photosynthetic least-cost theory predicts that, all else equal, an increase in
1187 soil nitrogen availability should decrease the cost of acquiring and using nitrogen
1188 relative to water (a ratio referred to herein as β), resulting in optimal photosyn-
1189 thetic rates achieved with greater N_{area} at lower stomatal conductance and lower
1190 leaf $C_i:C_a$ (Wright et al. 2003; Prentice et al. 2014; Paillassa et al. 2020). Alter-
1191 natively, an increase in soil moisture should reduce costs of water acquisition and
1192 use, increasing β (Lavergne et al. 2020), stomatal conductance, and leaf $C_i:C_a$,
1193 resulting in optimal photosynthetic rates achieved with decreased N_{area} . The the-
1194 ory also predicts variability in stomatal conductance and N_{area} in response to
1195 climatic factors, suggesting that the optimal response to increased vapor pressure
1196 deficit (VPD) should be a reduction in stomatal conductance and leaf $C_i:C_a$ that
1197 is counterbalanced by an increase in N_{area} to support the greater photosynthetic
1198 capacity needed to maintain high assimilation at lower conductance (Grossiord
1199 et al. 2020; Dong et al. 2020; López et al. 2021; Westerband et al. 2023).

1200 Leaf nitrogen allocation responses to changing climates or soil resource
1201 availability may also depend on their mode of nutrient acquisition or photo-

1202 synthetic pathway. For example, species that form associations with symbiotic
1203 nitrogen-fixing bacteria (referred as “N-fixing species” from this point forward)
1204 should, in theory, have access to less finite nitrogen supply than species not capa-
1205 ble of forming such associations (referred as “non-fixing species” from this point
1206 forward), which may result in lower β values in N-fixing species than non-fixing
1207 species. This result was previously shown in a greenhouse experiment, where a
1208 leguminous species generally had lower costs of nitrogen acquisition compared to a
1209 non-leguminous species, although these differences were generally stronger under
1210 increased nitrogen limitation (Perkowski et al. 2021). Lower β values could be an
1211 explanation for why N-fixing species commonly have greater leaf nitrogen content
1212 than non-fixing species (Adams et al. 2016; Dong et al. 2017).

1213 Similarly, leaf nitrogen allocation patterns across environmental gradients
1214 may be dependent on photosynthetic pathway. Lower leaf $C_i:C_a$ values in C₄
1215 species suggests that C₄ species should have lower β values than C₃ species (Scott
1216 and Smith 2022), a pattern that could be the result of increased costs associated
1217 with water acquisition and use or reduced costs of nitrogen acquisition and use
1218 relative to C₃ species. Theory predicts that this response in C₄ species will cause
1219 C₄ species to have higher leaf nitrogen content on average compared to C₃ species,
1220 though ample evidence exists documenting general lower leaf nitrogen content in
1221 C₄ species (Schmitt and Edwards 1981; Sage and Pearcy 1987; Ghannoum et al.
1222 2011). No study to date has directly quantified β in C₄ species aside from the
1223 initial parameterization of β in an optimality model for C₄ species (Scott and
1224 Smith 2022) using a global dataset of leaf $\delta^{13}\text{C}$ values (Cornwell et al. 2018).

1225 While photosynthetic least-cost theory provides a unified framework for

1226 understanding integrated effects of climate and soil resource availability on N_{area} ,
1227 empirical tests of the theory are sparse. Previous work shows that increasing
1228 soil nitrogen availability decreases costs of acquiring nutrients (Bae et al. 2015;
1229 Perkowski et al. 2021; Lu et al. 2022), which can induce predictable nutrient-
1230 water use tradeoffs expected from the theory across broad environmental gradients
1231 (Paillassa et al. 2020; Querejeta et al. 2022; Westerband et al. 2023) and in
1232 manipulation experiments (Bialic-Murphy et al. 2021). Additionally, increasing
1233 vapor pressure deficit has been shown to have a positive effect on N_{area} , which is
1234 commonly associated with reduced leaf $C_i:C_a$ (Dong et al. 2017; Dong et al. 2020;
1235 Firn et al. 2019; López et al. 2021).

1236 Despite evidence for patterns expected from photosynthetic least-cost the-
1237 ory, studies have been restricted to exploring these patterns in C₃ species and,
1238 while variance in N_{area} across environmental gradients has been shown to be driven
1239 by strong negative relationships with leaf $C_i:C_a$ (Dong et al. 2017; Paillassa et al.
1240 2020; Westerband et al. 2023), no study has explicitly investigated effects of soil
1241 resource availability or species identity on N_{area} using β as a direct predictor of
1242 leaf $C_i:C_a$. Furthermore, as N_{area} can be broken down into structural (leaf mass
1243 per area; M_{area} ; g m⁻²) and metabolic (mass-based leaf nitrogen content; N_{mass} ;
1244 gN g⁻¹) components (Dong et al. 2017), no study has investigated which compo-
1245 nent of N_{area} drives the hypothesized response of N_{area} to leaf $C_i:C_a$, which limits
1246 our ability to assess whether changes in N_{area} across environmental gradients are
1247 driven by changes in leaf morphology (i.e. M_{area}), leaf stoichiometry (i.e. N_{mass}),
1248 or both.

1249 In this study, I measured N_{area} , N_{mass} , M_{area} , leaf $\delta^{13}\text{C}$ -derived estimates

1250 of leaf $C_i:C_a$, and leaf $\delta^{13}\text{C}$ -derived estimates of β in 504 individuals spanning
1251 52 species scattered across 24 grassland sites in Texas, USA. The state of Texas
1252 contains a diverse climatic gradient, indicated by 2006-2020 mean annual precipi-
1253 tation totals ranging from 204 to 1803 mm and 2006-2020 mean annual tempera-
1254 ture ranging from 11.8° to 24.6°C within state boundaries (Fig. 4.1). Variability
1255 in soil nitrogen availability and soil moisture was expected across sites, owing to
1256 differences in soil texture and aboveground climate that would drive differential
1257 rates of water retention and nitrogen transformations to plant-available nitrogen
1258 substrate. I leveraged the expected climatic and soil resource variability across
1259 sites to test the following hypotheses:

- 1260 1. Soil nitrogen availability will decrease β through a reduction in costs of
1261 nitrogen acquisition and use, while soil moisture will increase β through a
1262 reduction in costs of water acquisition and use. Following previous results, I
1263 expected that N-fixing species would have lower β values and that C_4 species
1264 would have lower β values.
- 1265 2. Leaf $C_i:C_a$ will be positively related to β , a pattern that will result in a
1266 negative indirect effect of increasing soil nitrogen availability on leaf $C_i:C_a$,
1267 a positive indirect effect of increasing soil moisture on leaf $C_i:C_a$, and lower
1268 leaf $C_i:C_a$ in both N-fixing species and C_4 species. I expected that leaf
1269 $C_i:C_a$ would be negatively related to vapor pressure deficit, as increasing
1270 atmospheric dryness would cause plants to close stomata to minimize water
1271 loss.
- 1272 3. N_{area} will be negatively related to leaf $C_i:C_a$. This response will result in an
1273 indirect positive and negative effect of increasing soil nitrogen availability

1274 and soil moisture, respectively, on N_{area} , and larger N_{area} values in N-fixing
1275 species. While theory predicts that lower β values in C₄ species should
1276 yield larger N_{area} in C₄ species, I expected that C₄ species would have lower
1277 N_{area} than C₃ species due to greater nitrogen use efficiency in C₄ species.
1278 Additionally, I expected vapor pressure deficit to increase N_{area} , a pattern
1279 that would be directly mediated through the reduction in leaf $C_i:C_a$ with
1280 increasing vapor pressure deficit.

1281 4.2 Methods

1282 4.2.1 *Site descriptions and sampling methodology*

1283 Leaf and soil samples were collected from 24 open canopy grassland sites scattered
1284 across central and eastern Texas in summer 2020 and summer 2021 (Fig. 4.1).
1285 Twelve sites were visited between June and July 2020 and 14 sites (11 unique from
1286 2020) were visited between May and June 2021 (Table 4.1). Sites were chosen to
1287 maximize precipitation and edaphic variability across sites (Table 4.1). No site
1288 with personally communicated or anecdotal evidence of grazing or disturbance
1289 (e.g., mowing, feral hog activity, etc.) was used. Leaf material was collected
1290 from three individuals each of the five most abundant species at random locations
1291 at each site, only selecting species that were broadly classified as graminoid or
1292 forb/herb growth habits per the USDA PLANTS database (USDA NRCS 2022).
1293 All collected leaves were fully expanded with no visible herbivory or other external
1294 damage and also free from shading by nearby shrubs or trees. Five soil samples
1295 were collected from 0-15 cm below the soil surface at each site near the leaf
1296 collection sample locations. Soil samples were mixed together by hand to create

1297 one composite soil sample per site.

1298 4.2.2 *Leaf trait measurements*

1299 Images of each leaf were taken immediately following each site visit using a flat-
1300 bed scanner. Fresh leaf area was determined from each image using the ‘LeafArea’
1301 R package (Katabuchi 2015), which automates leaf area calculations using ImageJ
1302 software (Schneider et al. 2012). Each leaf was dried at 65°C for at least 48 hours
1303 to a constant mass, weighed, and manually ground in a mortar and pestle until
1304 homogenized. Leaf mass per area (M_{area} ; g m⁻²) was calculated as the ratio of
1305 dry leaf biomass to fresh leaf area. Subsamples of dried and homogenized leaf
1306 tissue were used to measure leaf nitrogen content (N_{mass} ; gN g⁻¹) through ele-
1307 mental combustion analysis (Costech-4010, Costech Instruments, Valencia, CA).
1308 Leaf nitrogen content per unit leaf area (N_{area} ; gN m⁻²) was calculated as the
1309 product of N_{mass} and M_{area} .

1310 Subsamples of dried and homogenized leaf tissue were sent to the University
1311 of California-Davis Stable Isotope Facility to determine leaf $\delta^{13}\text{C}$. Leaf $\delta^{13}\text{C}$ values
1312 were determined using an elemental analyzer (PDZ Europa ANCA-GSL; Sercon
1313 Ltd., Chestshire, UK) interfaced to an isotope ratio mass spectrometer (PDZ
1314 Europa 20-20 Isotope Ratio Mass Spectrometer, Sercon Ltd., Chestshire, UK).
1315 I used leaf $\delta^{13}\text{C}$ values (‰; relative to Vienna Pee Dee Belemnite international
1316 reference standard) to estimate the ratio of intercellular (C_i) to extracellular (C_a)
1317 CO₂ ratio (leaf $C_i:C_a$; unitless) following the approach of Farquhar et al. (1989)
1318 described in Cernusak et al. (2013). Specifically, I derived leaf $C_i:C_a$ as:

$$C_i : C_a = \frac{\Delta^{13}C - a}{b - a} \quad (4.1)$$

1319 where $\Delta^{13}C$ represents the relative difference between leaf $\delta^{13}\text{C}$ (\textperthousand) and air $\delta^{13}\text{C}$
1320 (\textperthousand), calculated as:

$$\Delta^{13}C = \frac{\delta^{13}C_{air} - \delta^{13}C_{leaf}}{1 + \delta^{13}C_{leaf}} \quad (4.2)$$

1321 $\delta^{13}\text{C}_{air}$, which is commonly assumed to be $-8\text{\textperthousand}$ (Keeling et al. 1979; Farquhar
1322 et al. 1989), was calculated as a function of calendar year t using an empirical
1323 equation derived in Feng (1999):

$$\delta^{13}C_{air} = -6.429 - 0.006e^{0.0217(t-1740)} \quad (4.3)$$

1324 Using this equation, $\delta^{13}\text{C}_{air}$ values were set to $-9.04\text{\textperthousand}$ and $-9.09\text{\textperthousand}$ for 2020 and
1325 2021, respectively. The parameter a represents the fractionation between ^{12}C
1326 and ^{13}C due to diffusion in air, assumed to be $4.4\text{\textperthousand}$, while b represents the
1327 fractionation caused by Rubisco carboxylation, assumed to be $27\text{\textperthousand}$ (Farquhar
1328 et al. 1989). For C_4 species, b in Eqn. 4.1 was set to $6.3\text{\textperthousand}$, and was derived from:

$$b = c + (d \cdot \phi) \quad (4.4)$$

1329 Where c was set to $-5.7\text{\textperthousand}$ and d was set to $30\text{\textperthousand}$ (Farquhar et al. 1989). ϕ , which
1330 is the bundle sheath leakiness term, was set to 0.4. All leaf $C_i:C_a$ values less than
1331 0.1 and greater than 0.95 were assumed to be incorrect and removed from the

1332 analysis.

1333 I derived the unit cost of resource use (β) using leaf $C_i:C_a$ and site climate

1334 data using equations first described in Prentice et al. (2014) and simplified in

1335 Lavergne et al. (2020):

$$\beta = 1.6\eta^*VPD \frac{\chi - (\frac{\Gamma^*}{C_a})^2}{(1 - \chi)^2(K_m + \Gamma^*)} \quad (4.5)$$

1336 where η^* is the viscosity of water relative to 25°C, calculated using elevation and

1337 mean air temperature of the seven days leading up to each site visit following equa-

1338 tions in Huber et al. (2009). VPD (Pa) was set to the mean vapor pressure deficit

1339 of the seven days leading up to each site visit, C_a represents atmospheric CO₂

1340 concentration, arbitrarily set to 420 μmol mol⁻¹ CO₂. K_m (Pa) is the Michaelis-

1341 Menten coefficient for Rubisco affinity to CO₂ and O₂, calculated as:

$$K_m = K_c \cdot \left(1 + \frac{O_i}{K_o}\right) \quad (4.6)$$

1342 where K_c (Pa) and K_o (Pa) are the Michaelis-Menten coefficients for Rubisco

1343 affinity to CO₂ and O₂, respectively, and O_i is the intercellular O₂ concentration.

1344 Γ^* (Pa) is the CO₂ compensation point in the absence of dark respiration. K_c , K_o ,

1345 and Γ^* were determined using equations described in Medlyn et al. (2002) and

1346 derived in Bernacchi et al. (2001), invoking an elevation correction for atmospheric

1347 pressure as explained in Stocker et al. (2020).

Table 4.1. Site locality information, sampling year, 2006-2020 mean annual precipitation (MAP; mm), mean annual temperature (MAT; °C), and water holding capacity (WHC; mm)*

Site	Latitude	Longitude	Sampling year	MAP	MAT	WHC
Edwards_2019_17	29.95	-100.36	2020	563.5	19.0	224.7
Uvalde_2020_02	29.59	-100.09	2020, 2021	648.5	19.5	224.7
Menard_2020_01	30.91	-99.59	2020	641.9	18.3	220.2
Kerr_2020_03	30.06	-99.34	2021	672.4	18.3	237.5
Bandera_2020_03	29.85	-99.30	2021	789.4	18.8	235.1
Sansaba_2020_01	31.29	-98.62	2020	733.0	18.8	234.3
Comal_2020_21	29.79	-98.43	2020	878.5	19.9	220.7
Blanco_2019_16	29.99	-98.43	2020	833.0	19.2	222.2
Bexar_2019_13	29.24	-98.43	2020	759.3	21.5	206.0
Burnet_2020_14	30.84	-98.34	2021	763.3	19.5	217.8
Comal_2020_19	30.01	-98.32	2021	845.0	19.3	220.4
Hays_2020_54	29.96	-98.17	2021	861.3	20.0	225.6
Burnet_2020_12	30.82	-98.06	2021	815.1	19.4	245.3
Williamson_2019_09	30.71	-97.86	2020	867.7	19.7	270.2
Williamson_2019_10	30.54	-97.77	2020	819.5	19.9	239.8
Bell_2021_08	31.06	-97.55	2021	937.3	19.6	232.3
Fayette_2021_12	29.86	-97.21	2021	985.7	20.4	165.6
Fayette_2019_04	30.09	-96.78	2020	1017.4	20.6	226.9
Fayette_2020_09	29.86	-96.71	2021	1002.7	20.8	187.6
Washington_2020_08	30.28	-96.41	2021	1077.4	20.4	203.9
Austin_2020_03	29.78	-96.24	2021	1108.7	20.6	253.0
Brazos_2020_16	30.93	-96.23	2021	1078.0	20.1	202.2
Brazos_2020_18	30.52	-96.21	2020, 2021	1099.4	20.4	233.5
Harris_2020_03	29.88	-95.31	2020, 2021	1492.0	21.6	265.6

1348 * Rows are arranged by longitude to visualize precipitation variability across sites

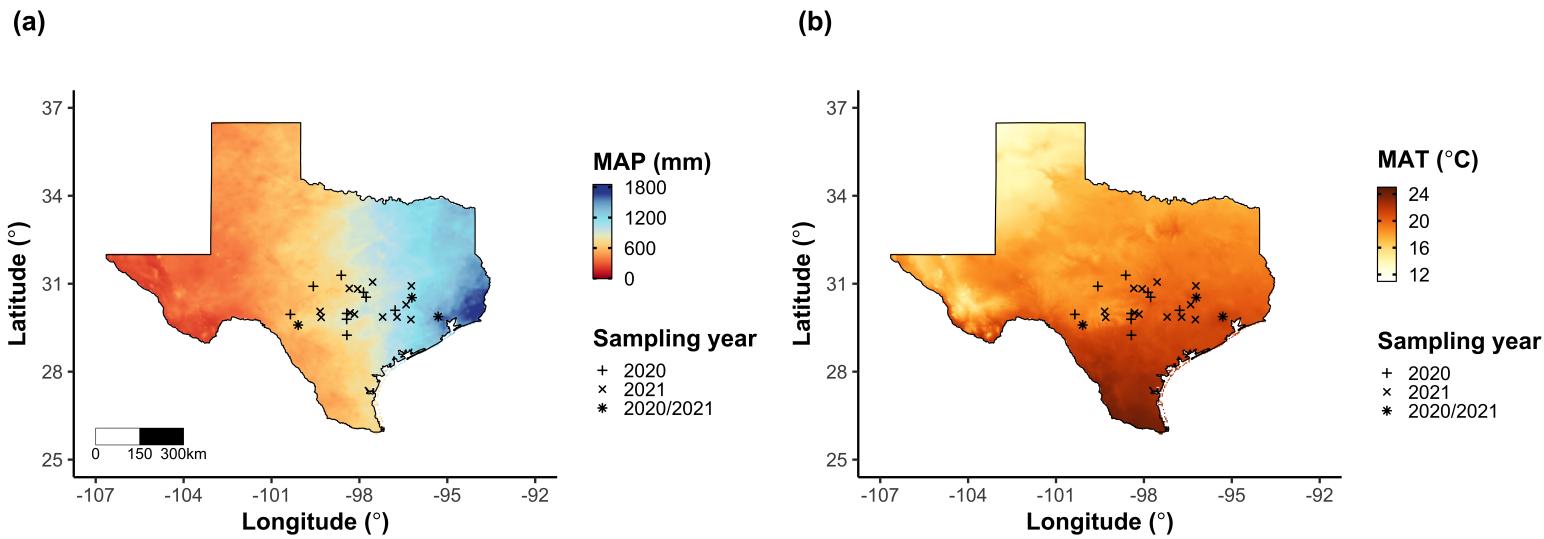


Figure 4.1. Site locations along 2006-2020 mean annual precipitation (a) and mean annual temperature (b) gradients in Texas, USA. Precipitation and temperature data were plotted using PRISM data at a 4-km grid resolution and are masked to include only grid cells that occur within the Texas state boundary of the United States. In both panels, addition signs refer to sites visited in 2020, multiplication signs to sites visited in 2021, and asterisks to sites visited in 2020 and 2021. The scale bar in (a) also applies to (b).

1349 4.2.3 *Site climate data*

1350 I used the Parameter elevation Regressions on Independent Slopes Model (PRISM)
1351 (Daly et al. 2008) climate product to access gridded daily temperature and precip-
1352 itation data for the coterminous United States at a 4-km grid resolution between
1353 January 1, 2006 and July 31, 2021 (PRISM Climate Group, Oregon State Uni-
1354 versity, <https://prism.oregonstate.edu>, data created 4 Feb 2014, accessed 24
1355 Mar 2022). Mean daily air temperature, mean daily VPD, and total daily pre-
1356 cipitation data were extracted from the grid cell that contained the latitude and
1357 longitude of each property using the ‘extract’ function in the ‘terra’ R package
1358 (Hijmans 2022). PRISM data were used in lieu of local weather station data
1359 because several rural sites did not have a local weather station present within a
1360 20-km radius of the site. Daily site climate data were used to estimate mean an-
1361 nual precipitation and mean annual temperature for each site between 2006 and
1362 2020 (Table 4.1). I calculated total precipitation and mean daily VPD for the
1363 prior 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 60, and 90 days leading up to each
1364 site visit. Temperature was not included in any analysis due to the close range in
1365 mean annual temperature between sites (mean \pm SD: $19.8\pm0.9^{\circ}\text{C}$; Table 4.1).

1366 4.2.4 *Site edaphic characteristics*

1367 Composted soil samples were sent to the Texas A&M Soil, Water and Forage
1368 Laboratory to quantify soil nitrate concentration ($\text{NO}_3\text{-N}$; ppm). Soil $\text{NO}_3\text{-N}$
1369 was determined by extracting composite soil samples in 1 M KCl, measuring
1370 absorbance values of extracts at 520 nm using the end product of a $\text{NO}_3\text{-N}$ to
1371 $\text{NO}_2\text{-N}$ cadmium reduction reaction (Kachurina et al. 2000; Keeney and Nelson

1372 1983). Soil texture data from 0-15 cm below the soil surface were accessed using
1373 the SoilGrids2.0 data product (Poggio et al. 2021) through the ‘fetchSoilGrids’
1374 function in the ‘soilDB’ R package (Beaudette et al. 2022). I used SoilGrids2.0
1375 to access soil texture data in lieu of analyses using the composite soil sample due
1376 to a lack of soil material from some sites after sending samples for soil NO₃-N.

1377 Soil moisture was not measured in the field, but was estimated using the
1378 ‘Simple Process-Led Algorithms for Simulating Habitats’ model (SPLASH) (Davis
1379 et al. 2017). This model, derived from the STASH model (Cramer and Prentice
1380 1988), spins up a bucket model using Priestley-Taylor equations (Priestley and
1381 Taylor 1972) to calculate daily soil moisture (W_n ; mm) as a function of the previous
1382 day’s soil moisture (W_{n-1} ; mm), daily precipitation (P_n ; mm), condensation (C_n ;
1383 mm), actual evapotranspiration (E_n^a ; mm), and runoff (RO; mm):

$$W_n = W_{n-1} + P_n + C_n - E_n^a - RO \quad (4.7)$$

1384 Models were spun up by equilibrating the previous day’s soil moisture using succes-
1385 sive model iterations with daily mean air temperature, daily precipitation total,
1386 the number of daily sunlight hours, and latitude as model inputs (Davis et al.
1387 2017). Daily sunlight hours were estimated for each day at each site using the
1388 ‘getSunlightTimes’ function in the ‘suncalc’ R package, which estimated sunrise
1389 and sunset times of each property using date and site coordinates (Thieurmel and
1390 Elmarhraoui 2019). Water holding capacity (mm), or bucket size, was estimated
1391 as a function of soil texture using pedotransfer equations explained in Saxton and
1392 Rawls (2006), as done in Stocker et al. (2020) and Bloomfield et al. (2023). A

1393 summary of these equations is included in Appendix C.1.

1394 Daily soil moisture outputs from the SPLASH model for each site were
1395 used to calculate mean daily soil moisture for the prior 1, 2, 3, 4, 5, 6, 7, 8, 9,
1396 10, 15, 20, 25, 30, 60, and 90 days leading up to each site visit. Mean daily
1397 soil moisture values were then expressed as a fraction of water holding capacity
1398 to normalize across sites with different bucket depths, as done in Stocker et al.
1399 (2018). Site water holding capacity values are referenced in Table 4.1.

1400 4.2.5 *Plant functional group assignments*

1401 Plant functional group was assigned to each species and used as the primary de-
1402 scriptor of species identity. Specifically, plant functional groups were assigned
1403 based on photosynthetic pathway (C_3 , C_4) and ability to form associations with
1404 symbiotic nitrogen-fixing bacteria (legume, nonlegume). The ability to form as-
1405 sociations with symbiotic nitrogen-fixing bacteria was assigned based on whether
1406 species were in the *Fabaceae* family, and photosynthetic pathway of each species
1407 was determined from past literature and confirmed through leaf $\delta^{13}C$ values. I
1408 chose these plant functional groups based on *a priori* hypotheses regarding the
1409 functional role of nitrogen fixation and photosynthetic pathway on the sensitivity
1410 of plant nitrogen uptake and leaf nitrogen allocation to soil nitrogen availability
1411 and aboveground growing conditions. These plant functional group classifications
1412 resulted in three distinct plant functional groups within our dataset: C_3 legumes
1413 (n=53), C_3 nonlegumes (n=350), and C_4 nonlegumes (n=117).

1414 4.2.6 *Data analysis*

1415 All analyses and plotting were conducted in R version 4.1.1 (R Core Team 2021).

1416 I constructed a series of separate linear mixed-effects models to investigate en-

1417 vironmental drivers of β , leaf $C_i:C_a$, N_{area} , N_{mass} , and M_{area} , followed by a path

1418 analysis using a piecewise structural equation model to investigate direct and

1419 indirect effects of climate and soil resource availability on N_{area} .

1420 To explore environmental drivers of β , I built a linear mixed-effects model

1421 that included soil moisture, soil nitrogen availability, and plant functional group

1422 as fixed effect coefficients. Species were designated as a random intercept term.

1423 Interaction coefficients between all possible combinations of the three fixed effect

1424 coefficients were also included. β was natural log transformed to linearize data.

1425 I used an information-theoretic model selection approach to determine whether

1426 90-, 60-, 30-, 20-, 15-, 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day mean daily soil

1427 moisture conferred the best model fit for β . To do this, I constructed 16 separate

1428 linear mixed-effects models where log-transformed β was included as the response

1429 variable and each soil moisture time step was separately included as a single

1430 continuous fixed effect. Species were included as a random intercept term for all

1431 models. I used corrected Akaike Information Criterion (AICc) to select the soil

1432 moisture timescale that conferred the best model fit, indicated by the model with

1433 the lowest AICc score (Table C3; Fig. C1).

1434 To explore environmental drivers of leaf $C_i:C_a$, I constructed a second linear

1435 mixed effects model that included VPD, soil moisture, soil nitrogen availability,

1436 and plant functional group as fixed effect coefficients. Two-way interactions be-

1437 tween plant functional group and VPD, soil nitrogen availability, or soil moisture

1438 were included as additional fixed effect coefficients, in addition to a three-way
1439 interaction between soil moisture, soil nitrogen availability, and plant functional
1440 group. Species were included as a random intercept term. I used an information-
1441 theoretic model selection approach to determine whether 90-, 60-, 30-, 20-, 15-,
1442 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day mean daily VPD conferred the best model
1443 fit for leaf $C_i:C_a$ using the same approach explained above for the soil moisture ef-
1444 fect on β . The soil moisture timescale was set to the same timescale that conferred
1445 the best fit for β .

1446 To explore environmental drivers of N_{area} , N_{mass} , and M_{area} , I constructed
1447 a linear mixed effects model for each trait, including leaf $C_i:C_a$, soil nitrogen
1448 availability, soil moisture, and plant functional group as fixed effect coefficients
1449 for each model. Two-way interactions between plant functional group and β , leaf
1450 $C_i:C_a$, soil nitrogen availability, or soil moisture were included as additional fixed
1451 effect coefficients, in addition to a three-way interaction between soil nitrogen
1452 availability, soil moisture, and plant functional group. Species were included as a
1453 random intercept term, with the soil moisture timescale set to the same timescale
1454 that conferred the best fit for β .

1455 In all linear mixed-effects models explained above, including those to select
1456 relevant timescales, I used the ‘lmer’ function in the ‘lme4’ R package (Bates et al.
1457 2015) to fit each model and the ‘Anova’ function in the ‘car’ R package (Fox and
1458 Weisberg 2019) to calculate Type II Wald’s χ^2 and determine the significance
1459 level ($\alpha=0.05$) of each fixed effect coefficient. I used the ‘emmeans’ R package
1460 (Lenth 2019) to conduct post-hoc comparisons using Tukey’s tests, where degrees
1461 of freedom were approximated using the Kenward-Roger approach (Kenward and

1462 Roger 1997). Trendlines and error ribbons for all plots were drawn using a series
1463 of ‘emmeans’ outputs across the range in plotted x-axis values.

1464 Finally, I conducted a path analysis using a piecewise structural equation
1465 model to examine direct and indirect pathways that determined variance in N_{area} .
1466 Six separate linear mixed effects models were loaded into the piecewise structural
1467 equation model. Models were constructed per *a priori* hypotheses following pat-
1468 terns expected from photosynthetic least-cost theory. The first model regressed
1469 N_{area} against N_{mass} and M_{area} . The second model regressed M_{area} against leaf
1470 $C_i:C_a$. The third model regressed N_{mass} against leaf $C_i:C_a$ and M_{area} (Dong et al.
1471 2017; Dong et al. 2020). The fourth model regressed leaf $C_i:C_a$ against β and
1472 VPD. The fifth model regressed β against soil nitrogen availability, soil moisture,
1473 ability to associate with symbiotic nitrogen-fixing bacteria, and photosynthetic
1474 pathway. The sixth model regressed soil nitrogen availability against soil mois-
1475 ture. All models included the relevant timescale selected in the individual linear
1476 mixed effect models explained above. Models included species as a random inter-
1477 cept term, were built using the ‘lme’ function in the ‘nlme’ R package (Pinheiro
1478 and Bates 2022), and subsequently loaded into the piecewise structural equation
1479 model using the ‘psem’ function in the ‘piecewiseSEM’ R package (Lefcheck 2016).

1480 4.3 Results

1481 4.3.1 *Cost to acquire nitrogen relative to water*

1482 Model selection indicated that 90-day mean soil moisture conferred the best model

1483 fit for β (AICc=1387.54; Table C3; Fig. C1).

1484 Increasing soil nitrogen availability generally decreased β ($p<0.001$; Table

1485 4.2; Fig. 4.2a), a pattern driven by a negative effect of increasing soil nitrogen on

1486 β in C₃ nonlegumes (Tukey: $p=0.005$) and C₃ legumes (Tukey: $p=0.035$) despite

1487 a null effect of increasing soil nitrogen on β in C₄ nonlegumes (Tukey: $p=0.856$).

1488 There was no effect of soil moisture on β ($p=0.872$; Table 4.2; Fig. 4.2b). A func-

1489 tional group effect ($p<0.001$; Table 4.2) indicated that C₄ nonlegumes generally

1490 had lower β values than both C₃ legumes and C₃ non-legumes (Tukey: $p<0.001$

1491 in both cases), while β values in C₃ legumes did not differ from C₃ nonlegumes

1492 (Tukey: $p=0.854$).

Table 4.2. Effects of soil moisture, soil nitrogen availability, and plant functional group on β (unitless)*

	df	Coefficient	χ^2	p
Intercept	-	3.39E+00	-	-
Soil moisture (SM ₉₀)	1	-1.96E-01	0.026	0.872
Soil N (N)	1	-1.42E-02	12.031	<0.001
PFT	2	-	199.617	<0.001
SM ₉₀ *N	1	-3.02E-03	1.000	0.317
SM ₉₀ *PFT	2	-	0.623	0.732
N*PFT	2	-	5.271	0.072
SM ₉₀ *N*PFT	2	-	5.271	0.182

1493 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values<0.05
1494 are in bold. Model coefficients are expressed on the natural-log scale and are only
1495 included for continuous fixed effects. Key: df=degrees of freedom; χ^2 =Wald Type
1496 II chi-square test statistic

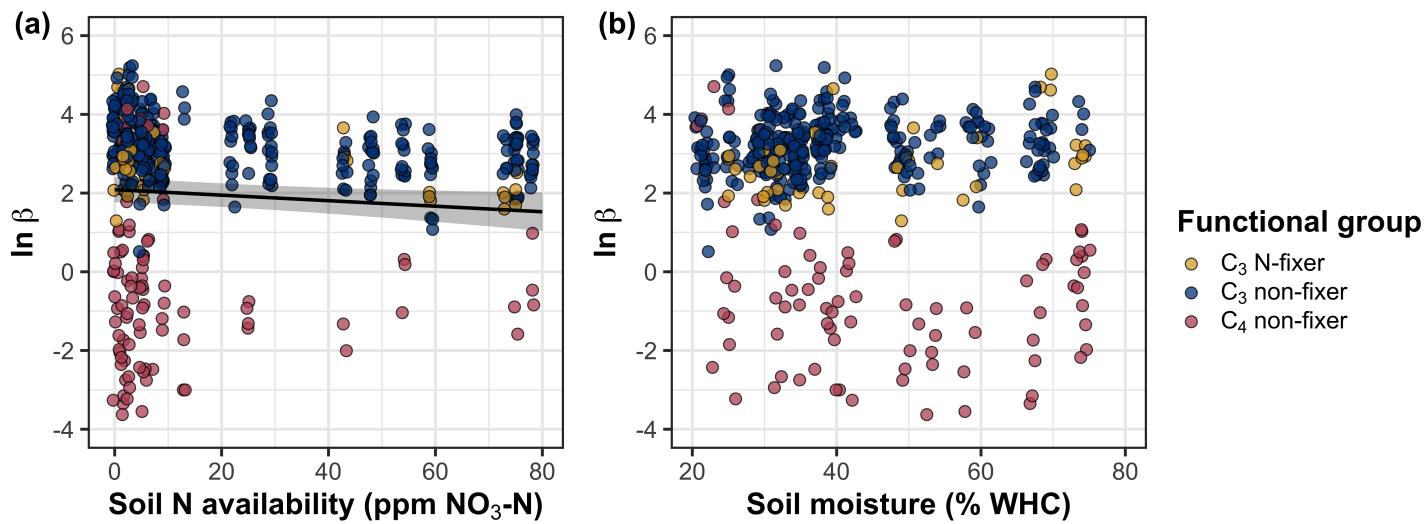


Figure 4.2. Effects of soil nitrogen availability (a) and soil moisture (b) on the cost of acquiring and using nitrogen (β ; unitless). Soil nitrogen availability is represented on the x-axis in (a), soil moisture is represented on the x-axis in (b) as a percent of site water holding capacity, and natural-log transformed β is represented on the y-axis for both panels. Yellow points represent C₃ legumes, blue points represent C₃ nonlegumes, and red points represent C₄ nonlegumes. Throughout, points are jittered for visibility. A black solid trendline is drawn to denote bivariate relationships where the slope is different from zero ($p<0.05$), with error ribbons representing the upper and lower 95% confidence intervals.

1497 4.3.2 *Leaf C_i:C_a*

1498 Model selection indicated that 4-day mean vapor pressure deficit was the timescale

1499 that conferred the best model fit for leaf $C_i:C_a$ (AICc=-755.81; Table C3; Fig. C1).

1500 Model results revealed that increasing vapor pressure deficit generally de-

1501 creased leaf $C_i:C_a$ ($p<0.001$; Table 4.3; Fig. 4.3a). There was no effect of soil

1502 moisture ($p=0.549$; Table 4.3; Fig. 4.3b) or soil nitrogen availability ($p=0.549$; Ta-

1503 ble 4.3; Fig. 4.3c) on leaf $C_i:C_a$. A strong plant functional group effect ($p<0.001$;

1504 Table 4.3) indicated that C₄ nonlegumes had lower leaf $C_i:C_a$ than C₃ legumes

1505 and C₃ nonlegumes (Tukey: $p<0.001$ in both cases), with no difference between

1506 C₃ legumes and C₃ nonlegumes (Tukey: $p=0.866$).

Table 4.3. Effects of soil moisture, soil nitrogen availability, and plant functional group on leaf $C_i:C_a$ (unitless)*

	df	Coefficient	χ^2	<i>p</i>
Intercept	-	1.32E+00	-	-
Vapor pressure deficit (VPD_4)	1	-4.53E-01	10.987	<0.001
Soil moisture (SM_{90})	1	-1.71E-01	0.039	0.843
Soil N (N)	1	-1.71E-03	0.043	0.549
PFT	2	-	205.274	<0.001
SM_{90}^*N	1	7.29E-03	2.266	0.132
VPD_4^*PFT	2	-	0.887	0.642
SM_{90}^*PFT	2	-	0.814	0.666
N^*PFT	2	-	4.158	0.125
$SM_{90}^*N^*PFT$	2	-	3.465	0.177

1507 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). *P*-values less
1508 than 0.05 are in bold and *p*-values where $0.05 < p < 0.1$ are italicized. Leaf $C_i:C_a$
1509 was not transformed prior to model fitting, so model coefficients are reported
1510 on the response scale. Model coefficients are only included for continuous fixed
1511 effects. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic

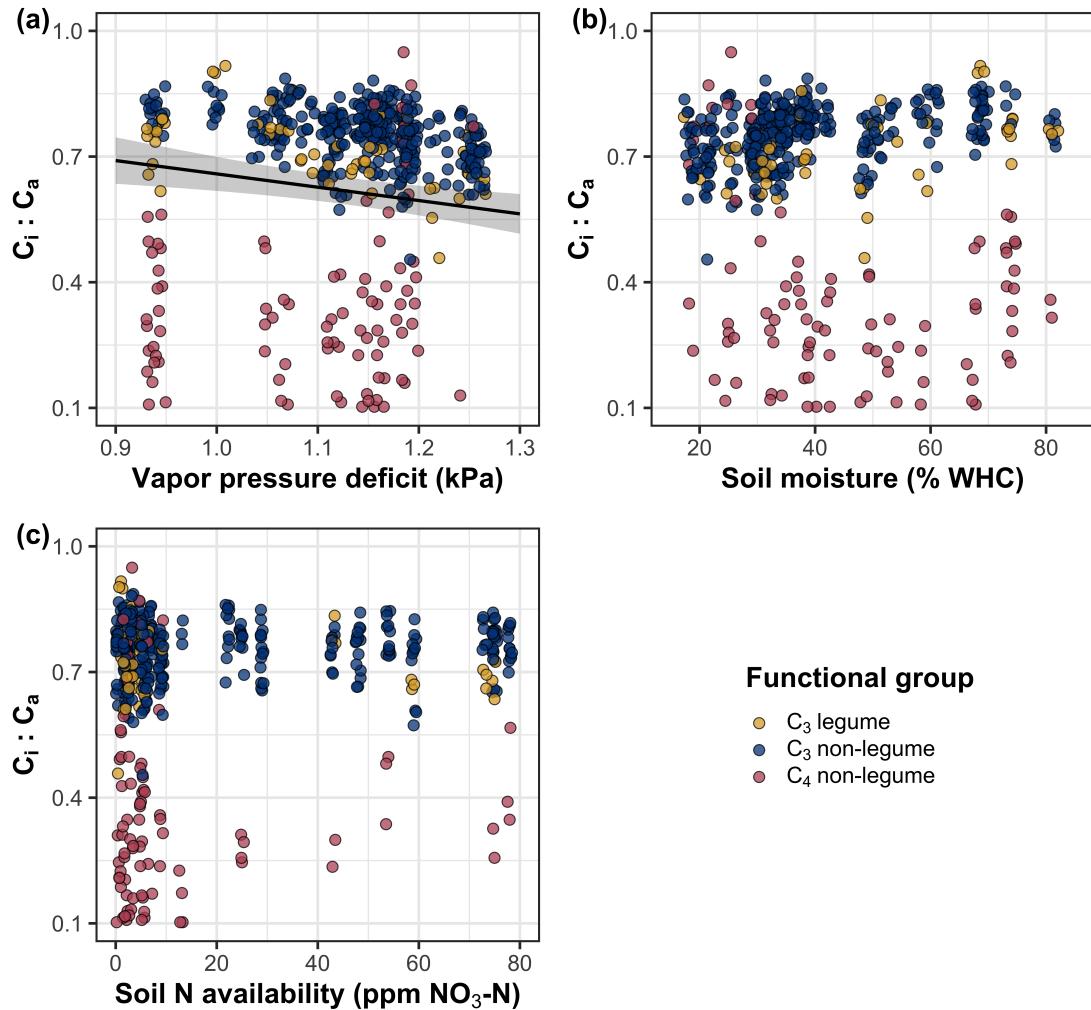


Figure 4.3. Effects of 4-day mean vapor pressure deficit (a), 90-day soil moisture (per water holding capacity; b), and soil nitrogen availability (c) on leaf $C_i:C_a$. Shading and trendlines are as explained in Figure 4.2. Points are jittered for visibility. Variably colored trendlines are only included if there is an interaction between the x-axis and plant functional group, where solid trendlines indicate slopes that are different from zero ($p < 0.05$) and dashed trendlines indicate slopes that are not different from zero ($p > 0.05$). Error ribbons represent the upper and lower 95% confidence intervals of each fitted trendline.

1512 4.3.3 *Leaf nitrogen content*

1513 An interaction between leaf $C_i:C_a$ and plant functional group ($p<0.001$; Table 4.4) revealed that the negative effect of increasing leaf $C_i:C_a$ on N_{area} ($p<0.001$; Table 4.4) was driven by a negative effect of increasing leaf $C_i:C_a$ on N_{area} in **1515** C_3 nonlegumes and C_3 legumes (Tukey: $p<0.001$ in both cases), but not C_4 **1517** nonlegumes (Tukey: $p=0.786$; Fig. 4.4a). A marginal interaction between soil **1518** nitrogen availability and plant functional group ($p=0.057$; Table 4.4) indicated **1519** that the positive effect of increasing soil nitrogen ($p=0.007$; Table 4.4) was only **1520** apparent in C_3 legumes (Tukey: $p<0.001$; Table 4.4; Fig. 4.4d), but not C_3 **1521** nonlegumes (Tukey: $p=0.329$) or C_4 nonlegumes (Tukey: $p=0.682$). Increasing **1522** soil moisture increased N_{area} ($p=0.011$, Table 4.4). A plant functional group effect **1523** ($p<0.001$; Table 4.4) indicated that C_4 nonlegumes had lower N_{area} compared to **1524** C_3 legumes and C_3 nonlegumes (Tukey: $p<0.001$ in both cases), while C_3 legumes **1525** had lower N_{area} compared to C_3 nonlegumes (Tukey: $p=0.024$).

1526 Leaf $C_i:C_a$ had no effect on N_{mass} ($p=0.455$; Table 4.4; Fig. 4.4b). Increasing **1527** soil nitrogen availability and soil moisture each had a positive effect on N_{mass} ($p<0.001$ in both cases; Table 4.4; Fig. 4.4h). A plant functional group effect **1528** ($p<0.001$; Table 4.4) indicated that C_4 nonlegumes had lower N_{mass} compared to **1529** C_3 legumes and C_3 nonlegumes (Tukey: $p=0.001$ in both cases), while N_{mass} did **1530** not differ between C_3 legumes and C_3 nonlegumes (Tukey: $p=0.323$).

1532 Variance in M_{area} was driven by a three-way interaction between soil nitrogen availability, soil moisture, and plant functional group ($p=0.018$; Table 4.4). **1533** This interaction indicated that increasing soil moisture increased the positive effect **1534** of increasing soil nitrogen availability on M_{area} in C_3 legumes (Tukey: $p=0.028$)

1536 but did not modify the negative effect of increasing soil nitrogen availability on
1537 M_{area} in C₄ nonlegumes (Tukey: $p=0.806$) or C₃ nonlegumes (Tukey: $p=0.998$).
1538 There was otherwise no effect of soil moisture on M_{area} ($p=0.436$; Table 4.4). An
1539 interaction between leaf $C_i:C_a$ and plant functional group ($p<0.001$; Table 4.4;
1540 Fig. 4.4c) indicated that the negative effect of increasing leaf $C_i:C_a$ on M_{area}
1541 ($p<0.001$; Table 4.4) was driven by a negative effect of increasing leaf $C_i:C_a$ on
1542 M_{area} in C₃ legumes (Tukey: $p<0.001$) and C₃ nonlegumes (Tukey: $p=0.003$), but
1543 not C₄ nonlegumes (Tukey: $p=0.257$; Fig. 4.4c).

Table 4.4. Effects of soil moisture, soil nitrogen availability, plant functional group, and leaf $C_i:C_a$ on leaf nitrogen content per unit leaf area (N_{area} ; gN m $^{-2}$), leaf nitrogen content per unit leaf biomass (N_{mass} ; gN g $^{-1}$), and leaf biomass per unit leaf area (M_{area} ; g m $^{-2}$)

		N_{area}			N_{mass}			M_{area}		
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	2.41E+00	-	-	7.72E-02	-	-	6.91E+00	-	-
$C_i:C_a$	1	-2.32E+00	6.841	0.009	7.91E-01	0.558	0.455	-3.13E+00	15.913	<0.001
Soil N (N)	1	1.26E-02	7.072	0.011	1.21E-02	87.457	<0.001	-2.66E-02	41.791	<0.001
Soil moisture (SM ₉₀)	1	5.60E-01	6.493	0.011	7.94E-01	10.889	<0.001	-2.54E-01	0.605	0.437
PFT	1		-	49.273	<0.001	-	21.786	<0.001	-	6.673
SM ₉₀ *N	1	5.45E-02	0.482	0.488	-2.18E-02	2.606	0.106	8.16E-02	0.791	0.374
$C_i:C_a$ *PFT	1		-	24.380	<0.001	-	5.367	0.068	-	30.073
N*PFT	1		-	5.713	0.057	-	1.286	0.526	-	19.405
SM ₉₀ *PFT	1		-	3.487	0.175	-	0.889	0.641	-	2.998
SM ₉₀ *N*PFT	1		-	3.523	0.172	-	0.161	0.923	-	7.996

96

1544 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold and p-values
 1545 where $0.05 < p < 0.1$ are italicized. Coefficients are reported on the natural-log scale for all traits and are only included
 1546 for continuous fixed effects. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic

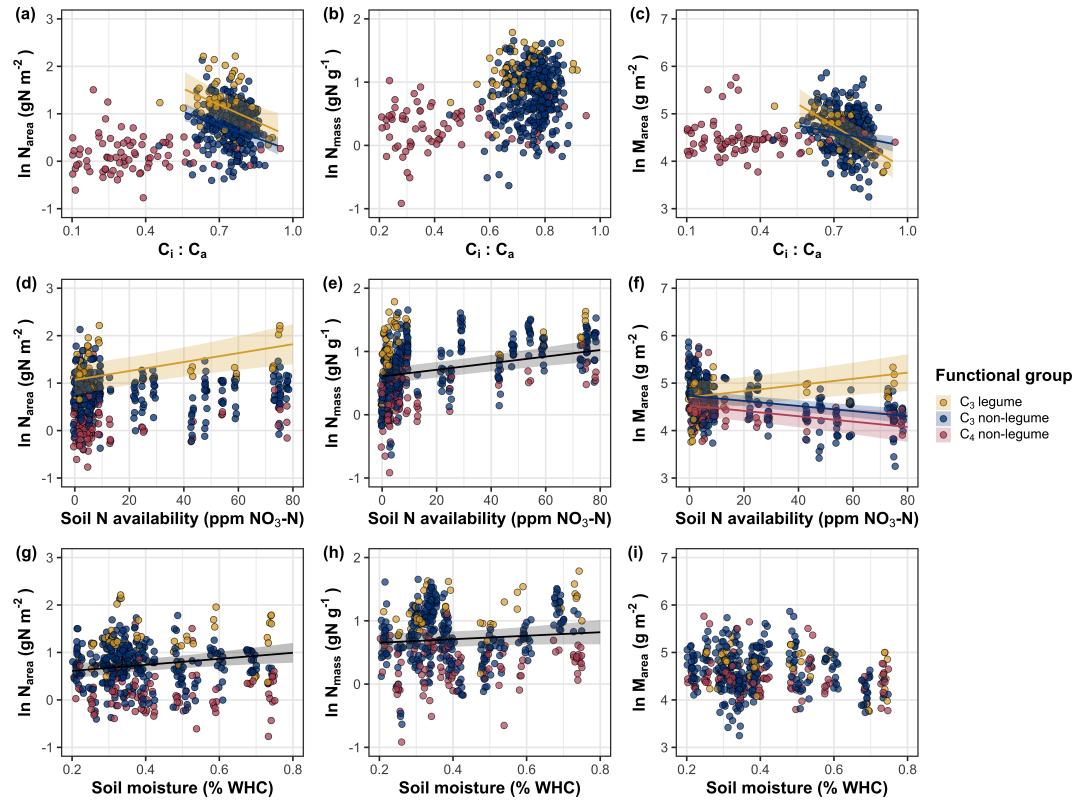


Figure 4.4. Effects of leaf $C_i:C_a$ (a-c), soil nitrogen availability (d-f), and soil moisture (g-i) on leaf nitrogen content per unit leaf area (a, d, g), leaf nitrogen content per unit leaf biomass (b, e, h), and leaf mass per area (c, f, i). Yellow points and trendlines indicate C_3 legumes, blue points and trendlines indicate C_3 nonlegumes, and red points and trendlines indicate C_4 nonlegumes. Points are jittered for visibility. Variably colored trendlines are only included if there is an interaction between plant functional group and the x-axis. Black solid trendlines denote bivariate slopes that are different from zero ($p < 0.05$) where there is no apparent interaction between plant functional group and the x-axis.

1547 4.3.4 *Structural equation model*

1548 The piecewise structural equation model explained 89%, 56%, 57%, 82%, and
1549 38% of variance in N_{area} , N_{mass} , M_{area} , leaf $C_i:C_a$, and β , respectively (Table
1550 4.5; Fig. 4.5). Variance in N_{area} was driven by a positive effect of increasing
1551 N_{mass} and M_{area} ($p < 0.001$ in both cases; Table 4.5; Fig. 4.5). Model results
1552 indicated that an indirect negative effect of $C_i:C_a$ on N_{area} was driven by a strong
1553 reduction in M_{area} with increasing leaf $C_i:C_a$ ($p < 0.001$; Table 4.5) paired with no
1554 effect of increasing $C_i:C_a$ on N_{mass} ($p = 0.153$; Table 4.5). However, there was a
1555 strong negative effect of increasing M_{area} on N_{mass} ($p < 0.001$; Table 4.5; Fig. 4.5).
1556 Leaf $C_i:C_a$ increased with increasing β ($p < 0.001$; Table 4.5) and decreased with
1557 increasing vapor pressure deficit ($p < 0.001$; Table 4.5; Fig. 4.5). Variance in β
1558 was driven by a negative effect of increasing soil nitrogen availability ($p < 0.001$;
1559 Table 4.5) and was generally higher in C₃ species ($p < 0.001$; Table 4.5; Fig. 4.5).
1560 However, β did not change with soil moisture ($p = 0.895$; Table 4.5) or with ability
1561 to acquire nitrogen via symbiotic nitrogen fixation ($p = 0.495$; Table 4.5). Finally,
1562 soil nitrogen availability was positively associated with increasing soil moisture
1563 ($p = 0.003$; Table 4.5; Fig. 4.5).

Table 4.5. Structural equation model results investigating direct effects of climatic and soil resource availability on leaf nitrogen content (N_{area} ; g m⁻²)*

Predictor	Coefficient	<i>p</i>
N_{area} ($R^2_c=0.89$)		
M_{area}	0.714	<0.001
N_{mass}	0.778	<0.001
N_{mass} ($R^2_c=0.56$)		
Leaf $C_i:C_a$	0.086	0.153
M_{area}	-0.256	<0.001
M_{area} ($R^2_c=0.57$)		
Leaf $C_i:C_a$	-0.250	<0.001
Leaf $C_i:C_a$ ($R^2_c=0.82$)		
β	0.308	<0.001
VPD_4	-0.111	<0.001
β ($R^2_c=0.38$)		
Soil N	-0.207	<0.001
SM_{90}	-0.006	0.895
Photo. pathway	0.446	<0.001
N-fixing ability	-0.059	0.519
Soil N ($R^2_c=0.35$)		
SM_{90}	-0.148	0.003

1564 *Coefficients are standardized across the structural equation model. *P*-values less
 1565 than 0.05 are noted in bold. Positive coefficients for photosynthetic pathway
 1566 indicate generally larger values in C₃ species, while positive coefficients for N-
 1567 fixing ability indicate generally larger values in N-fixing species. Key: df=degrees
 1568 of freedom; χ^2 =Wald Type II chi-square test statistic; R^2_c =conditional R² value;
 1569 N_{mass} =leaf nitrogen content per unit leaf biomass (gN g⁻¹); M_{area} =leaf mass per
 1570 unit leaf biomass (g m⁻²); β =cost of acquiring nitrogen relative to water (unitless);
 1571 VPD_4 =4-day mean vapor pressure deficit (kPa); SM_{90} =90-day mean soil moisture
 1572 (mm)

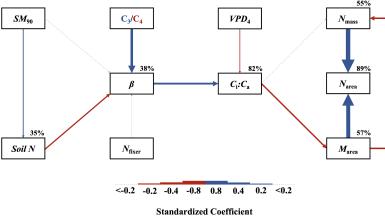


Figure 4.5. Structural equation model results exploring drivers of N_{area} . Boxes indicate measured edaphic factors, climatic factors, and leaf traits. Solid arrows indicate bivariate relationships where $p < 0.05$, while dashed arrows indicate relationships where $p > 0.05$. Positive model coefficients are indicated through blue arrows, negative model coefficients are indicated through red arrows, and insignificant coefficients are indicated through gray dashed arrows. Arrow thickness scales with the standardized model coefficient of each bivariate relationship. A positive coefficient for photosynthetic pathway indicates larger values in C_3 species, while a positive coefficient for N_{fixer} indicates larger values in N -fixing species. Standardized model coefficients and associated p -values are reported in Table 4.5, with conditional R^2 values for each response variable reported on the top right of each box.

1573 4.4 Discussion

1574 In this study, I quantified direct and indirect effects of edaphic and climatic char-
1575 acteristics on N_{area} and components of N_{area} (N_{mass} and M_{area}) in 520 individuals
1576 spanning across a soil resource availability and climate gradient in Texas, USA.
1577 Strong and consistent patterns emerged in support of those expected from photo-
1578 synthetic least-cost theory, a result driven by a strong direct negative relationship
1579 between leaf $C_i:C_a$ and N_{area} . In further support of patterns expected from theory,
1580 increasing soil nitrogen availability had a strong negative effect on β , resulting in
1581 an indirect stimulation in N_{area} mediated through a positive relationship between
1582 β and $C_i:C_a$. Increasing VPD also indirectly increased N_{area} through a direct
1583 negative effect of increasing VPD on leaf $C_i:C_a$, following hypotheses and pat-
1584 terns expected from theory. Interestingly, a strong positive association between
1585 soil moisture and N_{area} was driven by covariance between soil moisture and soil
1586 nitrogen availability and was not associated with a direct effect of soil moisture
1587 on β . Overall, results provide strong and consistent support for patterns expected
1588 from photosynthetic least-cost theory, showing that both soil resource availability
1589 and climate drive variance in N_{area} through changes in leaf $C_i:C_a$.

1590 4.4.1 *Negative effects of leaf $C_i:C_a$ on N_{area} are driven by reductions in M_{area} ,*
1591 *not N_{mass}*

1592 A strong negative effect of increasing leaf $C_i:C_a$ on N_{area} was detected in both
1593 the linear mixed effect and piecewise structural equation models. The negative
1594 response of N_{area} to increasing leaf $C_i:C_a$ is consistent with previous environmental
1595 gradient (Dong et al. 2017; Querejeta et al. 2022) and manipulation experiments

1596 (3.4c), showing strong support for the nitrogen-water use tradeoffs expected from
1597 photosynthetic least cost theory (Wright et al. 2003; Prentice et al. 2014). Neg-
1598 ative effects of increasing leaf $C_i:C_a$ on N_{area} were driven by a strong negative
1599 effect of increasing $C_i:C_a$ on M_{area} , with no apparent effect of leaf $C_i:C_a$ on N_{mass} ,
1600 suggesting that changes in N_{area} were driven by changes in leaf structure and not
1601 leaf chemistry. However, increasing M_{area} was negatively associated with N_{mass} ,
1602 indicating that an increase in N_{mass} was associated with larger, thinner leaves (i.e.
1603 lower M_{area}). These results are consistent with patterns reported from previous
1604 studies indicating that variance in N_{area} is driven by changes in M_{area} across envi-
1605 ronmental gradients, and that part of this response is due to negative covariance
1606 between M_{area} and N_{mass} (Dong et al. 2017; Dong et al. 2020). Negative co-
1607 variance between M_{area} and N_{mass} could be a response associated with tradeoffs
1608 between leaf longevity and leaf productivity (Wright et al. 2004; Dong et al. 2017;
1609 Dong et al. 2022; Querejeta et al. 2022; Wang et al. 2023).

1610 The negative relationship between leaf $C_i:C_a$ and M_{area} could also be a re-
1611 sponse that allows leaves to maximize productivity in shorter-lived leaves. Trade-
1612 offs between leaf longevity and leaf productivity are commonly observed and are
1613 included in a continuum of coordinated leaf traits that position individuals along
1614 a fast- or slow-growing leaf economics spectrum (Wright et al. 2003; Onoda et al.
1615 2004; Reich 2014; Onoda et al. 2017; Wang et al. 2023). Negative relationships
1616 between leaf $C_i:C_a$ and M_{area} indicate that increased stomatal conductance and
1617 reduced water use efficiency were associated with thinner, larger leaves (i.e., lower
1618 M_{area}). These patterns, combined with the negative covariance between M_{area} and
1619 N_{mass} mentioned above, may have allowed individuals to maximize light intercep-

tion and productivity by exploiting high light environments, though this comes
1621 at the expense of increased water loss and decreased water-use efficiency. This
1622 strategy may be especially advantageous for fast-growing species in open canopy
1623 systems. In this study, C₃ legumes and C₃ nonlegumes dominated the dataset
1624 (78% of total sampling effort), of which 22% (17% of total sampling effort) were
1625 classified as annual species with short growing seasons. We observed no effect of
1626 leaf C_i:C_a on N_{area} or M_{area} in C₄ nonlegumes, which made up 22% of the sampling
1627 effort and were generally classified as warm season graminoid species with slower
1628 growth rates and longer growing seasons. These patterns indicate that stronger
1629 tradeoffs between nitrogen and water use may be more apparent in fast-growing
1630 species with high demand for building and maintaining productive leaf tissues.

1631 4.4.2 *Soil nitrogen availability increases N_{area} through changes in β*

1632 The experimental and statistical approach used in this study allowed for N_{area} and
1633 components of N_{area} to be quantified as direct and indirect products of soil nitrogen
1634 availability. Linear mixed effect models revealed a positive effect of increasing soil
1635 nitrogen availability on N_{area}, a pattern that was driven by a stronger positive
1636 effect of increasing soil nitrogen availability on N_{mass} than the negative effect
1637 of increasing soil nitrogen availability on M_{area}. These patterns were associated
1638 with a reduction in β with increasing soil nitrogen availability. Similar patterns
1639 were observed in the structural equation model, where indirect positive effects
1640 of increasing soil nitrogen availability on N_{area} were driven by negative effects of
1641 increasing soil nitrogen availability on β , positive relationships between β and
1642 leaf C_i:C_a, and negative relationships between leaf C_i:C_a and M_{area}. However,

1643 structural equation model results revealed a null direct relationship between leaf
1644 $C_i:C_a$ and N_{mass} , and instead indicated an indirect positive effect of leaf $C_i:C_a$ on
1645 N_{mass} mediated through strong negative covariance between M_{area} and N_{mass} .

1646 Results reported here suggest that positive direct effects of increasing soil
1647 nitrogen availability on N_{area} are not necessarily the direct product of increased
1648 mass-based leaf nitrogen content, as has been previously suggested (Firn et al.
1649 2019; Liang et al. 2020). Instead, effects of increasing soil nitrogen availability on
1650 leaf nitrogen concentration may be driven by costs of nitrogen acquisition relative
1651 to water and negative covariance between M_{area} and N_{mass} , following patterns
1652 predicted by photosynthetic least-cost theory. Findings reported here suggest that
1653 studies quantifying variance in leaf nitrogen content across resource availability
1654 gradients may risk confounding covariance between M_{area} and N_{mass} if costs of
1655 nitrogen acquisition relative to water are not also quantified.

1656 4.4.3 *Soil moisture increases N_{area} by facilitating increases in soil nitrogen
1657 availability*

1658 Increasing soil moisture had a positive effect on N_{area} , a response that was asso-
1659 ciated with a null effect of soil moisture on β . These results contrast patterns
1660 expected from theory, where increasing soil moisture is expected to indirectly de-
1661 crease N_{area} through an increase in β due to a reduction in costs associated with
1662 water acquisition and use (Wright et al. 2003; Prentice et al. 2014; Lavergne
1663 et al. 2020). Interestingly, structural equation model results revealed a strong
1664 positive association between soil moisture and soil nitrogen availability, indicat-
1665 ing an indirect positive effect of increasing soil moisture on N_{area} mediated by
1666 the negative effect of increasing soil nitrogen availability on β . In Texan grass-

1667 lands, productivity and nutrient uptake are often co-limited by precipitation and
1668 nutrient availability (Yahdjian et al. 2011; Wang et al. 2017). Thus, increases
1669 in soil moisture may have facilitated more favorable and productive environments
1670 for soil microbial communities (Reichman et al. 1966; Stark and Firestone 1995;
1671 Paul et al. 2003), or alternatively greater nitrogen mobility in soil solution. As
1672 discussed above, the positive indirect response of N_{area} to increasing soil nitrogen
1673 availability as mediated through reductions in β follow patterns expected from
1674 theory.

1675 4.4.4 *Indirect effects of climate on N_{area} are mediated through changes in leaf*
1676 *$C_i:C_a$ and β*

1677 In support of hypotheses and patterns expected from theory, increasing vapor
1678 pressure deficit indirectly increased N_{area} , mediated through the negative effect
1679 of increasing vapor pressure deficit on leaf $C_i:C_a$. These responses are consistent
1680 with previous work noting strong reductions in stomatal conductance with increas-
1681 ing vapor pressure deficit (Oren et al. 1999; Novick et al. 2016; Sulman et al.
1682 2016; Grossiord et al. 2020; López et al. 2021), a response that allows plants
1683 to minimize water loss as a result of high atmospheric water demand. Results
1684 also support findings from previous experiments across environmental gradients,
1685 where increasing vapor pressure deficit generally increases N_{area} at lower stomatal
1686 conductance across environmental gradients (Dong et al. 2017; Dong et al. 2022;
1687 Paillassa et al. 2020; Westerband et al. 2023). These responses provide another
1688 line of evidence that suggests leaf nitrogen content is a deterministic acclima-
1689 tion response to changing aboveground climate, allowing plants to satisfy demand
1690 to build and maintain photosynthetic enzymes and optimize photosynthetic pro-
1691 cesses by maximizing resource use efficiency (Paillassa et al. 2020; Peng et al.

1692 2021; Dong et al. 2022; Westerband et al. 2023).

1693 4.4.5 *Species identity traits modify effects of the environment on β , leaf $C_i:C_a$,*
1694 *and N_{area}*

1695 N-fixing species had greater N_{area} values on average compared to non-fixing species,

1696 a pattern driven by a stronger stimulation in N_{mass} in N-fixing species coupled with

1697 no change in M_{area} between species with different N-fixation ability. There was

1698 no evidence to suggest that N-fixing species had different β or leaf $C_i:C_a$ values

1699 compared to non-fixing species across the environmental gradient. These results

1700 follow patterns from previous environmental gradient experiments that investi-

1701 gate variance in leaf nitrogen allocation in N-fixing species (Adams et al. 2016;

1702 Dong et al. 2017; Dong et al. 2020), and that increases in N_{mass} and N_{area} in

1703 N-fixing species are not necessarily correlated to increases in water use efficiency

1704 or reductions in leaf $C_i:C_a$ (Adams et al. 2016). While results are consistent with

1705 results from previous environmental gradient experiments, they do not support

1706 hypotheses presented here or patterns expected from theory, which predicts that

1707 stimulations in N_{area} by N-fixing species should be driven by a reduction in β

1708 relative to non-fixing species, and that this response should decrease stomatal

1709 conductance and leaf $C_i:C_a$.

1710 C₄ species had reduced β , leaf $C_i:C_a$, and N_{area} than C₃ species. Reduced

1711 β and leaf $C_i:C_a$ values in C₄ species follow hypotheses listed above, a pattern

1712 that could be the result of either reduced costs of nitrogen acquisition and use,

1713 increased costs of water acquisition and use, or both (Wright et al. 2003; Prentice

1714 et al. 2014). Results also indicate that β in C₄ nonlegumes was unresponsive to

1715 changes in soil nitrogen availability despite an apparent negative effect of increas-

1716 ing soil nitrogen availability on β in C₃ legumes and C₃ nonlegumes. Combined
1717 with a general null response of β to soil moisture regardless of plant functional
1718 group, these patterns imply that reduced β values in C₄ species may be the re-
1719 sult of lower costs of nitrogen acquisition and use relative to C₃ species. While
1720 lower β values in C₄ species provides a possible explanation for why C₄ species
1721 often have lower leaf $C_i:C_a$ and greater water use efficiency, theory predicts that
1722 this response should cause C₄ species to have greater N_{area} values compared to
1723 C₃ species, though C₄ species commonly exhibit lower N_{area} and higher nitrogen
1724 use efficiency than C₃ species (Schmitt and Edwards 1981; Sage and Pearcy 1987;
1725 Ghannoum et al. 2011). We speculate that lowered costs of nitrogen acquisition
1726 and use in C₄ species could be driven by more efficient Rubisco carboxylation effi-
1727 ciency in C₄ species associated with CO₂ concentrating mechanisms that eliminate
1728 photorespiration (Ghannoum et al. 2011), which could reduce or eliminate the
1729 need to sacrifice inefficient nitrogen use for efficient water use to achieve optimal
1730 photosynthesis rates.

1731 4.4.6 *Next steps for optimality model development*

1732 Optimality models for both C₃ and C₄ species have been developed using principles
1733 from photosynthetic least-cost theory (Prentice et al. 2014; Wang et al. 2017;
1734 Smith et al. 2019; Stocker et al. 2020; Scott and Smith 2022). In both C₃ and C₄
1735 model variants, β values are held constant using global datasets of leaf $\delta^{13}\text{C}$ (Wang
1736 et al. 2017; Cornwell et al. 2018). Specifically, the C₃ optimality model initially
1737 assumed a constant β value of 240 (Wang et al. 2017), later corrected to 146
1738 (Stocker et al. 2020), while the C₄ optimality model assumes a constant β value of
1739 166 (Scott and Smith 2022). These results, which build on findings from Paillassa

1740 et al. (2020) and Lavergne et al. (2020), demonstrate high variability in calculated
1741 β values across the environmental gradient. Specifically, β values in C₃ species
1742 ranged from 1.7 to 188.0 (mean: 30.2; median: 23.1; standard deviation: 25.4),
1743 while ranged from 0.1 to 110.6 in C₄ species (mean: 7.2; median: 0.7; standard
1744 deviation: 18.6). Mean β values in both C₃ and C₄ species were consistently lower
1745 than values currently implemented in optimality models, though this was likely
1746 the result of increased water limitation across sites relative to global averages.
1747 Regardless, the high degree of β variability across this environmental gradient,
1748 together with findings from Lavergne et al. (2020) and Paillassa et al. (2020),
1749 suggests that the use of constant β values may contribute to erroneous errors when
1750 conducting optimality model simulations. Results from this experiment build
1751 on suggestions from Wang et al. (2017), suggesting that future photosynthetic
1752 least-cost optimality model developments should consider adopting frameworks
1753 for dynamically calculating β .

1754 4.4.7 *Conclusions*

1755 To summarize, variability in N_{area} across an environmental gradient in Texan
1756 grasslands was driven by indirect effects of climate and soil resource availability
1757 mediated by changes in β and leaf $C_i:C_a$. Results from this experiment provide
1758 strong and consistent support for patterns expected from photosynthetic least-
1759 cost theory, demonstrating that negative relationships between $C_i:C_a$ and N_{area}
1760 unify expected effects of climatic and edaphic characteristics on N_{area} across en-
1761 vironmental gradients. Results reported here also demonstrate a need to consider
1762 the dynamic nature of the relative cost of nitrogen versus water uptake (β) across

1763 environmental gradients in optimality models that leverage principles of photo-

1764 synthetic least-cost theory.

1765

Chapter 5

1766
1767

Optimal resource investment to photosynthetic capacity maximizes
nutrient allocation to whole plant growth under elevated CO₂

1768 5.1 Introduction

1769 Terrestrial ecosystems are regulated by complex carbon and nitrogen cycles. As
1770 a result, terrestrial biosphere models, which are beginning to include coupled
1771 carbon and nitrogen cycles (Shi et al. 2016; Davies-Barnard et al. 2020; Braghieri
1772 et al. 2022), must accurately represent these cycles under different environmental
1773 scenarios to reliably simulate carbon and nitrogen atmosphere-biosphere fluxes
1774 (Hungate et al. 2003; Prentice et al. 2015). While the inclusion of coupled carbon
1775 and nitrogen cycles tends to reduce model uncertainty (Arora et al. 2020), large
1776 uncertainty in role of soil nitrogen availability and nitrogen acquisition strategy
1777 on leaf and whole plant acclimation responses to CO₂ remains (Smith and Dukes
1778 2013; Terrer et al. 2018; Smith and Keenan 2020). This source of uncertainty
1779 likely contributes to the widespread divergence in future carbon and nitrogen flux
1780 simulations across terrestrial biosphere models (Friedlingstein et al. 2014; Zaehle
1781 et al. 2014; Meyerholt et al. 2020).

1782 Plants grown under elevated CO₂ generally have less leaf nitrogen content
1783 than those grown under ambient CO₂, a response that often corresponds with
1784 reductions in photosynthetic capacity and stomatal conductance at the leaf-level
1785 and biomass stimulation over time at the whole plant level (Curtis 1996; Drake
1786 et al. 1997; Ainsworth et al. 2002; Makino 2003; Morgan et al. 2004; Ainsworth
1787 and Long 2005; Ainsworth and Rogers 2007; Smith and Dukes 2013; Poorter et al.
1788 2022). As net primary productivity is generally limited by nitrogen availability

1789 (Vitousek and Howarth 1991; LeBauer and Treseder 2008; Fay et al. 2015), and
1790 soil nitrogen availability is often positively correlated with leaf nitrogen content
1791 and photosynthetic capacity (Field and Mooney 1986; Evans and Seemann 1989;
1792 Evans 1989; Walker et al. 2014; Firn et al. 2019; Liang et al. 2020), some
1793 have hypothesized that leaf and whole plant acclimation responses to CO₂ are
1794 constrained by soil nitrogen availability.

1795 The progressive nitrogen limitation hypothesis predicts that elevated CO₂
1796 will increase plant nitrogen demand, which will increase plant nitrogen uptake and
1797 progressively deplete soil nitrogen if soil nitrogen supply does not exceed plant
1798 nitrogen demand (Luo et al. 2004). The hypothesis predicts that this response
1799 should result in strong acute stimulations in whole plant growth and primary
1800 productivity that diminish over time as nitrogen becomes more limiting. Assuming
1801 a positive relationship between soil nitrogen availability, leaf nitrogen content, and
1802 photosynthetic capacity, this hypothesis also implies that progressive reductions in
1803 soil nitrogen availability should be the mechanism that drives the downregulation
1804 of leaf nitrogen content and photosynthetic capacity under elevated CO₂. This
1805 hypothesis has received some support from free air CO₂ enrichment experiments
1806 (Reich et al. 2006; Norby et al. 2010), although is not consistently observed across
1807 experiments (Finzi et al. 2006; Moore et al. 2006; Liang et al. 2016).

1808 While possible that progressive nitrogen limitation may determine leaf and
1809 whole plant acclimation responses to CO₂, growing evidence indicates that leaf ni-
1810 trogen and photosynthetic capacity are more strongly determined through above-
1811 ground growing conditions than by soil resource availability (Dong et al. 2017;
1812 Dong et al. 2020; Dong et al. 2022; Smith et al. 2019; Smith and Keenan 2020;

1813 Paillassa et al. 2020; Peng et al. 2021; Querejeta et al. 2022; Westerband et al.
1814 2023), and satellite-derived chlorophyll fluorescence data indicate that increasing
1815 atmospheric CO₂ may decrease leaf and canopy demand for nitrogen (Dong et al.
1816 2022). Together, results from these studies suggest that the downregulation in
1817 leaf nitrogen content and photosynthetic capacity due to increasing CO₂ may not
1818 be as tightly linked to progressive nitrogen limitation as previously hypothesized.

1819 A unification of optimal coordination and least-cost theories predicts that
1820 leaves acclimate to elevated CO₂ by downregulating nitrogen allocation to Ribulose-
1821 1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) to optimize resource
1822 use efficiencies at the leaf level, which allows for greater resource allocation to
1823 whole plant growth (Drake et al. 1997; Wright et al. 2003; Prentice et al. 2014;
1824 Smith et al. 2019). The theory predicts that the downregulation in nitrogen
1825 allocation to Rubisco results in a stronger downregulation in the maximum rate
1826 of Rubisco carboxylation (V_{cmax}) than the maximum rate of RuBP regeneration
1827 (J_{max}), which maximizes photosynthetic efficiency by allowing net photosynthesis
1828 rates to be equally co-limited by Rubisco carboxylation and RuBP regeneration
1829 (Chen et al. 1993; Maire et al. 2012). This acclimation response allows plants to
1830 make more efficient use of available light while avoiding overinvestment in Rubisco,
1831 which has high nitrogen and energetic costs of building and maintaining (Evans
1832 1989; Evans and Clarke 2019). Instead, additional acquired resources not needed
1833 to optimize leaf photosynthesis are allocated to the maintenance of structures that
1834 support whole plant growth (e.g., total leaf area, whole plant biomass, etc.) or
1835 to allocation processes not related to leaf photosynthesis or growth, such as plant
1836 defense mechanisms. Regardless, optimized resource allocation at the leaf level

1837 should allow for greater resource allocation to whole plant growth. The theory
1838 indicates that leaf acclimation responses to CO₂ should be independent of changes
1839 in soil nitrogen availability. While this leaf acclimation response maximizes nitro-
1840 gen allocation to structures that support whole plant growth, the theory suggests
1841 that the positive effect of elevated CO₂ on whole plant growth may be further
1842 stimulated by soil nitrogen availability through reductions in the cost of acquiring
1843 nitrogen (Bae et al. 2015; Perkowski et al. 2021; Lu et al. 2022).

1844 Plants acquire nitrogen by allocating photosynthetically derived carbon be-
1845 lowground in exchange for nitrogen through different nitrogen acquisition strate-
1846 gies. These nitrogen acquisition strategies can include direct uptake pathways
1847 such as mass flow or diffusion (Barber 1962), symbioses with mycorrhizal fungi or
1848 symbiotic nitrogen-fixing bacteria (Vance and Heichel 1991; Marschner and Dell
1849 1994; Smith and Read 2008; Udvardi and Poole 2013), or through the release
1850 of root exudates that prime free-living soil microbial communities (Phillips et al.
1851 2011; Wen et al. 2022). Plants cannot acquire nitrogen without first allocating
1852 carbon belowground, which implies an inherent carbon cost to the plant for acquir-
1853 ing nitrogen regardless of nitrogen acquisition strategy. Carbon costs to acquire
1854 nitrogen often vary in species with different nitrogen acquisition strategies and
1855 are dependent on external environmental factors such as atmospheric CO₂, light
1856 availability, and soil nitrogen availability (Brzostek et al. 2014; Terrer et al. 2016;
1857 Terrer et al. 2018; Allen et al. 2020; Perkowski et al. 2021; Lu et al. 2022). These
1858 patterns suggest that acquisition strategy may at least partially determine the net
1859 effect of soil nitrogen availability on leaf and whole plant acclimation responses to
1860 elevated CO₂.

1861 A recent meta-analysis using data across 20 grassland and forest CO₂ en-
1862 richment experiments suggested that species which acquire nitrogen from sym-
1863 biotic nitrogen-fixing bacteria had reduced costs of nitrogen acquisition under
1864 elevated CO₂ (Terrer et al. 2018). Though these analyses only included data
1865 from two experimental sites, findings from this meta-analysis indicated that re-
1866 ductions in costs of nitrogen acquisition in species that form associations with
1867 symbiotic nitrogen-fixing bacteria under elevated CO₂ may drive stronger stim-
1868 ulations in whole plant growth and downregulations in V_{cmax} than species that
1869 associate with arbuscular mycorrhizal fungi (Smith and Keenan 2020), which gen-
1870 erally have greater costs of nitrogen acquisition under elevated CO₂ (Terrer et al.
1871 2018). However, plant investments in symbiotic nitrogen fixation generally de-
1872 cline with increasing nitrogen availability (Dovrat et al. 2018; Perkowski et al.
1873 2021), a response that has been previously inferred to be the result of a shift in
1874 the dominant mode of nitrogen acquisition to direct uptake pathways as costs of
1875 direct uptake decrease with increasing soil nitrogen availability (Rastetter et al.
1876 2001; Perkowski et al. 2021). Thus, effects of symbiotic nitrogen fixation on plant
1877 acclimation responses to CO₂ should decline with increasing soil nitrogen avail-
1878 ability, although manipulative experiments that directly test these patterns are
1879 rare.

1880 Here, I conducted a 7-week growth chamber experiment using *Glycine max*
1881 L. (Merr.) to examine the effects of soil nitrogen fertilization and inoculation with
1882 symbiotic nitrogen-fixing bacteria on leaf and whole plant acclimation responses
1883 to elevated CO₂. Following patterns expected from theory, I hypothesized that in-
1884 dividual leaves should acclimate to elevated CO₂ by more strongly downregulating

1885 V_{cmax} relative to J_{max} , allowing leaf photosynthesis to approach optimal coordi-
1886 nation. I expected this response to correspond with a stronger downregulation in
1887 leaf nitrogen content than V_{cmax} and J_{max} , which would increase the fraction of
1888 leaf nitrogen content allocated to photosynthesis and photosynthetic nitrogen use
1889 efficiency. At the whole-plant level, I hypothesized that plants would acclimate
1890 to elevated CO₂ by stimulating whole plant growth and productivity, a response
1891 that would be driven by a strong positive response of total leaf area and above-
1892 ground biomass to elevated CO₂. I predicted that leaf acclimation responses to
1893 elevated CO₂ would be independent of soil nitrogen fertilization and inoculation
1894 with symbiotic nitrogen-fixing bacteria; however, I expected that increasing soil
1895 nitrogen fertilization would increase the positive effect of elevated CO₂ on mea-
1896 sures of whole plant growth due to a stronger reduction in the cost of acquiring
1897 nitrogen under elevated CO₂ with increasing fertilization. I also expected stronger
1898 stimulations in whole plant growth due to inoculation, but that this effect would
1899 only be apparent under low fertilization due to a reduction in root nodulation
1900 with increasing fertilization.

1901 5.2 Methods

1902 5.2.1 *Seed treatments and experimental design*

1903 *Glycine max* L. (Merr) seeds were planted in 144 6-liter surface sterilized pots (NS-
1904 600, Nursery Supplies, Orange, CA, USA) containing a steam-sterilized 70:30 v:v
1905 mix of Sphagnum peat moss (Premier Horticulture, Quakertown, PA, USA) to
1906 sand (Pavestone, subsidiary of Quikrete Companies, Atlanta, GA, USA). Before
1907 planting, all *G. max* seeds were surface sterilized in 2% sodium hypochlorite for 3

1908 minutes, followed by three separate 3-minute washes with ultrapure water (MilliQ
1909 7000; MilliporeSigma, Burlington, MA USA). A subset of surface sterilized seeds
1910 were inoculated with *Bradyrhizobium japonicum* (Verdesian N-DureTM Soybean,
1911 Cary, NC, USA) in a slurry following manufacturer recommendations (3.12 g
1912 inoculant and 241 g deionized water per 1 kg seed).

1913 Seventy-two pots were randomly planted with surface-sterilized seeds inoc-
1914 ulated with *B. japonicum*, while the remaining 72 pots were planted with surface-
1915 sterilized uninoculated seeds. Thirty-six pots within each inoculation treatment
1916 were randomly placed in one of two atmospheric CO₂ treatments (ambient and
1917 1000 $\mu\text{mol mol}^{-1}$ CO₂). Pots within each unique inoculation-by-CO₂ treatment
1918 combination randomly received one of nine soil nitrogen fertilization treatments
1919 equivalent to 0, 35, 70, 105, 140, 210, 280, 350, or 630 ppm N. Nitrogen fertil-
1920 ization treatments were created using a modified Hoagland solution (Hoagland
1921 and Arnon 1950) designed to keep concentrations of other macronutrients and
1922 micronutrients equivalent across treatments (Table D1). Pots received the same
1923 fertilization treatment throughout the entire duration experiment, which were ap-
1924 plied twice per week in 150 mL doses as topical agents to the soil surface. This
1925 experimental design yielded a fully factorial experiment with four replicates per
1926 unique fertilization-by-inoculation-by-CO₂ combination.

1927 5.2.2 *Growth chamber conditions*

1928 Upon experiment initiation, pots were randomly placed in one of six Percival
1929 LED-41L2 growth chambers (Percival Scientific Inc., Perry, IA, USA) over two
1930 experimental iterations due to chamber space limitation. Two iterations were

1931 conducted such that one iteration included all elevated CO₂ pots and the second
1932 iteration included all ambient CO₂ pots. Mean (\pm SD) CO₂ concentrations across
1933 chambers throughout the experiment were $439 \pm 5 \mu\text{mol mol}^{-1}$ CO₂ for the ambient
1934 CO₂ treatment and $989 \pm 4 \mu\text{mol mol}^{-1}$ CO₂ for the elevated CO₂ treatment.

1935 Daytime growing conditions were simulated using a 16-hour photoperiod,
1936 with incoming light radiation set to chamber maximum (mean \pm SD: 1240 ± 32
1937 $\mu\text{mol m}^{-2} \text{s}^{-1}$ across chambers), air temperature set to 25°C, and relative humid-
1938 ity set to 50%. The remaining 8 hours simulated nighttime growing conditions,
1939 with incoming light radiation set to 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$, chamber temperature set
1940 to 17°C, and relative humidity set to 50%. Transitions between daytime and
1941 nighttime growing conditions were simulated by ramping incoming light radiation
1942 in 45-minute increments and temperature in 90-minute increments over a 3-hour
1943 period (Table D2).

1944 Including the two, 3-hour ramping periods, pots grew under average (\pm SD)
1945 daytime light intensity of $1049 \pm 27 \mu\text{mol m}^{-2} \text{s}^{-1}$. In the elevated CO₂ iteration,
1946 pots grew under $24.0 \pm 0.2^\circ\text{C}$ during the day, $16.4 \pm 0.8^\circ\text{C}$ during the night, and
1947 51.6 $\pm 0.4\%$ relative humidity. In the ambient CO₂ iteration, pots grew under
1948 $23.9 \pm 0.2^\circ\text{C}$ during the day, $16.0 \pm 1.4^\circ\text{C}$ during the night, and 50.3 $\pm 0.2\%$ relative
1949 humidity. I accounted for any climatic differences across the six chambers by
1950 shuffling the same group of pots daily throughout the growth chambers. This
1951 process was done by iteratively moving the group of pots on the top rack of a
1952 chamber to the bottom rack of the same chamber, while simultaneously moving
1953 the group of pots on the bottom rack of a chamber to the top rack of the adjacent
1954 chamber. I moved pots within and across chambers every day throughout the

1955 course of each experiment iteration.

1956 5.2.3 *Leaf gas exchange measurements*

1957 Gas exchange measurements were collected for all individuals on the seventh week

1958 of development. All gas exchange measurements were collected on the center leaf

1959 of the most recent fully expanded trifoliate leaf set. Specifically, I measured net

1960 photosynthesis (A_{net} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_{sw} ; $\text{mol m}^{-2} \text{s}^{-1}$),

1961 and intercellular CO_2 (C_i ; $\mu\text{mol mol}^{-1}$) concentrations across a range of atmo-

1962 spheric CO_2 concentrations (i.e., an A_{net}/C_i curve) using the Dynamic Assimila-

1963 tion TechniqueTM. The Dynamic Assimilation TechniqueTM has been shown to

1964 correspond well with traditional steady-state CO_2 response curves in *G. max*

1965 (Saathoff and Welles 2021). A_{net}/C_i curves were generated along a reference CO_2

1966 ramp down from $420 \mu\text{mol mol}^{-1} \text{CO}_2$ to $20 \mu\text{mol mol}^{-1} \text{CO}_2$, followed by a ramp

1967 up from $420 \mu\text{mol mol}^{-1} \text{CO}_2$ to $1620 \mu\text{mol mol}^{-1} \text{CO}_2$ after a 90-second wait

1968 period at $420 \mu\text{mol mol}^{-1} \text{CO}_2$. The ramp rate for each curve was set to 200

1969 $\mu\text{mol mol}^{-1} \text{min}^{-1}$, logging every five seconds, which generated 96 data points per

1970 response curve. All A_{net}/C_i curves were generated after A_{net} and g_{sw} stabilized

1971 in a LI-6800 cuvette set to a 500 mol s^{-1} , 10,000 rpm mixing fan speed, 1.5 kPa

1972 vapor pressure deficit, 25°C leaf temperature, $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ incoming light

1973 radiation, and initial reference CO_2 set to $420 \mu\text{mol mol}^{-1}$.

1974 With the same focal leaf used to generate A_{net}/C_i curves, I measured dark

1975 respiration (R_{d25} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) following at least a 30-minute period of darkness.

1976 Measurements were collected on a 5-second log interval for 60 seconds after stabi-

1977 lizing in a LI-6800 cuvette set to a 500 mol s^{-1} , 10,000 rpm mixing fan speed, 1.5

1978 kPa vapor pressure deficit, 25°C leaf temperature, and 420 $\mu\text{mol mol}^{-1}$ reference
1979 CO₂ concentration (for both CO₂ concentrations), with incoming light radiation
1980 set to 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A single dark respiration value was determined for each
1981 focal leaf by calculating the mean dark respiration value (i.e. the absolute value
1982 of A_{net} during the logging period) across the logging interval.

1983 5.2.4 *Leaf trait measurements*

1984 The focal leaf used to generate A_{net}/C_i curves and dark respiration was harvested
1985 immediately following gas exchange measurements. Images of each focal leaf were
1986 curated using a flat-bed scanner to determine wet leaf area using the ‘LeafArea’ R
1987 package (Katabuchi 2015), which automates leaf area calculations using ImageJ
1988 software (Schneider et al. 2012). Each leaf was dried at 65°C for at least 48
1989 hours, and subsequently weighed and ground until homogenized. Leaf mass per
1990 area (M_{area} ; g m^{-2}) was calculated as the ratio of dry leaf biomass to fresh leaf
1991 area. Using subsamples of ground and homogenized leaf tissue, I measured leaf
1992 nitrogen content (N_{mass} ; gN g^{-1}) through elemental combustion analysis (Costech-
1993 4010, Costech, Inc., Valencia, CA, USA). Leaf nitrogen content per unit leaf area
1994 (N_{area} ; gN m^{-2}) was calculated by multiplying N_{mass} and M_{area} . Subsamples of
1995 ground and homogenized leaf tissue were also sent to the UC-Davis Stable Isotope
1996 Facility to quantify leaf $\delta^{15}\text{N}$, later used to estimate the fraction of leaf nitrogen
1997 derived from the atmosphere.

1998 I extracted chlorophyll content from a second leaf in the same trifoliolate
1999 leaf set as the focal leaf used to generate A_{net}/C_i curves. Prior to chlorophyll
2000 extraction, I used a cork borer to punch between 3 and 5 0.6 cm² disks from the

2001 leaf. Separate images of each punched leaf and set of leaf disks were curated using
2002 a flat-bed scanner to determine wet leaf area, again quantified using the ‘LeafArea’
2003 R package (Katabuchi 2015). The punched leaf was dried and weighed after at
2004 least 65°C in the drying oven to determine M_{area} of the chlorophyll leaf.

2005 Leaf disks were shuttled into a test tube containing 10mL dimethyl sulfoxide, vortexed, and incubated at 65°C for 120 minutes (Barnes et al. 1992). Incubated test tubes were vortexed again before loaded in 150 μL triplicate aliquots to a 96-well plate. Dimethyl sulfoxide was also loaded in a 150 μL triplicate aliquot as a blank. Absorbance measurements at 649.1 nm ($A_{649.1}$) and 665.1 nm ($A_{665.1}$) were read in each well using a plate reader (Bioteck Synergy H1; Bioteck Instruments, Winooski, VT USA) (Wellburn 1994), with triplicates subsequently averaged and corrected by the mean of the blank absorbance value. Blank-corrected absorbance values were used to estimate Chl_a ($\mu\text{g mL}^{-1}$) and Chl_b ($\mu\text{g mL}^{-1}$) following equations from Wellburn (1994):

$$Chl_a = 12.47A_{665.1} - 3.62A_{649.1} \quad (5.1)$$

2015 and

$$Chl_b = 25.06A_{665.1} - 6.50A_{649.1} \quad (5.2)$$

2016 Chl_a and Chl_b were converted to mmol mL^{-1} using the molar mass of chlorophyll a (893.51 g mol^{-1}) and the molar mass of chlorophyll b (907.47 g mol^{-1}), then added together to calculate total chlorophyll content in the dimethyl sulfoxide extractant (mmol mL^{-1}). Total chlorophyll content was multiplied by the volume of the dimethyl sulfoxide extractant (10 mL) and converted to area-based chlorophyll

2021 content by dividing by the total area of the leaf disks (Chl_{area} ; mmol m⁻²). Mass-
2022 based chlorophyll content (Chl_{mass} ; mmol g⁻¹) was calculated by dividing Chl_{area}
2023 by the leaf mass per area of the punched leaf.

2024 5.2.5 *A/C_i curve fitting and parameter estimation*

2025 I fit A_{net}/C_i curves of each individual using the ‘fitaci’ function in the ‘plante-
2026 cophys’ R package (Duursma 2015). This function estimates the maximum rate
2027 of Rubisco carboxylation (V_{cmax} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) and maximum rate of electron
2028 transport for RuBP regeneration (J_{max} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) based on the Farquhar
2029 biochemical model of C₃ photosynthesis (Farquhar et al. 1980). Triose phosphate
2030 utilization (TPU) limitation was included in all curve fits, and all curve fits in-
2031 cluded measured dark respiration values. As A_{net}/C_i curves were generated using
2032 a common leaf temperature, curves were fit using Michaelis-Menten coefficients
2033 for Rubisco affinity to CO₂ (K_c ; $\mu\text{mol mol}^{-1}$) and O₂ (K_o ; $\mu\text{mol mol}^{-1}$), and the
2034 CO₂ compensation point (Γ^* ; $\mu\text{mol mol}^{-1}$) reported in Bernacchi et al. (2001).
2035 Specifically, K_c was set to 404.9 $\mu\text{mol mol}^{-1}$, K_o was set to 278.4 $\mu\text{mol mol}^{-1}$,
2036 and Γ^* was set to 42.75 $\mu\text{mol mol}^{-1}$. All curve fits were visually examined for
2037 goodness-of-fit. The use of a common leaf temperature across curves and dark
2038 respiration measurements eliminated the need to temperature standardize rate
2039 estimates. For clarity, I reference V_{cmax} , J_{max} , and R_d estimates throughout the
2040 rest of the chapter as V_{cmax25} , J_{max25} , and R_{d25} .

2041 5.2.6 *Stomatal limitation*

2042 I quantified the extent by which stomatal conductance limited photosynthesis (l ;

2043 unitless) following equations originally described in Farquhar and Sharkey (1982).

2044 Stomatal limitation was calculated as:

$$l = 1 - \frac{A_{net}}{A_{mod}} \quad (5.3)$$

2045 where A_{mod} represents the photosynthetic rate where $C_i = C_a$. A_{mod} was calculated

2046 as:

$$A_{mod} = V_{cmax25} - \frac{420 - \Gamma^*}{420 + K_m} - R_{d25} \quad (5.4)$$

2047 K_m is the Michaelis-Menten coefficient for Rubisco-limited photosynthesis, calcu-

2048 lated as:

$$K_m = K_c \cdot \left(1 + \frac{O_i}{K_o}\right) \quad (5.5)$$

2049 where O_i refers to leaf intercellular O_2 concentrations, set to $210 \mu\text{mol mol}^{-1}$.

2050 5.2.7 *Proportion of leaf nitrogen allocated to photosynthesis and structure*

2051 I used equations from Niinemets and Tenhunen (1997) to estimate the proportion

2052 of leaf nitrogen content allocated to Rubisco, bioenergetics, and light harvesting

2053 proteins. The proportion of leaf nitrogen allocated to Rubisco ($\rho_{rubisco}$; gN g^{-1})

2054 was calculated as a function of V_{cmax25} and N_{area} :

$$\rho_{rubisco} = \frac{V_{cmax25} N_r}{V_{cr} N_{area}} \quad (5.6)$$

2055 where N_r is the amount of nitrogen in Rubisco, set to 0.16 gN (gN in Rubisco) $^{-1}$
 2056 and V_{cr} is the maximum rate of RuBP carboxylation per unit Rubisco protein,
 2057 set to 20.5 $\mu\text{mol CO}_2$ (g Rubisco) $^{-1}$. The proportion of leaf nitrogen allocated to
 2058 bioenergetics (ρ_{bioe} ; gN gN $^{-1}$) was similarly calculated as a function of J_{max25} and
 2059 N_{area} :

$$\rho_{bioe} = \frac{J_{max25} N_b}{J_{mc} N_{area}} \quad (5.7)$$

2060 where N_b is the amount of nitrogen in cytochrome f, set to 0.12407 gN (μmol
 2061 cytochrome f) $^{-1}$ assuming a constant 1: 1: 1.2 cytochrome f: ferredoxin NADP
 2062 reductase: coupling factor molar ratio (Evans and Seemann 1989; Niinemets and
 2063 Tenhunen 1997), and J_{mc} is the capacity of electron transport per cytochrome f,
 2064 set to 156 $\mu\text{mol electron}$ ($\mu\text{mol cytochrome f}$) $^{-1}\text{s}^{-1}$.

2065 The proportion of leaf nitrogen allocated to light harvesting proteins (ρ_{light} ;
 2066 gN gN $^{-1}$) was calculated as a function of Chl_{mass} and N_{mass} :

$$\rho_{light} = \frac{Chl_{mass}}{N_{mass} c_b} \quad (5.8)$$

2067 where c_b is the stoichiometry of the light-harvesting chlorophyll complexes of
 2068 photosystem II, set to 2.75 mmol chlorophyll (gN in chlorophyll) $^{-1}$. I used the
 2069 N_{mass} value of the focal leaf used to generate A_{net}/C_i curves instead of the leaf
 2070 used to extract chlorophyll content, as the two leaves are from the same trifoliolate
 2071 leaf set and are highly correlated (Figure D1).

2072 The proportion of leaf nitrogen content allocated to photosynthetic tissue
 2073 (ρ_{photo} ; gN gN $^{-1}$) was estimated as the sum of $\rho_{rubisco}$, ρ_{bioe} , and ρ_{light} . Finally,
 2074 the proportion of leaf nitrogen content allocated to structural tissue ($\rho_{structure}$; gN

2075 gN^{-1}) was estimated as:

$$\rho_{structure} = \frac{N_{cw}}{N_{area}} \quad (5.9)$$

2076 where N_{cw} is the leaf nitrogen content allocated to cell walls (gN m^{-2}), calculated

2077 as a function of M_{area} using an empirical equation from Onoda et al. (2017):

$$N_{cw} = 0.000355 * M_{area}^{1.39} \quad (5.10)$$

2078 5.2.8 *Whole plant traits*

2079 Seven weeks after experiment initiation and immediately following gas exchange
2080 measurements, I harvested all experimental individuals and separated biomass of
2081 each experimental individual into major organ types (leaves, stems, roots, and
2082 nodules when present). Fresh leaf area of all harvested leaves was measured using
2083 an LI-3100C (Li-COR Biosciences, Lincoln, Nebraska, USA). Total fresh leaf area
2084 (cm^2) was calculated as the sum of all leaf areas, including the focal leaf used to
2085 collect gas exchange data and the focal leaf used to extract chlorophyll content. All
2086 harvested material was dried in an oven set to 65°C for at least 48 hours, weighed,
2087 and ground to homogeneity. Leaves and nodules were manually ground with a
2088 mortar and pestle, while stems and roots were ground using a Wiley mill (E3300
2089 Mini Mill; Eberbach Corp., MI, USA). Total dry biomass (g) was calculated as
2090 the sum of dry leaf (including focal leaf for both the A_{net}/C_i curve and leaf used
2091 to extract chlorophyll content), stem, root, and root nodule biomass. I quantified
2092 carbon and nitrogen content of each respective organ type through elemental
2093 combustion (Costech-4010, Costech, Inc., Valencia, CA, USA) using subsamples
2094 of ground and homogenized organ tissue.

Following the approach explained in the first experimental chapter, I calculated structural carbon costs to acquire nitrogen as the ratio of total belowground carbon biomass to whole plant nitrogen biomass (N_{cost} ; gC gN⁻¹). Belowground carbon biomass (C_{bg} ; gC) was calculated as the sum of root carbon biomass and root nodule carbon biomass. Root carbon biomass and root nodule carbon biomass was calculated as the product of the organ biomass and the respective organ carbon content. Whole plant nitrogen biomass (N_{wp} ; gN) was similarly calculated as the sum of total leaf, stem, root, and root nodule nitrogen biomass, including the focal leaf used for A_{net}/C_i curve and chlorophyll extractions. Leaf, stem, root, and root nodule nitrogen biomass was calculated as the product of the organ biomass and the respective organ nitrogen content. This calculation only quantifies plant structural carbon costs to acquire nitrogen and does not include any additional costs of nitrogen acquisition associated with 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).

Finally, plant investments in nitrogen fixation were calculated 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; Dovrat et al. 2020; Perkowski et al. 2021). I also calculated the percent of leaf nitrogen acquired from the atmosphere (% N_{dfa} ; %) using leaf δ¹⁵N and the following equation from Andrews et al. (2011):

$$\%N_{\text{dfa}} = \frac{\delta^{15}N_{\text{reference}} - \delta^{15}N_{\text{sample}}}{\delta^{15}N_{\text{reference}} - B} \quad (5.11)$$

2117 where $\delta^{15}\text{N}_{\text{reference}}$ refers to a reference plant that exclusively acquires nitrogen via
2118 direct uptake, $\delta^{15}\text{N}_{\text{sample}}$ refers to an individual's leaf $\delta^{15}\text{N}$, and B refers to individuals
2119 that are entirely reliant on nitrogen fixation. Within each unique nitrogen
2120 fertilization treatment-by-CO₂ treatment combination, I calculated the mean leaf
2121 $\delta^{15}\text{N}$ for individuals growing in the non-inoculated treatment for $\delta^{15}\text{N}_{\text{reference}}$. Any
2122 individuals with visual confirmation of root nodule formation were omitted from
2123 the calculation of $\delta^{15}\text{N}_{\text{reference}}$. Following recommendations from Andrews et al.
2124 (2011) I calculated B within each CO₂ treatment using the mean leaf $\delta^{15}\text{N}$ of
2125 inoculated individuals that received 0 ppm N. I did not calculate B within each
2126 unique soil nitrogen-by-CO₂ treatment combination, as previous studies suggest
2127 decreased reliance on nitrogen fixation with increasing soil nitrogen availability
2128 (Perkowski et al. 2021).

2129 5.2.9 *Statistical analyses*

2130 Uninoculated pots that had substantial root nodule formation (nodule biomass:
2131 root biomass values greater than 0.05 g g⁻¹) were removed from all analyses, as
2132 pots were assumed to have been colonized by symbiotic nitrogen-fixing bacteria
2133 from outside sources. This decision resulted in the removal of sixteen pots from
2134 analyses: two pots in the elevated CO₂ treatment that received 35 ppm N, three
2135 pots in the elevated CO₂ treatment that received 70 ppm N, one pot in the elevated
2136 CO₂ treatment that received 210 ppm N, two pots in the elevated CO₂ treatment
2137 that received 280 ppm N, two pots in the ambient CO₂ treatment that received
2138 0 ppm N, three pots in the ambient CO₂ treatment that received 70 ppm N, two
2139 pots in the ambient CO₂ treatment that received 105 ppm N, and one pot in the

2140 ambient CO₂ treatment that received 280 ppm N.

2141 I built a series of linear mixed effects models to investigate the impacts of
2142 CO₂ concentration, soil nitrogen fertilization, and inoculation with *B. japonicum*
2143 on *G. max* gas exchange, tradeoffs between nitrogen and water use, whole plant
2144 growth, and investment in nitrogen fixation. All models included CO₂ treatment
2145 as a categorical fixed effect, inoculation treatment as a categorical fixed effect,
2146 soil nitrogen fertilization as a continuous fixed effect, with interaction terms be-
2147 tween all three fixed effects. All models also accounted for climatic difference
2148 between chambers across experiment iterations by including a random intercept
2149 term that nested starting chamber rack by CO₂ treatment. Models with this
2150 independent variable structure were created for each of the following dependent
2151 variables: N_{area} , M_{area} , N_{mass} , Chl_{area} , V_{cmax25} , J_{max25} , $J_{\text{max25}}:V_{\text{cmax25}}$, R_{d25} , g_{sw} ,
2152 stomatal limitation, ρ_{rubisco} , ρ_{bioe} , ρ_{light} , ρ_{photo} , $\rho_{\text{structure}}$, total biomass, total leaf
2153 area, N_{cost} , C_{bg} , N_{wp} , nodule biomass, the ratio of nodule biomass to root biomass,
2154 and % N_{dfa} .

2155 I used Shapiro-Wilk tests of normality to determine whether linear mixed
2156 effects models satisfied residual normality assumptions. If residual normality as-
2157 sumptions were not met (Shapiro-Wilk: $p < 0.05$), then models were fit using de-
2158 pendent variables that were natural log transformed. If residual normality as-
2159 sumptions were still not met (Shapiro-Wilk: $p < 0.05$), then models were fit using
2160 dependent variables that were square root transformed. All residual normality
2161 assumptions that did not originally satisfy residual normality assumptions were
2162 met with either a natural log or square root data transformation (Shapiro-Wilk:
2163 $p > 0.05$ in all cases). Specifically, models for N_{area} , N_{mass} , Chl_{area} , V_{cmax25} , J_{max25} ,

2164 $J_{\max25}$: $V_{\text{cmax}25}$, R_{d25} , g_{sw} , stomatal limitation, ρ_{rubisco} , ρ_{bioe} , ρ_{light} , ρ_{photo} , total leaf
2165 area, N_{cost} satisfied residual normality assumptions without data transformation.
2166 Models for M_{area} , $\rho_{\text{structure}}$, C_{bg} , and total biomass satisfied residual normality as-
2167 sumptions with a natural log data transformation, while models for N_{wp} , nodule
2168 biomass, nodule biomass: root biomass, and $\%N_{dfa}$ satisfied residual normality
2169 assumptions with a square root data transformation.

2170 In all statistical models, I used the ‘lmer’ function in the ‘lme4’ R package
2171 (Bates et al. 2015) to fit each model and the ‘Anova’ function in the ‘car’ R
2172 package (Fox and Weisberg 2019) to calculate Type II Wald’s χ^2 and determine
2173 the significance ($\alpha=0.05$) of each fixed effect coefficient. I used the ‘emmeans’
2174 R package (Lenth 2019) to conduct post-hoc comparisons using Tukey’s tests,
2175 where degrees of freedom were approximated using the Kenward-Roger approach
2176 (Kenward and Roger 1997). All analyses and plots were conducted in R version
2177 4.2.0 (R Core Team 2021).

2178 5.3 Results

2179 5.3.1 Leaf nitrogen and chlorophyll content

2180 Elevated CO₂ reduced N_{area} , N_{mass} , and Chl_{area} by 29%, 50%, and 31%, respec-
2181 tively, and stimulated M_{area} by 44% ($p<0.001$ in all cases; Table 5.1). An inter-
2182 action between fertilization and CO₂ (CO₂-by-fertilization interaction: $p_{N_{\text{area}}}=$
2183 0.017, $p_{N_{\text{mass}}}<0.001$, $p_{Chl_{\text{area}}}=0.083$; Table 5.1) indicated that the positive effect
2184 of increasing fertilization on N_{area} , N_{mass} , and Chl_{area} ($p<0.001$ in all cases; Table
2185 5.1) was stronger under ambient CO₂ (Tukey _{N_{area}} : $p=0.026$; Tukey _{N_{mass}} : $p<0.001$;
2186 Tukey _{Chl_{area}} : $p=0.065$; Table 5.1; Figs. 5.1a, 5.1b, 5.1d). An interaction between

2187 fertilization and CO₂ on M_{area} (CO₂-by-fertilization interaction: $p=0.006$; Ta-
2188 ble 5.1) indicated that the positive effect of increasing fertilization on M_{area} was
2189 stronger under elevated CO₂ (Tukey: $p=0.009$; Fig. 5.1c). Overall, interactions
2190 between fertilization and CO₂ resulted in stronger reductions in N_{area} , N_{mass} , and
2191 Chl_{area} , and a stronger stimulation in M_{area} under elevated CO₂ with increasing
2192 fertilization.

2193 An interaction between inoculation and CO₂ on N_{area} (CO₂-by-inoculation
2194 interaction: $p=0.030$; Table 5.1) indicated that the positive effect of inoculation
2195 on N_{area} ($p<0.001$; Table 5.1) was stronger under elevated CO₂ (45% increase;
2196 Tukey: $p<0.001$) than under ambient CO₂ (18% increase; Tukey: $p<0.001$), a
2197 result that increased the reduction in N_{area} in inoculated pots under elevated
2198 CO₂. Inoculation treatment did not modify the downregulation in N_{mass} (CO₂-
2199 by-inoculation interaction: $p=0.148$; Table 5.1) and Chl_{area} ($p = 0.147$; Table
2200 5.1) or the stimulation in M_{area} ($p=0.866$; Table 5.1) under elevated CO₂. How-
2201 ever, interactions between fertilization and inoculation on N_{area} , N_{mass} , M_{area} ,
2202 and Chl_{area} (fertilization-by-inoculation interaction: $p_{N_{\text{area}}}<0.001$, $p_{N_{\text{mass}}}=0.001$,
2203 $p_{M_{\text{area}}}=0.025$, $p_{Chl_{\text{area}}}=0.083$; Table 5.1) indicated that the positive effect of in-
2204 creasing fertilization on each trait was stronger in uninoculated pots (Tukey _{N_{area}} :
2205 $p<0.001$; Tukey _{N_{mass}} : $p=0.001$; Tukey _{M_{area}} : $p=0.031$; Tukey _{Chl_{area}} : $p<0.001$;
2206 Figs. 5.1a-d).

Table 5.1. Effects of soil nitrogen fertilization, inoculation, and CO₂ treatments on leaf nitrogen content per unit leaf area (N_{area} ; gN m⁻²), leaf nitrogen content per unit leaf mass (N_{mass} , gN g⁻¹), leaf mass per unit leaf area (M_{area} ; g m⁻²), and chlorophyll content per unit leaf area (Chl_{area} ; mmol m⁻²)^{*}

	N_{area}			N_{mass}			M_{area}^a			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	1.10E+00	-	-	3.05E-02	-	-	3.64E+00	-	-
CO ₂	1	-5.67E-01	155.908	<0.001	-1.80E-02	272.362	<0.001	3.04E-01	151.319	<0.001
Inoculation (I)	1	6.21E-01	86.029	<0.001	7.54E-03	15.576	<0.001	1.81E-01	19.158	<0.001
Fertilization (N)	1	3.06E-03	316.408	<0.001	5.78E-05	106.659	<0.001	3.10E-04	21.440	<0.001
CO ₂ *I	1	2.63E-01	4.729	0.030	3.96E-03	2.025	0.155	-3.37E-02	0.029	0.866
CO ₂ *N	1	-3.68E-04	5.723	0.017	-2.85E-05	22.542	<0.001	2.80E-04	7.619	0.006
I*N	1	-1.36E-03	43.381	<0.001	-2.00E-05	11.137	0.001	-3.36E-04	5.022	0.025
CO ₂ *I*N	1	-3.23E-04	0.489	0.484	-2.59E-06	0.041	0.839	1.15E-04	0.208	0.649
Chl_{area}										
	df	Coefficient	χ^2	p						
(Intercept)	-	2.13E-02	-	-						
CO ₂	1	-1.33E-02	69.233	<0.001						
Inoculation (I)	1	1.24E-01	136.341	<0.001						
Fertilization (N)	1	3.35E-04	163.111	<0.001						
CO ₂ *I	1	-3.18E-02	2.102	0.147						
CO ₂ *N	1	-8.79E-05	2.999	0.083						
I*N	1	-2.65E-04	75.769	<0.001						
CO ₂ *I*N	1	7.68E-05	2.144	0.147						

2207 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2208 between 0.05 and 0.1 are italicized. A superscript “a” is included after trait labels to indicate if models were fit with
 2209 natural log transformed response variables. Key: df=degrees of freedom, χ^2 =Wald Type II chi-square test statistic.

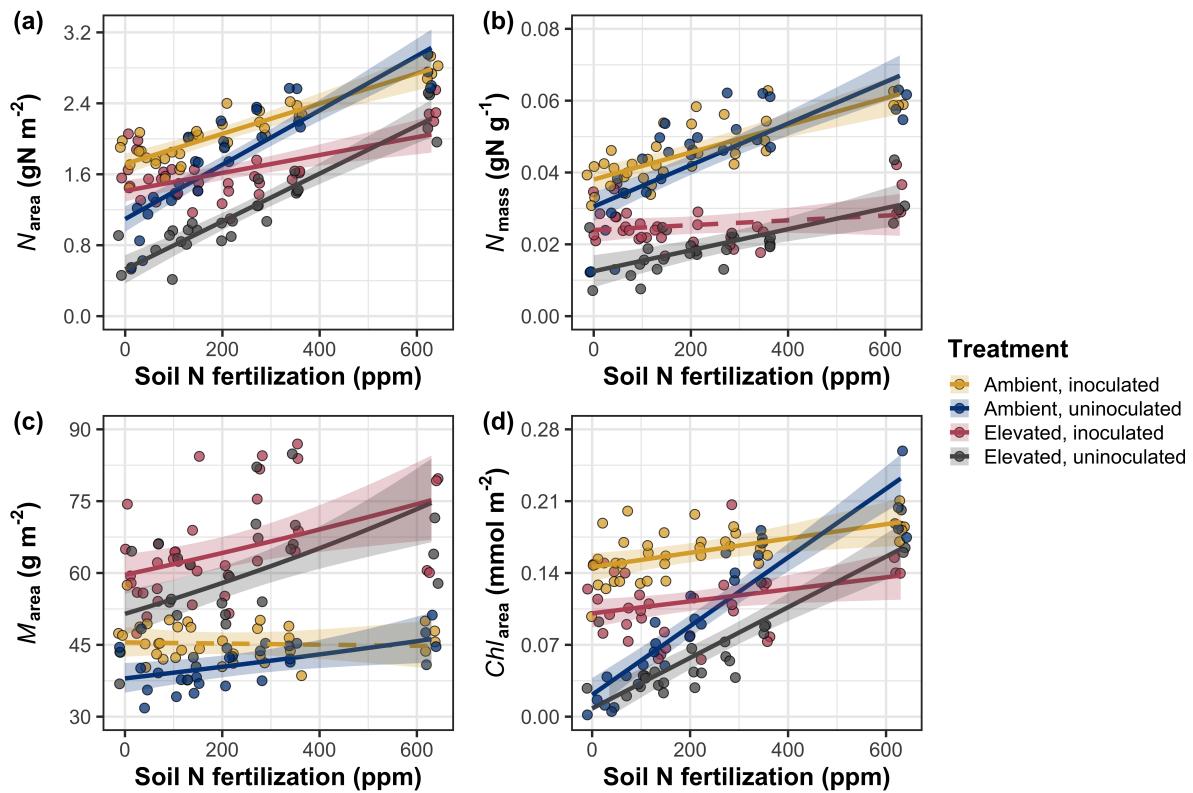


Figure 5.1. Effects of CO_2 , fertilization, and inoculation on leaf nitrogen per unit leaf area (a), leaf nitrogen content (b), leaf mass per unit leaf area (c), and chlorophyll content per unit leaf area (d). Soil nitrogen fertilization is represented on the x-axis in all panels. Yellow points and trendlines indicate inoculated individuals grown under ambient CO_2 , blue points and trendlines indicate uninoculated individuals grown under ambient CO_2 , red points and trendlines indicate inoculated individuals grown under elevated CO_2 , and grey points indicate uninoculated individuals grown under elevated CO_2 . Solid trendlines indicate regression slopes that are different from zero ($p < 0.05$), while dashed trendlines indicate slopes that are not distinguishable from zero ($p > 0.05$).

2210 5.3.2 *Leaf biochemistry and stomatal conductance*

2211 Elevated CO₂ resulted in plants with 16% lower V_{cmax25} ($p<0.001$; Table 5.2) and
2212 10% lower J_{max25} ($p=0.014$; Table 5.2) compared to those grown under ambient
2213 CO₂. However, CO₂ concentration did not influence R_{d25} ($p=0.613$; Table 5.2;
2214 Fig. 5.2d). A relatively stronger downregulation in V_{cmax25} than J_{max25} resulted
2215 in an 8% stimulation in $J_{max25}:V_{cmax25}$ under elevated CO₂ ($p<0.001$; Table 5.2).
2216 The downregulatory effect of CO₂ on V_{cmax25} and J_{max25} was not modified across
2217 the fertilization gradient (CO₂-by-fertilization interaction: $p=0.185$ and $p=0.389$
2218 for V_{cmax25} and J_{max25} , respectively; Table 5.2; Figs. 5.2a, 5.2b) or between in-
2219 oculation treatments (CO₂-by-inoculation interaction: $p=0.799$ and $p=0.714$ for
2220 V_{cmax25} and J_{max25} , respectively; Table 5.2). However, a strong interaction between
2221 fertilization and inoculation (fertilization-by-inoculation interaction: $p\leq0.001$ in
2222 all cases; Table 5.2) indicated that the positive effect of increasing fertilization
2223 on V_{cmax25} ($p<0.001$; Table 5.2), J_{max25} ($p<0.001$; Table 5.2), and R_{d25} ($p=0.015$;
2224 Table 5.2) was only observed in uninoculated pots (Tukey: $p\leq0.001$ in all cases;
2225 Figs. 5.2a, 5.2b). A stronger positive effect of increasing fertilization on V_{cmax25}
2226 than J_{max25} resulted in a reduction in $J_{max25}:V_{cmax25}$ with increasing fertilization
2227 ($p<0.001$; Table 5.2), though this pattern was only observed in uninoculated pots
2228 (fertilization-by-inoculation interaction: $p=0.002$; Table 5.2; Fig. 5.2c).

2229 Elevated CO₂ reduced stomatal conductance by 20% ($p<0.001$; Table 5.2;
2230 Fig. 5.2e), but this downregulation did not influence stomatal limitation of pho-
2231 tosynthesis ($p=0.355$; Table 5.2; Fig. 5.2f). As with V_{cmax25} and J_{max25} , the down-
2232 regulation of stomatal conductance due to elevated CO₂ was not modified across
2233 the fertilization gradient (CO₂-by-fertilization interaction: $p=0.141$; Table 5.2) or

2234 between inoculation treatments (CO_2 -by-inoculation interaction: $p=0.179$; Table
2235 5.2). Fertilization also did not modify the null effect of CO_2 on stomatal limitation
2236 (CO_2 -by-fertilization interaction: $p=0.554$; Table 5.2), although an interaction
2237 between CO_2 and inoculation (CO_2 -by-inoculation interaction: $p=0.043$; Table
2238 5.2) indicated that inoculation increased stomatal limitation under ambient CO_2
2239 (Tukey: $p=0.021$), but not under elevated CO_2 (Tukey: $p>0.999$). An interaction
2240 between inoculation and fertilization on stomatal conductance (fertilization-by-
2241 inoculation interaction: $p<0.001$; Table 5.2) indicated that increasing fertilization
2242 increased stomatal conductance in uninoculated pots (Tukey: $p=0.003$) but de-
2243 creased stomatal conductance in inoculated pots (Tukey: $p=0.021$). The similar
2244 in magnitude, but opposite direction, trend in the effect of increasing fertiliza-
2245 tion on stomatal conductance between inoculation treatments likely drove a null
2246 response of stomatal conductance to increasing fertilization ($p=0.642$; Table 5.2).

Table 5.2. Effects of soil nitrogen fertilization, inoculation, and CO₂ on the maximum rate of Rubisco carboxylation ($V_{\text{cmax}25}$; $\mu\text{mol m}^{-2} \text{s}^{-1}$), the maximum rate of RuBP regeneration ($J_{\text{max}25}$; $\mu\text{mol m}^{-2} \text{s}^{-1}$), dark respiration ($R_{\text{d}25}$; $\mu\text{mol m}^{-2} \text{s}^{-1}$), the ratio of the maximum rate of RuBP regeneration to the maximum rate of Rubisco carboxylation ($J_{\text{max}25}:V_{\text{cmax}25}$; unitless), stomatal conductance (g_{sw} ; $\text{mol m}^{-2} \text{s}^{-1}$), and stomatal limitation (unitless)*

	$V_{\text{cmax}25}$			$J_{\text{max}25}$			$R_{\text{d}25}$			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	4.36E+01	-	-	8.30E+01	-	-	1.69E+00	-	-
CO ₂	1	-7.05E+00	18.039	<0.001	-9.11E+00	6.042	0.014	4.53E-01	0.256	0.613
Inoculation (I)	1	5.87E+01	98.579	<0.001	9.62E+01	85.064	<0.001	1.04E+00	3.094	0.079
Fertilization (N)	1	1.32E-01	37.053	<0.001	2.09E-01	25.356	<0.001	2.86E-03	5.965	0.015
CO ₂ *I	1	-4.65E+00	0.065	0.799	7.84E-01	0.667	0.414	-5.71E-01	2.563	0.109
CO ₂ *N	1	-3.58E-02	1.758	0.185	-4.33E-02	0.742	0.389	-1.55E-03	2.675	0.102
I*N	1	-1.35E-01	60.394	<0.001	-2.30E-01	57.410	<0.001	-2.84E-03	12.083	0.001
CO ₂ *I*N	1	2.73E-02	0.748	0.387	3.46E-02	0.377	0.539	7.21E-04	0.244	0.622

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	$J_{\text{max}25}:V_{\text{cmax}25}$			g_{sw}			Stomatal limitation			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	1.92E+00	-	-	1.95E-01	-	-	2.12E-01	-	-
CO ₂	1	5.71E-02	92.010	<0.001	-6.23E-02	9.718	0.002	3.91E-02	0.856	0.355
Inoculation (I)	1	-1.79E-01	27.768	<0.001	1.30E-01	22.351	<0.001	7.87E-02	4.582	0.032
Fertilization (N)	1	-4.61E-04	28.147	<0.001	2.50E-04	0.066	0.797	2.60E-04	32.218	<0.001
CO ₂ *I	1	8.94E-02	2.916	0.088	6.69E-02	1.810	0.179	-7.84E-02	4.093	0.043
CO ₂ *N	1	2.35E-04	3.210	0.073	-8.50E-05	2.165	0.141	-1.24E-04	0.350	0.554
I*N	1	3.27E-04	9.607	0.002	-3.09E-04	14.696	<0.001	-1.67E-04	2.547	0.110
CO ₂ *I*N	1	-1.66E-04	1.102	0.294	-8.89E-05	0.234	0.629	1.67E-04	2.231	0.135

2247 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2248 between 0.05 and 0.1 are italicized. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

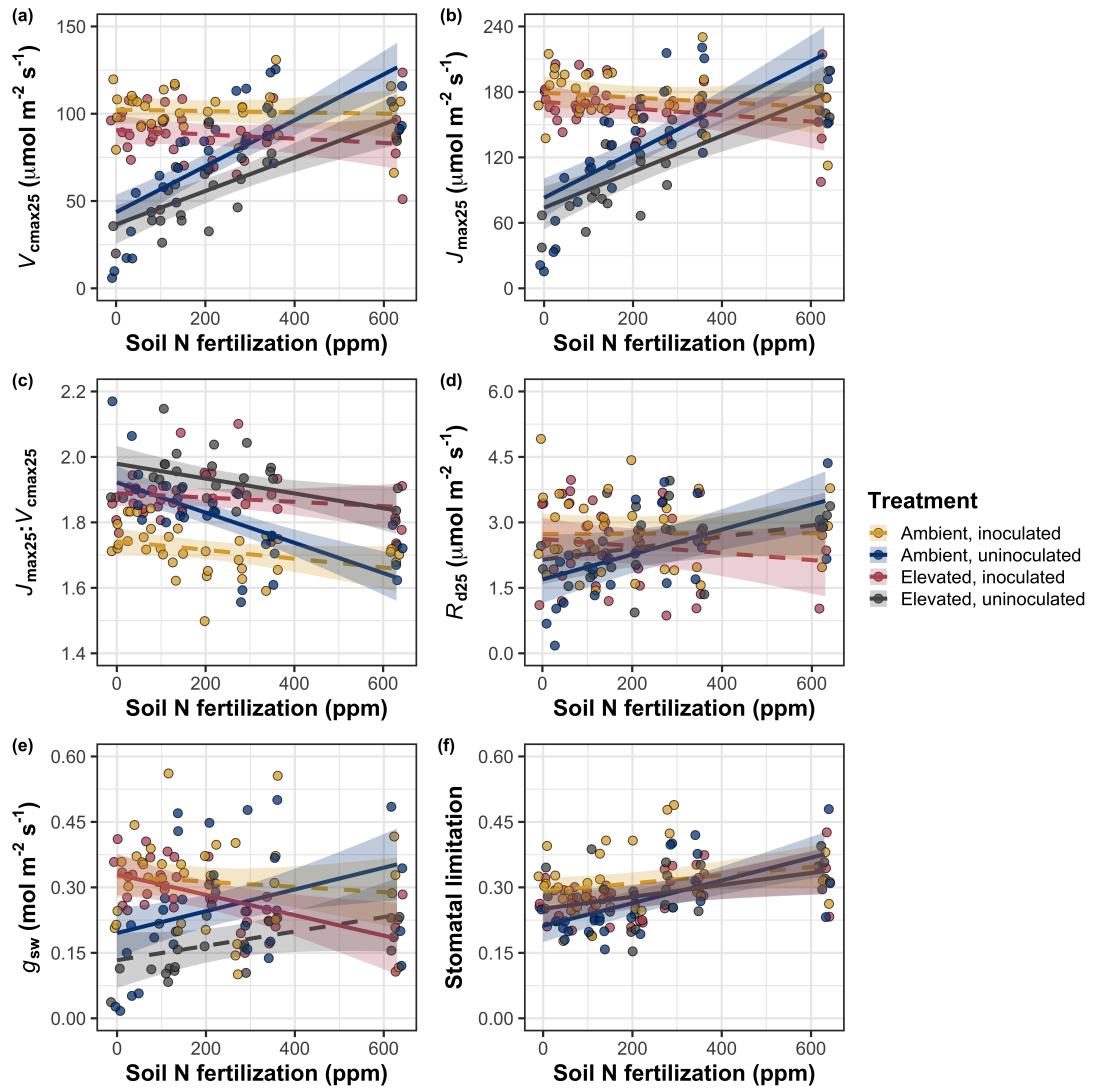


Figure 5.2. Effects of CO₂, fertilization, and inoculation on maximum rate of Rubisco carboxylation (a), the maximum rate of RuBP regeneration (b), and the ratio of the maximum rate of RuBP regeneration to the maximum rate of Rubisco carboxylation leaf mass per unit leaf area (c), dark respiration (d), stomatal conductance (e), and stomatal limitation (f). Soil nitrogen fertilization is represented on the x-axis in all panels. Colored points and trendlines are as explained in Figure 5.1.

2249 5.3.3 *Leaf nitrogen allocation*

2250 A relatively stronger downregulation in N_{area} than $V_{\text{cmax}25}$ and $J_{\text{max}25}$ resulted in
2251 an 20% and 29% respective stimulation in ρ_{rubisco} and ρ_{bioe} under elevated CO₂
2252 ($p<0.001$ in both cases; Table 5.3). There was no effect of CO₂ on ρ_{light} ($p=0.700$;
2253 Table 5.3), but the stimulation in ρ_{rubisco} and ρ_{bioe} resulted in a 21% stimulation
2254 of ρ_{photo} under elevated CO₂ ($p<0.001$; Table 5.3; Fig. 5.3a). The stimulation
2255 of ρ_{rubisco} , ρ_{bioe} , and ρ_{photo} under elevated CO₂ was not modified across the fer-
2256 tilization gradient (CO₂-by-fertilization interaction: $p_{\text{rubisco}}=0.269$, $p_{\text{bioe}}=0.298$,
2257 $p_{\text{photo}}=0.281$; Table 5.3). A marginal interaction between inoculation and CO₂ on
2258 ρ_{rubisco} and ρ_{photo} (CO₂-by-inoculation interaction: $p_{\text{rubisco}}=0.057$, $p_{\text{photo}}=0.055$;
2259 Table 5.3) indicated that the positive effect of inoculation on ρ_{rubisco} and ρ_{photo}
2260 ($p<0.001$ in both cases; Table 5.3) was only apparent under ambient CO₂ (Tukey:
2261 $p<0.001$ in both cases). Inoculation did not modify the stimulation of ρ_{bioe} un-
2262 der elevated CO₂ (CO₂-by-inoculation interaction: $p=0.122$; Table 5.3) or the
2263 null effect of CO₂ on ρ_{bioe} (CO₂-by-inoculation interaction: $p=0.298$; Table 5.3).
2264 An interaction between fertilization and inoculation on ρ_{rubisco} , ρ_{bioe} , and ρ_{photo}
2265 (fertilization-by-inoculation interaction: $p<0.001$ in all cases; Table 5.3) indicated
2266 that the negative effect of increasing fertilization on each trait ($p<0.001$ in all
2267 cases; Table 5.3) was only observed in inoculated pots (Tukey: $p<0.001$ in all
2268 cases). An additional interaction between fertilization and inoculation on ρ_{light}
2269 (fertilization-by-inoculation interaction: $p<0.001$; Table 5.3) indicated a negative
2270 effect of increasing fertilization on ρ_{light} in inoculated pots (Tukey: $p=0.041$), but
2271 a positive effect of increasing fertilization in uninoculated pots (Tukey: $p<0.001$).
2272 The stimulation in M_{area} under elevated CO₂ resulted in an 133% stimu-

2273 lation of $\rho_{\text{structure}}$ ($p<0.001$; Table 5.3; Fig 5.3b). An interaction between fertil-
2274 ization and CO₂ (CO₂-by-fertilization interaction: $p=0.039$; Table 5.3) indicated
2275 that the negative effect of increasing fertilization ($p<0.001$; Table 5.3) on $\rho_{\text{structure}}$
2276 was marginally stronger under ambient CO₂ (Tukey: $p=0.055$). A marginal inter-
2277 action between inoculation and CO₂ (CO₂-by-inoculation interaction: $p=0.057$;
2278 Table 5.3) indicated that the positive effect of inoculation on $\rho_{\text{structure}}$ ($p<0.001$;
2279 Table 5.3) was only observed under elevated CO₂ (Tukey: $p<0.001$), with no ap-
2280 parent inoculation effect observed under ambient CO₂ (Tukey: $p=0.513$). Finally,
2281 an interaction between fertilization and inoculation (fertilization-by-inoculation
2282 interaction: $p<0.001$; Table 5.3) indicated that, while increasing fertilization in-
2283 creased $\rho_{\text{structure}}$ ($p<0.001$; Table 5.3), this response was stronger in uninoculated
2284 pots (Tukey: $p=0.001$; Fig. 5.3b).

Table 5.3. Effects of soil nitrogen fertilization, inoculation, and CO₂ on the fraction of leaf nitrogen allocated to Rubisco (ρ_{rubisco} ; gN gN⁻¹), bioenergetics (ρ_{bioe} ; gN gN⁻¹), light harvesting proteins (ρ_{light} ; gN gN⁻¹), photosynthesis (ρ_{photo} ; gN gN⁻¹), and structure ($\rho_{\text{structure}}$; gN gN⁻¹)*

	ρ_{rubisco}			ρ_{bioe}			ρ_{light}			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	2.70E-01	-	-	5.26E-02	-	-	8.48E-03	-	-
CO ₂	1	1.42E-01	23.510	<0.001	3.00E-02	53.899	<0.001	2.03E-03	0.149	0.700
Inoculation (I)	1	1.83E-01	23.475	<0.001	2.80E-02	13.860	<0.001	2.04E-02	147.234	<0.001
Fertilization (N)	1	1.35E-04	16.609	<0.001	1.22E-05	26.827	<0.001	3.22E-05	19.378	<0.001
CO ₂ *I	1	-1.07E-01	3.629	0.057	-1.67E-02	2.390	0.122	-5.33E-03	0.684	0.408
CO ₂ *N	1	-2.16E-04	1.223	0.269	-3.59E-05	1.085	0.298	-7.01E-06	0.351	0.553
I*N	1	-4.26E-04	20.045	<0.001	-6.87E-05	15.458	<0.001	-4.37E-05	64.042	<0.001
CO ₂ *I*N	1	2.50E-04	3.327	0.068	4.08E-05	2.651	0.103	1.74E-05	3.735	0.053

	ρ_{photo}			$\rho_{\text{structure}}^a$				
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	
(Intercept)	-	3.32E-01	-	-	-2.93E+00	-	-	
CO ₂	1	1.81E-01	27.651	<0.001	8.77E-01	229.571	<0.001	
Inoculation (I)	1	2.31E-01	26.238	<0.001	-2.55E-01	13.872	<0.001	
Fertilization (N)	1	1.76E-04	15.899	<0.001	-1.51E-03	38.128	<0.001	
CO ₂ *I	1	-1.36E-01	3.671	0.055	-2.99E-01	3.622	0.057	
CO ₂ *N	1	-2.72E-04	1.163	0.281	3.14E-04	4.266	0.039	
I*N	1	-5.37E-04	21.355	<0.001	7.00E-04	11.025	0.001	
CO ₂ *I*N	1	3.29E-04	4.009	0.045	4.52E-04	0.669	0.413	

2285 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2286 between 0.05 and 0.1 are italicized. A superscript “a” is included after trait labels to indicate if models were fit with
 2287 natural log transformed response variable. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

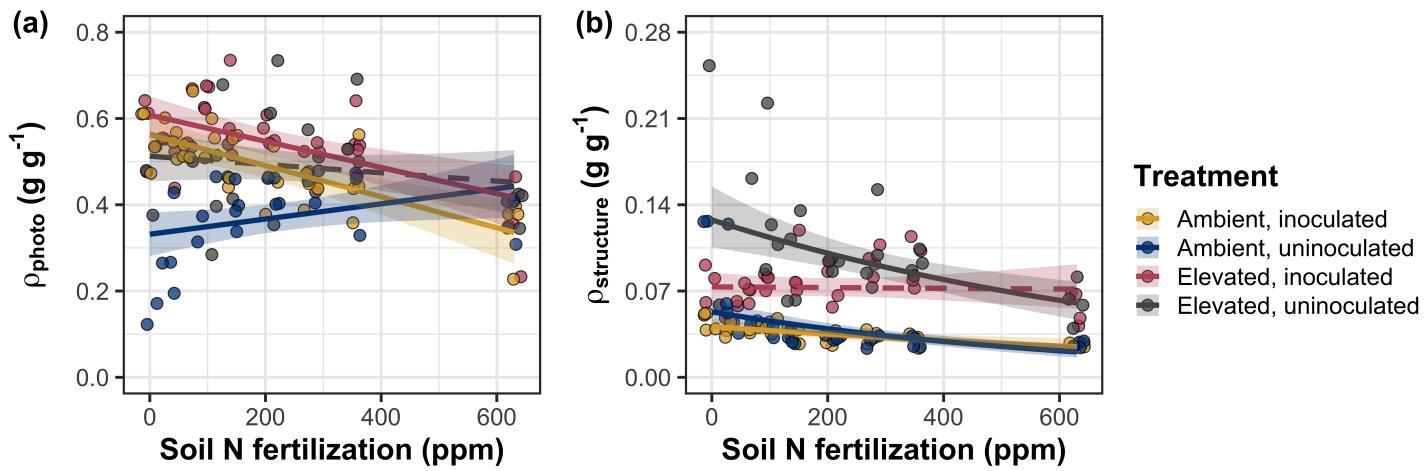


Figure 5.3. Effects of CO_2 , fertilization, and inoculation on the relative fraction of leaf nitrogen allocated to photosynthesis (a) and the fraction of leaf nitrogen allocated to structure (b). Soil nitrogen fertilization is represented on the x-axis in both panels. Colored points and trendlines are as explained in Figure 5.1.

2288 5.3.4 *Whole plant traits*

2289 Total leaf area and total biomass were 51% and 102% greater under elevated CO₂,
2290 respectively ($p<0.001$ in both cases; Table 5.4). The stimulation in total leaf area
2291 and total biomass under elevated CO₂ was enhanced by increasing fertilization
2292 (CO₂-by-fertilization interaction: $p<0.001$ in both cases; Table 5.4; Figs. 5.4a,
2293 5.4b) but was not modified across inoculation treatments (CO₂-by-inoculation
2294 interaction: $p_{total_leaf_area}=0.151$, $p_{total_biomass}=0.472$; Table 5.4). The positive
2295 effect of increasing fertilization on total leaf area and total biomass was modified by
2296 inoculation treatment (fertilization-by-inoculation interaction: $p<0.001$ in both
2297 cases; Table 5.4), indicating a stronger positive effect of increasing fertilization in
2298 uninoculated pots (Tukey: $p_{total_leaf_area}=0.002$, $p_{total_biomass}=0.001$, Figs. 5.4a,
2299 5.4b).

2300 A 62% stimulation in N_{cost} under elevated CO₂ was modified through a
2301 strong three-way interaction between CO₂, fertilization, and inoculation (CO₂-
2302 by-inoculation-by-fertilization interaction: $p<0.001$; Table 5.4; Fig. 5.4). This
2303 interaction revealed a general negative effect of increasing fertilization on N_{cost}
2304 ($p<0.001$; Table 5.4) that was observed in all treatment combinations (Tukey:
2305 $p<0.001$ in all cases) except for inoculated pots grown under elevated CO₂ (Tukey:
2306 $p=0.779$; Fig. 5.4c). This response also resulted in stronger negative effects of in-
2307 creasing fertilization on N_{cost} in uninoculated pots grown under elevated CO₂ than
2308 uninoculated pots grown under ambient CO₂ (Tukey: $p=0.001$) and inoculated
2309 pots grown under either ambient CO₂ (Tukey: $p<0.001$) or elevated CO₂ (Tukey:
2310 $p<0.001$), while uninoculated pots grown under ambient CO₂ had stronger nega-
2311 tive effects of increasing fertilization on N_{cost} than inoculated pots grown under

2312 elevated CO₂ (Tukey: $p=0.002$), but not inoculated pots grown under ambient
2313 CO₂ (Tukey: $p=0.216$; Fig. 5.4). The reduction in N_{cost} with increasing fertiliza-
2314 tion and in uninoculated pots were driven by a stronger positive effect of increasing
2315 fertilization on N_{wp} (denominator of N_{cost}) than C_{bg} (numerator of N_{cost}), while
2316 the stimulation in N_{cost} under elevated CO₂ was driven by a stronger positive
2317 effect of elevated CO₂ on C_{bg} than N_{wp} (Table 5.4).

Table 5.4. Effects of CO₂, fertilization, and inoculation on total leaf area (cm²), whole plant biomass (g), carbon costs to acquire nitrogen (N_{cost} ; gC gN⁻¹), belowground carbon biomass (C_{bg} ; gC), and whole plant nitrogen biomass (N_{wp} ; gN)*

	Total leaf area			Total biomass ^b			<i>N</i> _{cost}			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	8.78E+01	-	-	9.96E-01	-	-	8.67E+00	-	-
CO ₂	1	3.36E+01	69.291	<0.001	5.07E-01	131.477	<0.001	8.75E+00	88.189	<0.001
Inoculation (I)	1	1.88E+02	35.715	<0.001	7.96E-01	34.264	<0.001	-1.68E+00	136.343	<0.001
Fertilization (N)	1	9.35E-01	274.199	<0.001	3.14E-03	269.046	<0.001	-8.50E-03	80.501	<0.001
CO ₂ *I	1	6.44E+01	2.064	0.151	-7.69E-02	0.518	0.472	-8.38E+00	85.237	<0.001
CO ₂ *N	1	5.05E-01	18.655	<0.001	1.61E-03	16.877	<0.001	-9.17E-03	1.050	0.306
I*N	1	-3.84E-01	10.804	0.001	-1.45E-03	15.779	<0.001	4.20E-03	46.489	<0.001
CO ₂ *I*N	1	-2.97E-03	<0.001	0.990	-1.14E-04	0.023	0.880	1.32E-02	18.125	<0.001

	C_{bg}^{a}		N_{wp}^{b}				
	df	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	-1.70E+00	-	-	1.24E-01	-	-
CO ₂	1	9.21E-01	84.134	<0.001	-3.41E-03	23.890	<0.001
Inoculation (I)	1	1.18E+00	41.030	<0.001	1.68E-01	134.460	<0.001
N fertilization (N)	1	3.38E-03	152.248	<0.001	6.69E-04	529.021	<0.001
CO ₂ * I	1	-6.18E-01	8.965	0.003	3.68E-02	1.190	0.275
CO ₂ * N	1	-3.66E-05	1.188	0.276	1.58E-04	5.915	0.015
I * N	1	-2.22E-03	22.648	<0.001	-3.20E-04	55.562	<0.001
CO ₂ * I * N	1	8.09E-04	1.109	0.292	-7.54E-05	0.620	0.431

2318 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). *P*-values less than 0.05 are in bold. Superscripts
2319 included after trait labels indicate if models were fit with natural log (^a) or square root (^b) transformed response
2320 variables. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

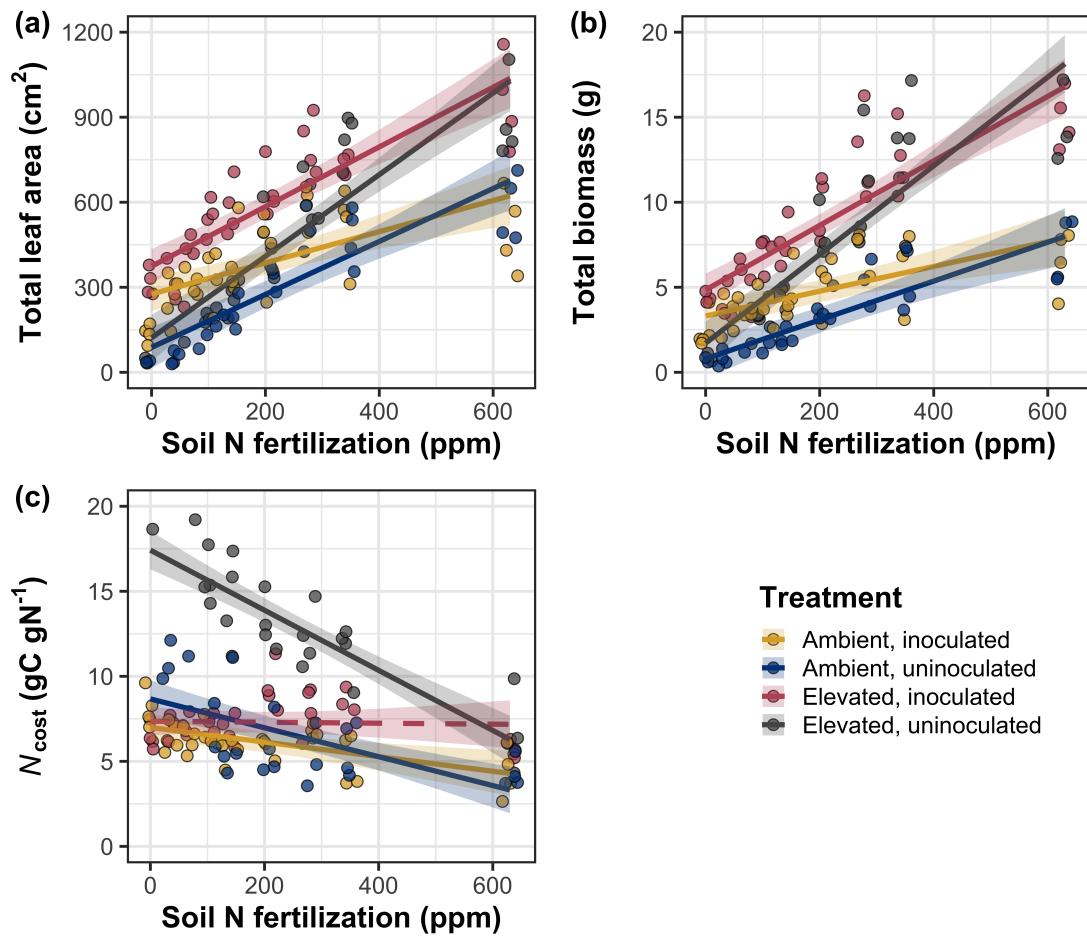


Figure 5.4. Effects of CO₂, fertilization, and inoculation on total leaf area (a), total biomass (b), and structural carbon costs to acquire nitrogen (c). Soil nitrogen fertilization is represented on the x-axis in all panels. Colored points and trendlines are as explained in Figure 5.1.

2321 5.3.5 *Nitrogen fixation*

2322 Nodule biomass was stimulated by 30% under elevated CO₂ ($p<0.001$; Table 5.5),
2323 a pattern that was modified across the fertilization gradient (CO₂-by-fertilization
2324 interaction: $p=0.479$; Table 5.5), but not between inoculation treatments (CO₂-
2325 by-inoculation interaction: $p=0.404$; Table 5.5). Specifically, the negative effect
2326 of increasing fertilization on nodule biomass ($p<0.001$; Table 5.5) was stronger
2327 under elevated CO₂ (Tukey: $p<0.001$; Fig. 5.5a). An interaction between fertil-
2328 ization and inoculation (fertilization-by-inoculation interaction: $p<0.001$; Table
2329 5.5) indicated a stronger negative effect of increasing fertilization in inoculated
2330 pots (Tukey: $p<0.001$; Fig. 5.5a).

2331 There was no effect of CO₂ on nodule: root biomass ($p=0.767$; Table 5.5),
2332 although an interaction between CO₂ and inoculation (CO₂-by-inoculation in-
2333 teraction: $p<0.001$; Table 5.5) indicated that the positive effect of inoculation
2334 on nodule: root biomass ($p<0.001$; Table 5.5) was stronger under ambient CO₂
2335 (3129% increase; Tukey: $p<0.001$) than elevated CO₂ (379% increase; Tukey:
2336 $p<0.001$; Fig. 5.5b). The null effect of CO₂ on nodule: root biomass was consis-
2337 tently observed across the fertilization gradient (CO₂-by-fertilization interaction:
2338 $p=0.183$; Table 5.5; Fig. 5.5b). An interaction between fertilization and inocula-
2339 tion (fertilization-by-inoculation interaction: $p<0.001$; Table 5.5) indicated that
2340 the negative effect of increasing fertilization on nodule: root biomass ($p<0.001$;
2341 Table 5.5) was stronger in inoculated pots (Tukey: $p<0.001$; Fig. 5.5b).

2342 There was no effect of CO₂ on %N_{dfa} ($p=0.472$; Table 5.5), a pattern
2343 that was not modified by inoculation (CO₂-by-inoculation interaction: $p=0.156$;
2344 Table 5.5) or fertilization (CO₂-by-fertilization interaction: $p=0.099$; Table 5.5).

- 2345** An interaction between fertilization and inoculation (fertilization-by-inoculation
2346 interaction: $p<0.001$; Table 5.5) indicated that the negative effect of increasing
2347 fertilization on $\%N_{dfa}$ ($p<0.001$; Table 5.5) was only observed in inoculated pots
2348 (Tukey: $p<0.001$; Fig. 5.5c).

Table 5.5. Effects of CO₂, fertilization, and inoculation on root nodule biomass (g), plant investments in symbiotic nitrogen fixation (unitless), and percent nitrogen fixed from the atmosphere (%N_{dfa}; unitless)*

	Root nodule biomass ^b			Root nodule: root biomass ^b			%N _{dfa} ^b			
	df	Coefficient	χ^2	p	Coefficient	χ^2	p	Coefficient	χ^2	p
(Intercept)	-	9.41E-03	-	-	1.33E-02	-	-	7.48E-01	-	-
CO ₂	1	1.20E-01	19.258	<0.001	9.94E-02	0.087	0.768	-1.00E-01	0.518	0.472
Inoculation (I)	1	5.74E-01	755.020	<0.001	5.40E-01	903.691	<0.001	9.01E+00	955.570	<0.001
Fertilization (N)	1	7.71E-06	84.376	<0.001	-5.99E-06	258.099	<0.001	3.64E-04	292.938	<0.001
CO ₂ *I	1	-4.68E-02	0.950	0.330	-1.38E-01	20.614	<0.001	-1.44E-01	2.010	0.156
CO ₂ *N	1	-1.59E-04	2.106	0.147	-1.73E-04	1.773	0.183	-6.21E-05	2.716	0.099
I*N	1	-5.82E-04	44.622	<0.001	-7.45E-04	133.918	<0.001	-1.58E-02	231.290	<0.001
CO ₂ *I*N	1	7.26E-05	0.196	0.658	1.76E-04	2.359	0.125	2.77E-03	2.119	0.145

2349 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2350 between 0.05 and 0.1 are italicized. Superscript letters indicate model coefficients fit to square-root (^b) transformed
 2351 data. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

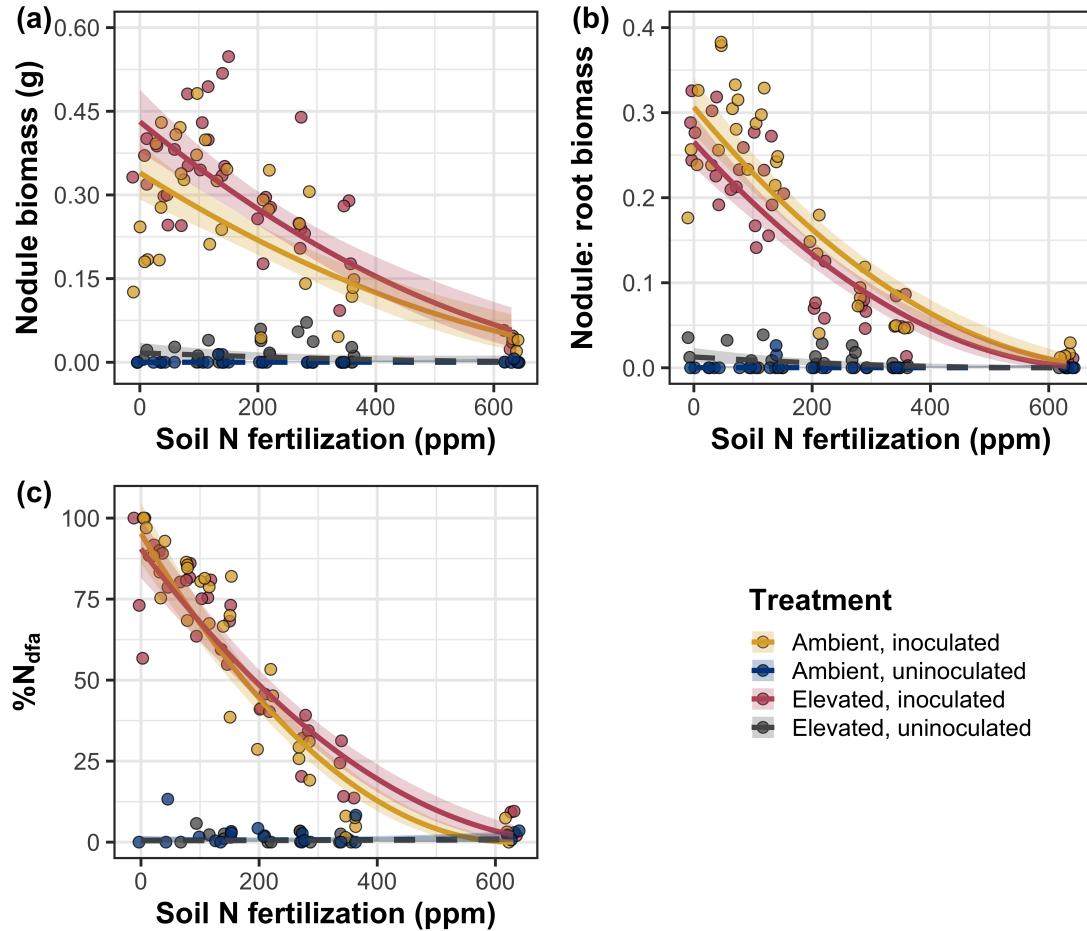


Figure 5.5. Effects of CO₂, fertilization, and inoculation on nodule biomass (a), nodule biomass: root biomass (b), and percent nitrogen fixed from the atmosphere (c). Soil nitrogen fertilization is represented on the x-axis. Colored points and trendlines are as explained in Figure 5.1. Curvilinear trendlines occur as a result of back-transforming models where response variables received either a natural log or square root transformation prior to fitting.

2352 5.4 Discussion

2353 In this study, I determined leaf and whole plant acclimation responses of 7-week *G.*
2354 *max* seedlings grown under two CO₂ concentrations, two inoculation treatments,
2355 and nine soil nitrogen fertilization treatments in a full-factorial growth chamber
2356 experiment. In support of hypotheses and patterns expected from theory, elevated
2357 CO₂ reduced N_{area} , V_{cmax25} , and J_{max25} . The relatively stronger downregulation in
2358 V_{cmax25} than J_{max25} under elevated CO₂ resulted in a stimulation in $J_{\text{max25}}:V_{\text{cmax25}}$
2359 under elevated CO₂. The downregulation of V_{cmax25} and J_{max25} under elevated
2360 CO₂ was similar across fertilization and inoculation treatments, indicating that
2361 the CO₂ responses were not associated with nitrogen limitation. Interestingly,
2362 results indicate that elevated CO₂ increased the fraction of leaf nitrogen allocated
2363 to photosynthesis and structure, leading to a stimulation in nitrogen use efficiency
2364 under elevated CO₂ despite the apparent downregulation in N_{area} , V_{cmax25} , and
2365 J_{max25} .

2366 The downregulation in leaf photosynthetic processes under elevated CO₂
2367 corresponded with a strong stimulation in total leaf area and total biomass. Strong
2368 stimulations in whole plant growth due to elevated CO₂ were generally enhanced
2369 with increasing fertilization and were negatively related to structural carbon costs
2370 to acquire nitrogen. Inoculation generally did not modify whole plant responses
2371 to elevated CO₂ across the fertilization gradient, likely due to a strong reduc-
2372 tion in root nodulation with increasing fertilization. However, strong positive
2373 effects of inoculation on whole plant growth were observed under low fertilization,
2374 consistent with hypotheses. Overall, observed leaf and whole plant acclimation
2375 responses to CO₂ support hypotheses and patterns expected from photosynthetic

2376 least-cost theory, showing that leaf acclimation responses to CO₂ were decoupled
2377 from soil nitrogen availability and ability to acquire nitrogen via symbiotic nitro-
2378 gen fixation. Instead, leaf and whole plant acclimation responses to CO₂ were
2379 driven by optimal resource investment to photosynthetic capacity, where optimal
2380 resource investment at the leaf level maximized nitrogen allocation to structures
2381 that support whole plant growth.

2382 5.4.1 *Soil nitrogen fertilization has divergent effects on leaf and whole plant*
2383 *acclimation responses to CO₂*

2384 Elevated CO₂ reduced N_{area} , V_{cmax25} , J_{max25} , and stomatal conductance by 29%,
2385 16%, 10%, and 20%, respectively. The larger downregulation of V_{cmax25} than
2386 J_{max25} led to an 8% stimulation in $J_{\text{max25}}:V_{\text{cmax25}}$, while the larger downregulation
2387 of N_{area} than V_{cmax25} resulted in a 21% stimulation in the fraction of leaf nitro-
2388 gen allocated to photosynthesis under elevated CO₂. These acclimation responses
2389 are directionally consistent with previous studies that have investigated or re-
2390 viewed leaf acclimation responses to CO₂ (Drake et al. 1997; Makino et al. 1997;
2391 Ainsworth et al. 2002; Ainsworth and Long 2005; Ainsworth and Rogers 2007;
2392 Smith and Dukes 2013; Smith and Keenan 2020; Poorter et al. 2022), and fol-
2393 low patterns expected from photosynthetic least-cost theory (Wright et al. 2003;
2394 Prentice et al. 2014; Smith et al. 2019; Smith and Keenan 2020). Together, the
2395 stimulation in $J_{\text{max25}}:V_{\text{cmax25}}$ and the fraction of leaf nitrogen allocated to pho-
2396 tosynthesis under elevated CO₂ provide strong support for the idea that leaves
2397 were downregulating V_{cmax25} in response to elevated CO₂ in order to optimally co-
2398 ordinate photosynthesis such that net photosynthesis rates approached becoming

2399 equally co-limited by Rubisco carboxylation and RuBP regeneration (Chen et al.
2400 1993; Maire et al. 2012) while optimizing resource use efficiency.

2401 Increasing fertilization and inoculation induced strong positive effects on
2402 N_{area} , $V_{\text{cmax}25}$, $J_{\text{max}25}$. The general positive response of N_{area} to increasing fertiliza-
2403 tion and in inoculated pots was enhanced under ambient CO₂, which, paired with
2404 the general downregulation of N_{area} under elevated CO₂, resulted in a stronger
2405 downregulation of N_{area} under elevated CO₂ with increasing fertilization and in
2406 inoculated pots. These patterns suggest that N_{area} responses to CO₂ were at least
2407 partially dependent on soil nitrogen fertilization and nitrogen acquisition strat-
2408 egy. However, the general stimulation in the fraction of leaf nitrogen allocated to
2409 Rubisco, bioenergetics, or photosynthesis under elevated CO₂ was not modified
2410 across the fertilization gradient and was only marginally enhanced in inoculated
2411 pots. These patterns suggest that the increased downregulation of N_{area} under
2412 elevated CO₂ with increasing fertilization was not necessarily associated with a
2413 change in relative investment to photosynthetic tissue, providing another line of
2414 evidence suggesting that leaf acclimation responses to CO₂ are decoupled from
2415 changes in soil nitrogen availability.

2416 Leaf acclimation responses to elevated CO₂ corresponded with a 62% and
2417 100% stimulation in total leaf area and total biomass, respectively. The stimula-
2418 tion in total leaf area and total biomass under elevated CO₂ corresponded with
2419 generally larger structural carbon costs to acquire nitrogen, a pattern driven by
2420 a stimulation in belowground carbon biomass and reduction in whole plant ni-
2421 trogen biomass. This result suggests that elevated CO₂ reduces plant nitrogen
2422 uptake efficiency, which does not explain why plants grown under elevated CO₂

2423 generally had higher biomass and total leaf area, unless growth stimulations un-
2424 der elevated CO₂ were driven by reductions in per-tissue nitrogen demand (Dong
2425 et al. 2022). Interestingly, strong negative effects of increasing fertilization on
2426 structural carbon costs to acquire nitrogen, which were generally similar between
2427 CO₂ concentrations, were driven by stronger increases in whole plant nitrogen
2428 biomass than belowground carbon biomass. This response allowed plants to in-
2429 crease nitrogen uptake efficiency with increasing fertilization, which could be the
2430 mechanism that drove the enhanced growth stimulation under elevated CO₂ with
2431 increasing fertilization.

2432 Interestingly, results indicate that the stimulation in total leaf area and
2433 whole plant growth under elevated CO₂ was not modified by inoculation despite
2434 an apparent general negative effect of inoculation on N_{cost} . This response could
2435 have been due to strong negative effect of increasing fertilization on nodulation,
2436 which may have caused the strong increase in the positive effect of elevated CO₂ on
2437 whole plant growth with increasing fertilization to mask any increase in the posi-
2438 tive effect of elevated CO₂ on whole plant growth due to inoculation. Reductions
2439 in nodulation with increasing fertilization are commonly observed patterns that
2440 have been inferred to be a response that allows species optimize nitrogen uptake
2441 efficiency as costs to acquire nitrogen via direct uptake become more similar (Gib-
2442 son and Harper 1985; Rastetter et al. 2001). In this study, pairwise comparisons
2443 indicated strong positive effects of inoculation on total leaf area and total biomass
2444 (158% increase in total leaf area, 119% increase in total biomass) under elevated
2445 CO₂ at 0 ppm N ($p < 0.05$ in both cases), but no observable inoculation effect on
2446 total leaf area or total biomass under elevated CO₂ at 350 ppm N or 630 ppm N

2447 ($p>0.05$ in both cases). While these responses did not generally differ from those
2448 observed under ambient CO₂, they do confirm the hypothesis that positive effects
2449 of inoculation on whole plant growth responses to elevated CO₂ would decrease
2450 with increasing fertilization.

2451 Combined, results reported here suggest that soil nitrogen availability plays
2452 divergent roles in shaping leaf and whole plant acclimation responses to CO₂. Leaf
2453 acclimation responses were generally decoupled from fertilization, while whole
2454 plant acclimation responses relied heavily on an increase in nitrogen uptake ef-
2455 ficiency and consequent reduction in costs of acquiring nitrogen associated with
2456 increasing fertilization. Whole plant responses to CO₂ indicated that fertilization
2457 may play a more important role in determining whole plant acclimation responses
2458 to CO₂ than nitrogen acquisition strategy, although any inoculation effect was
2459 likely masked by the strong reduction in root nodulation with increasing fertil-
2460 ization. These results suggest that plants acclimate to CO₂ in nitrogen-limited
2461 systems by minimizing the number of optimally coordinated leaves, and that
2462 downregulations in leaf nitrogen content under elevated CO₂ are not driven by
2463 changes in soil nitrogen availability as has been previously implied.

2464 5.4.2 *Implications for future model development*

2465 Many terrestrial biosphere models predict photosynthetic capacity through plant
2466 functional group-specific linear regressions between N_{area} and V_{cmax} (Rogers 2014;
2467 Rogers et al. 2017), which assumes that leaf nitrogen-photosynthesis relation-
2468 ships are constant across growing environments. These results build on previ-
2469 ous work suggesting that leaf nitrogen-photosynthesis relationships dynamically
2470 change across growing environments (Luo et al. 2021; Dong et al. 2022), showing

2471 that CO₂ concentration increases the fraction of leaf nitrogen content allocated to
2472 photosynthesis independent of fertilization or acquisition strategy. Additionally,
2473 increasing fertilization strongly decreased the fraction of leaf nitrogen allocated
2474 to photosynthesis, a response that was largely determined by acquisition strategy.
2475 Specifically, reductions in the fraction of leaf nitrogen allocated to photosynthesis
2476 with increasing fertilization were only observed in inoculated pots that had less
2477 finite access to nitrogen, suggesting that constant leaf nitrogen-photosynthesis
2478 relationships may only be apparent in environments where nitrogen is limiting.
2479 Terrestrial biosphere models that parameterize photosynthetic capacity through
2480 linear relationships between N_{area} and V_{cmax} (Rogers 2014; Rogers et al. 2017) may
2481 therefore be overestimating photosynthetic capacity in systems where nitrogen is
2482 not as limiting. Such models are also not capable of detecting stimulations in the
2483 fraction of leaf nitrogen allocated to photosynthesis with increasing CO₂ concen-
2484 tration. The inability of models to predict these responses likely contributes to the
2485 widespread divergence of model simulations under future environmental scenarios
2486 (Friedlingstein et al. 2014; Davies-Barnard et al. 2020), and should therefore be
2487 a target for resolving in future generations of terrestrial biosphere models.

2488 These results demonstrate that optimal resource investment to photosyn-
2489 thetic capacity defines leaf acclimation responses to elevated CO₂, and that these
2490 responses were independent of fertilization or inoculation treatment. Current
2491 model approaches for simulating photosynthetic responses to CO₂ generally invoke
2492 patterns expected from progressive nitrogen limitation, where the downregulation
2493 in N_{area} , and therefore photosynthetic capacity, due to elevated CO₂ is formu-
2494 lated as a function of progressive reductions in soil nitrogen availability. Results

2495 reported here contradict this formulation, suggesting that the leaf acclimation re-
2496 sponse is driven by optimal resource investment to photosynthetic capacity and
2497 is independent of soil resource supply. Optimality models that leverage prin-
2498 ciples from optimal coordination and photosynthetic least-cost theories (Wang
2499 et al. 2017; Stocker et al. 2020; Scott and Smith 2022) are capable of capturing
2500 such acclimation responses to CO₂ (Smith and Keenan 2020), suggesting that the
2501 implementation of these models may improve the simulation of photosynthetic
2502 processes in terrestrial biosphere models under increasing CO₂ concentrations.

2503 5.4.3 *Study limitations and future directions*

2504 There are two study limitations that must be addressed to contextualize patterns
2505 observed in this study. First, restricting the volume of belowground substrate
2506 via a potted experiment does not adequately replicate belowground environments
2507 of natural systems, and therefore may modify effects of soil resource availability
2508 and inoculation on plant nitrogen uptake. This limitation may be particularly
2509 relevant if pot size limits whole plant growth (Poorter et al. 2012). I attempted
2510 to minimize the extent of pot size limitation experienced in the first experimen-
2511 tal chapter while accounting for the expected stimulation in whole plant growth
2512 under elevated CO₂ by using 6-liter pots. Despite attempts to minimize growth
2513 limitation imposed by pot volume, fertilization and CO₂ treatments increased the
2514 biomass: pot volume ratio such that all treatment combinations to exceed 1 g L⁻¹
2515 biomass: pot volume under high fertilization (Table D3; Fig. D2). The 1 g L⁻¹
2516 biomass: pot volume recommendation from Poorter et al. (2012) was designated
2517 to avoid growth limitation imposed by pot volume. However, if pot size limita-

2518 tion indeed limited whole plant growth, then structural carbon costs to acquire
2519 nitrogen, belowground carbon biomass, whole plant nitrogen biomass, and whole
2520 plant biomass should each exhibit strong saturation points with increasing fertil-
2521 ization, which was not observed here. Importantly, leaf acclimation responses to
2522 CO₂ observed in this study are consistent with findings reported in (Smith and
2523 Keenan 2020), who used data from field manipulation experiments that did not
2524 have any belowground space limitation.

2525 Second, this study evaluated leaf and whole plant responses to CO₂ in 7-
2526 week seedlings. Given the long-term scale of the progressive nitrogen limitation
2527 hypothesis, patterns observed here should be validated in longer-term nitrogen
2528 manipulation experiments. Previous work in free air CO₂ enrichment experiments
2529 show some support for patterns expected from the progressive nitrogen limitation
2530 hypothesis (Reich et al. 2006; Norby et al. 2010), although results are not consis-
2531 tent across experimental sites (Finzi et al. 2006; Moore et al. 2006; Liang et al.
2532 2016). I found some support for patterns expected by the progressive nitrogen
2533 limitation hypothesis, namely the increase in plant nitrogen uptake under elevated
2534 CO₂ (Luo et al. 2004), though leaf acclimation responses to CO₂ were strongly
2535 indicative of optimal resource investment to photosynthetic capacity as expected
2536 from photosynthetic least-cost theory (Prentice et al. 2014; Smith et al. 2019;
2537 Smith and Keenan 2020).

2538 5.4.4 *Conclusions*

2539 This study provides strong evidence suggesting that leaf acclimation responses
2540 to elevated CO₂ did not vary with soil nitrogen fertilization or ability to acquire
2541 nitrogen through symbiotic nitrogen fixation. However, whole plant acclimation

2542 responses to CO₂ were dependent on fertilization, where increasing fertilization
2543 increased the positive effect of whole plant growth under elevated CO₂. Results
2544 also indicate that fertilization played a relatively more important role in modify-
2545 ing whole plant responses to CO₂ than inoculation with symbiotic nitrogen-fixing
2546 bacteria, perhaps due to a reduction in nodulation across the fertilization gra-
2547 dient. These patterns strongly support the hypothesis that leaf and whole plant
2548 acclimation responses are driven by optimal resource investment to photosynthetic
2549 capacity, and that leaf acclimation responses to CO₂ were not modified by changes
2550 in soil nitrogen availability. These results build on previous work suggesting that
2551 constant leaf nitrogen-photosynthesis relationships are dynamic and change across
2552 growing environments, calling the current formulation of photosynthetic processes
2553 used in many terrestrial biosphere models into question.

2554

Chapter 6

2555

Conclusions

2556 The experiments included in this dissertation test mechanisms that drive patterns
2557 expected from photosynthetic least-cost theory across various edaphic and climatic
2558 gradients. Specifically, I investigate environmental drivers of carbon costs to ac-
2559 quire nitrogen, tradeoffs between nitrogen and water use, and plant acclimation
2560 responses to CO₂. These experiments provide important empirical data needed to
2561 test assumptions made in optimality models that leverage photosynthetic least-
2562 cost frameworks, and are among the first manipulative experiments to show sup-
2563 port for patterns expected from theory. Below, I summarize main findings of each
2564 chapter, synthesize common patterns observed across experiments, and conclude
2565 with a few study ideas that I think will help refine our understanding of plant
2566 nutrient acquisition and allocation responses to environmental change leveraging
2567 patterns predicted by photosynthetic least-cost theory.

2568 In the first experimental chapter, I quantified carbon costs to acquire ni-
2569 trogen in a species capable of forming associations with symbiotic nitrogen-fixing
2570 bacteria (*Glycine max*) and a species not capable of forming such associations
2571 (*Gossypium hirsutum*) grown under four soil nitrogen fertilization treatments and
2572 four light availability treatments in a full factorial greenhouse experiment. Sup-
2573 porting hypotheses, increasing light availability increased carbon costs to acquire
2574 nitrogen in both species due to a larger increase in belowground carbon biomass
2575 than whole plant nitrogen biomass. In further support of hypotheses, increasing
2576 fertilization decreased carbon costs to acquire nitrogen due to a larger increase in

2577 whole plant nitrogen biomass than belowground carbon biomass. Root nodulation
2578 data indicated that *G. max* shifted relative carbon allocation from nitrogen fixa-
2579 tion to direct uptake with increasing fertilization, which may explain the reduced
2580 responsiveness of *G. max* carbon costs to acquire nitrogen across the fertilization
2581 gradient.

2582 Despite evidence that reductions in the response of *G. max* carbon costs
2583 to acquire nitrogen to increasing fertilization may have been driven by shifts away
2584 from nitrogen fixation with increasing fertilization, I urge caution in assigning
2585 causality to the differential response of carbon costs to acquire nitrogen between
2586 species. This is because *G. max* and *G. hirsutum* are not phylogenetically related
2587 and have different life histories. Differences in life history between the two species
2588 limit my ability to assess whether reductions in the negative effect of increasing
2589 fertilization on carbon costs to acquire nitrogen in *G. max* were driven by shifts
2590 to direct uptake with increasing fertilization. However, these patterns were later
2591 confirmed in the fourth experimental chapter, where similar weaker negative ef-
2592 fects of increasing fertilization on carbon costs to acquire nitrogen were observed
2593 in *G. max* that were inoculated with symbiotic nitrogen-fixing bacteria compared
2594 to *G. max* that were left uninoculated across a similar soil nitrogen fertilization
2595 gradient.

2596 In the second experimental chapter, I assessed whether changes in soil
2597 nitrogen availability or soil pH drove changes in nitrogen-water use tradeoffs pre-
2598 dicted by photosynthetic least-cost theory. I measured leaf traits of mature upper
2599 canopy deciduous trees growing in a nine-year nitrogen-by-sulfur field manipula-
2600 tion experiment, where experimental sulfur additions were added with intent to

2601 acidify plots. Following patterns expected from the theory, increasing soil nitrogen
2602 availability was associated with increased leaf nitrogen content, but not net photo-
2603 synthesis, resulting in an increase in photosynthetic nitrogen use efficiency. In
2604 further support of theory, increasing soil nitrogen availability exhibited slight, but
2605 nonsignificant, decreases in leaf $C_i:C_a$ and increases in measures of photosynthetic
2606 capacity. Perhaps the strongest evidence for the theory was a strong negative
2607 relationship between leaf nitrogen content and leaf $C_i:C_a$, of which increased with
2608 increasing soil nitrogen availability through a stronger increase in leaf nitrogen
2609 content than leaf $C_i:C_a$.

2610 I found no effect of soil pH on nitrogen-water use tradeoffs aside from a
2611 marginal reduction in net photosynthesis rates that marginally reduced photosyn-
2612 thetic nitrogen use efficiency with increasing soil pH. Directionally, reductions in
2613 photosynthetic nitrogen use efficiency with increasing soil pH were expected per
2614 theory; however, this response was driven by no change in leaf nitrogen content
2615 and a reduction in net photosynthesis. Theory predicts that these tradeoffs should
2616 be driven by no change in net photosynthesis and an increase in leaf nitrogen con-
2617 tent. The general null leaf response to changing soil pH may have been due to
2618 experimental treatments directly increased soil nitrogen availability and affected
2619 soil pH in opposite patterns, suggesting that soil nitrogen availability may be more
2620 important in dictating nitrogen-water use tradeoffs than soil pH per se.

2621 In the third experimental chapter, I quantified variance in leaf nitrogen
2622 content across a precipitation and soil resource availability gradient in Texan
2623 grasslands. Specifically, I measured area-based leaf nitrogen content, components
2624 of area-based leaf nitrogen content (leaf mass per unit leaf area, leaf nitrogen per

2625 unit dry biomass), leaf $C_i:C_a$, and the unit cost of acquiring nitrogen relative to
2626 water in 520 individuals comprising 57 species. I found that variance in area-
2627 based leaf nitrogen content was positively associated with increasing soil nitrogen
2628 availability, soil moisture, vapor pressure deficit, and was negatively related to
2629 increasing leaf $C_i:C_a$. Following patterns expected from theory, a path analysis
2630 revealed that the positive soil nitrogen-leaf nitrogen relationship was driven by a
2631 positive relationship between soil nitrogen availability and the unit cost of acquir-
2632 ing and using nitrogen relative to water, a positive relationship between the unit
2633 cost of acquiring and using nitrogen relative to water, and negative relationship
2634 between leaf $C_i:C_a$ and leaf mass per unit leaf area. Interestingly, there was no
2635 effect of $C_i:C_a$ on leaf nitrogen content per unit dry biomass, indicating that vari-
2636 ance in area-based leaf nitrogen content across the environmental gradient was
2637 driven by a change in leaf morphology and not leaf chemistry.

2638 In the fourth experimental chapter, I quantified leaf and whole plant accli-
2639 mation responses in *G. max* grown under two atmospheric CO₂ levels, with and
2640 without inoculation with *Bradyrhizobium japonicum*, and across nine nitrogen fer-
2641 tilization treatments in a full factorial growth chamber experiment. I found strong
2642 evidence that leaf nitrogen content, V_{cmax} , and J_{max} were each downregulated un-
2643 der elevated CO₂. A stronger downregulation in V_{cmax} than J_{max} and stronger
2644 downregulation in leaf nitrogen content than V_{cmax} or J_{max} provided strong sup-
2645 port suggesting that leaves were acclimating to elevated CO₂ by optimizing leaf
2646 photosynthetic resource use efficiency to achieve optimal coordination. In striking
2647 support of my hypotheses, I find strong evidence suggesting that leaf acclimation
2648 responses to elevated CO₂ were decoupled from soil nitrogen fertilization and in-

2649 oculation treatment, despite apparent strong increases in leaf nitrogen content,
2650 V_{cmax} , and J_{max} with increasing fertilization and in inoculated pots. These find-
2651 ings contrast the current formulation of photosynthetic processes in terrestrial
2652 biosphere models, where many models simulate downregulations in leaf nitrogen
2653 content under elevated CO₂ as a function of progressive nitrogen limitation.

2654 There are currently two iterations of optimality models that employ the
2655 use of patterns expected from photosynthetic least-cost theory, one for C₃ species
2656 (Wang et al. 2017; Smith et al. 2019; Stocker et al. 2020) and one more recently
2657 developed for C₄ species (Scott and Smith 2022). In both model variants, costs
2658 to acquire and use nitrogen relative to water are held constant using a global
2659 dataset of δ¹³C (Cornwell et al. 2018). Throughout experiments, I show strong
2660 evidence suggesting that costs to acquire and use nitrogen are dynamic and vary
2661 predictably across environmental gradients, and that changes in these costs scale
2662 to alter leaf nitrogen-water use tradeoffs and acclimation responses to changing
2663 environments in ways predicted through photosynthetic least-cost theory. Thus,
2664 while optimality model simulations show good agreement with measured data
2665 (Smith et al. 2019; Stocker et al. 2020), such models may not be capturing an
2666 important source of variability in leaf nitrogen-water use tradeoffs by holding costs
2667 of resource use constant across environmental gradients.

2668 First principles of photosynthetic least-cost theory suggest that, in a given
2669 environment, plants optimize photosynthesis rates by sacrificing inefficient use of
2670 a relatively more abundant (and less costly to acquire) resource for more efficient
2671 use of a relatively less abundant (and more costly to acquire) resource. Through-
2672 out experimental chapters, I show strong support for these patterns across ex-

2673 periments, where increasing soil nitrogen fertilization generally decreased the cost
2674 of acquiring nitrogen relative to water, a pattern that scaled to influence leaf
2675 nitrogen-water use tradeoffs. I did not find evidence to suggest that soil moisture
2676 influenced nitrogen-water use tradeoffs, though this was due to strong covariation
2677 between soil moisture and soil nitrogen availability. Overall, findings across exper-
2678 iments provide empirical validation of photosynthetic least-cost theory needed to
2679 further develop optimality models and eventually implement such models in ter-
2680 restrial biosphere model products. Many terrestrial biosphere model products do
2681 not include robust frameworks for simulating acclimation responses to changing
2682 environmental conditions, and empirical findings shown here provide some support
2683 that optimality models that leverage photosynthetic least-cost theory predictions
2684 may improve the ability of terrestrial biosphere models to accurately simulate
2685 photosynthetic processes.

2686 Many terrestrial biosphere models predict photosynthetic capacity through
2687 plant functional group-specific linear regressions between area-based leaf nitrogen
2688 content and V_{cmax} (Rogers 2014; Rogers et al. 2017), which assumes that leaf
2689 nitrogen-photosynthesis relationships are constant across growing environments.
2690 I found constant leaf nitrogen-photosynthesis relationships with increasing soil ni-
2691 trogen availability in the nitrogen-by-sulfur field manipulation experiment. How-
2692 ever, results from the CO₂-by-nitrogen-by-inoculation manipulation experiment
2693 indicated that leaf nitrogen-photosynthesis responses to soil nitrogen availability
2694 were dependent on whether nitrogen was limiting. Further investigation regard-
2695 ing the effect of soil nitrogen availability in modifying leaf nitrogen-photosynthesis
2696 relationships is warranted to better understand the generality of leaf nitrogen pho-

2697 tosynthesis relationships across environmental gradients. However, findings from
2698 these experiments suggest that representing photosynthetic processes through pos-
2699 itive relationships between soil nitrogen availability, leaf nitrogen, and photosyn-
2700 thetic capacity are likely contributing to erroneous errors in model simulations and
2701 may explain the high degree of divergence in simulated processes across terrestrial
2702 biosphere models (Friedlingstein et al. 2014; Davies-Barnard et al. 2020).

2703 The experiments included in this dissertation have provided a strong foun-
2704 dation for me to continue growing as a plant physiological ecologist. I envision
2705 five primary avenues for future research that build on the work presented here,
2706 which are briefly summarized below:

- 2707** 1. Manipulative and environmental gradient experiments included here were
2708 designed to provide empirical data needed to test photosynthetic least-cost
2709 theory assumptions. While these results show promising patterns for pat-
2710 terns expected from photosynthetic least-cost theory, they do not necessarily
2711 address whether these patterns follow those simulated by optimality models
2712 that leverage photosynthetic least-cost principles. Thus, a clear future di-
2713 rection of these experiments would be to conduct model-data comparisons
2714 using data collected here (or similar experiments) to compare against opti-
2715 mality model simulations.

- 2716** 2. Experiments included here explicitly quantify effects of symbiotic nitrogen
2717 fixation on carbon costs to acquire nitrogen, nitrogen-water use tradeoffs,
2718 and leaf nitrogen-photosynthesis relationships. However, carbon costs to ac-
2719 quire nitrogen also vary in species that associate with different mycorrhizal
2720 types (Brzostek et al. 2014; Terrer et al. 2018), and dominant mycorrhizal

2721 type in an ecosystem has been shown to determine net biogeochemical cycle
2722 dynamics in deciduous forests of the northeastern United States (Phillips
2723 et al. 2013). Thus, future work should consider conducting similar experi-
2724 ments while manipulating mycorrhizal association to better understand how
2725 microbial symbioses modify leaf and whole plant acclimation responses to
2726 changing environments.

2727 3. Recent work indicates a high degree of variance in symbiotic nitrogen fixa-
2728 tion rates across terrestrial biosphere models (Meyerholt et al. 2016; Davies-
2729 Barnard et al. 2020), perhaps due to nitrogen fixation rates that are im-
2730 plemented across terrestrial biosphere models as a function of temperature
2731 (Houlton et al. 2008). While energetic costs of nitrogen fixation are de-
2732 pendent on temperature, I show that structural carbon costs to acquire
2733 nitrogen via symbiotic nitrogen fixation are driven by factors that influence
2734 demand to acquire nitrogen (i.e. CO₂, light) and are modified by soil ni-
2735 tragen supply. The light-by-nitrogen greenhouse experiment was published
2736 in *Journal of Experimental Botany*, and a reviewer encouraged future work
2737 to include a model-data comparison comparing structural carbon costs to
2738 acquire nitrogen measured in the experiment to carbon costs to acquire ni-
2739 tragen simulated by the FUN biogeochemical model (Fisher et al. 2010;
2740 Brzostek et al. 2014; Allen et al. 2020). Conveniently, FUN calculates car-
2741 bon costs to acquire nitrogen following the same calculation used in the first
2742 and fourth experimental chapter. Conducting such a model-data comparison
2743 would be a useful step toward identifying biases in the FUN biogeochemi-
2744 cal model, which is currently coupled to several terrestrial biosphere models

2745 (Clark et al. 2011; Shi et al. 2016; Lawrence et al. 2019; Davies-Barnard
2746 et al. 2020).

2747 4. Carbon costs to acquire nitrogen relative to water were quantified at the
2748 leaf level as a function of $\delta^{13}\text{C}$ and vapor pressure deficit, while structural
2749 carbon costs to acquire nitrogen were quantified at the whole plant level
2750 as the ratio of belowground carbon allocation per unit whole plant nitro-
2751 gen biomass. As increasing soil nitrogen availability decreases both leaf and
2752 whole plant estimates of costs to acquire and use nitrogen, one might expect
2753 leaf and whole plant carbon cost to acquire nitrogen estimates to covary. Fu-
2754 ture work should consider investigating if leaf and whole plant estimates of
2755 carbon costs to acquire nitrogen covary and evaluate whether environmental
2756 conditions (or species acquisition strategy) modifies any of this possible co-
2757 variance. Strong covariance between leaf and whole plant costs of nitrogen
2758 acquisition could be a possible avenue to implement frameworks for allowing
2759 costs of nitrogen acquisition to vary in optimality models, as the FUN model
2760 calculates carbon costs of nitrogen acquisition at the whole plant level.

2761 5. While experiments included here target effects of soil nitrogen availability
2762 on carbon costs to acquire nitrogen and associated leaf nitrogen-water use
2763 tradeoffs, photosynthetic least-cost theory predicts that plants acclimate
2764 their photosynthetic processes by minimizing the summed cost of nutrient
2765 (not just nitrogen) and water use. Therefore, the theory would predict
2766 similar leaf acclimation responses across soil phosphorus or other nutrient
2767 availability gradients. Recent iterations of the FUN biogeochemical cycle
2768 includes a framework for determining the carbon and nitrogen cost of ac-

2769 quiring and using phosphorus, which similarly varies in species with different
2770 nutrient acquisition strategies (Allen et al. 2020). The implementation of
2771 this model in a terrestrial biosphere model (E3SM) was also recently shown
2772 to improve model performance of ecosystem nutrient limitation (Braghieri
2773 et al. 2022). As nitrogen and phosphorus commonly co-limit leaf photo-
2774 synthesis and primary productivity, extending experiments reported here to
2775 investigate carbon and nitrogen costs of phosphorus use, and whether these
2776 patterns scale to leaf nutrient-water use tradeoffs would be a useful next
2777 step in understanding extensions and limitations of photosynthetic least-
2778 cost theory.

2779 The experiments included in this dissertation and the proposed experiments sum-
2780 marized above provide a snapshot view of the things that I have learned through-
2781 out my time as a graduate student. I am excited to continue learning and growing
2782 as a plant ecophysiologicalist, ecologist, and scientist, and look forward to continuing
2783 along my journey of investigating nutrient acquisition and allocation responses to
2784 global change.

2785

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3636 Appendix A: Supplemental material for "Structural carbon costs to
3637 acquire nitrogen are determined by nitrogen and light availability in
3638 two species with different nitrogen acquisition strategies"

Table A1. Summary table containing volumes of compounds used to create modified Hoagland's solutions for each soil nitrogen fertilization treatment. All volumes are expressed as milliliters per liter (mL/L)

Compound	0 ppm N	70 ppm N	210 ppm N	630 ppm N
1 M NH ₄ H ₂ PO ₄	0	0.33	1	1
2 M KNO ₃	0	0.67	2	2
2 M Ca(NO ₃) ₂	0	0.67	2	2
1 M NH ₄ NO ₃	0	0.33	1	0
8 M NH ₄ NO ₃	0	0	0	2
1 M KH ₂ PO ₄	1	0.67	0	0
1 M KCl	4	1.33	0	0
1 M CaCO ₃	4	3	0	0
2 M MgSO ₄	1	1	1	1
10% Fe-EDTA	1	1	1	1
Trace Elements	1	1	1	1

Table A2. Analysis of variance results exploring species-specific effects of light availability, nitrogen fertilization, and their interactions on the ratio of whole plant biomass to pot volume (g L^{-1})^{*}

	df	Coefficient	χ^2	p
<i>G. hirsutum</i>				
Intercept		0.740	-	-
Light (L)	1	-4.23E-03	189.581	<0.001
Nitrogen (N)	1	7.86E-04	17.927	<0.001
L*N	1	-6.61E-06	4.709	0.030
<i>G. max</i>				
Intercept		-0.233	-	-
Light (L)	1	-1.12E-02	69.500	<0.001
Nitrogen (N)	1	8.29E-04	40.297	<0.001
L*N	1	-8.51E-06	5.548	0.019

3639 *Significance determined using Wald's χ^2 tests ($p=0.05$). *P*-values less than 0.05
3640 are in bold and *p*-values between 0.05 and 0.1 are italicized. Negative coefficients
3641 for light treatments indicate a positive effect of increasing light availability on
3642 all response variables, as light availability is treated as percent shade cover in all
3643 linear mixed-effects models.

Table A3. Slopes of the regression line describing the relationship between each dependent variable and nitrogen fertilization at each light level*

Shade cover	Slope
<i>G. hirsutum</i>	
0%	8.29E-04^a
30%	5.74E-04^a
50%	4.03E-04^a
80%	1.48E-04 ^a
<i>G. max</i>	
0%	7.86E-04
30%	5.87E-04
50%	4.55E-04
80%	<i>2.57E-05</i>

3644 *Slopes represent estimated marginal mean slopes from linear mixed-effects models described in the Methods. Slopes
3645 were calculated using the ‘emmeans’ R package (Lenth 2019). Superscripts indicate slopes fit to natural-log (^a) or
3646 square root (^b) transformed data. Slopes statistically different from zero (Tukey: $p < 0.05$) are indicated in bold.
3647 Marginally significant slopes (Tukey: $0.05 < p < 0.1$) are italicized.

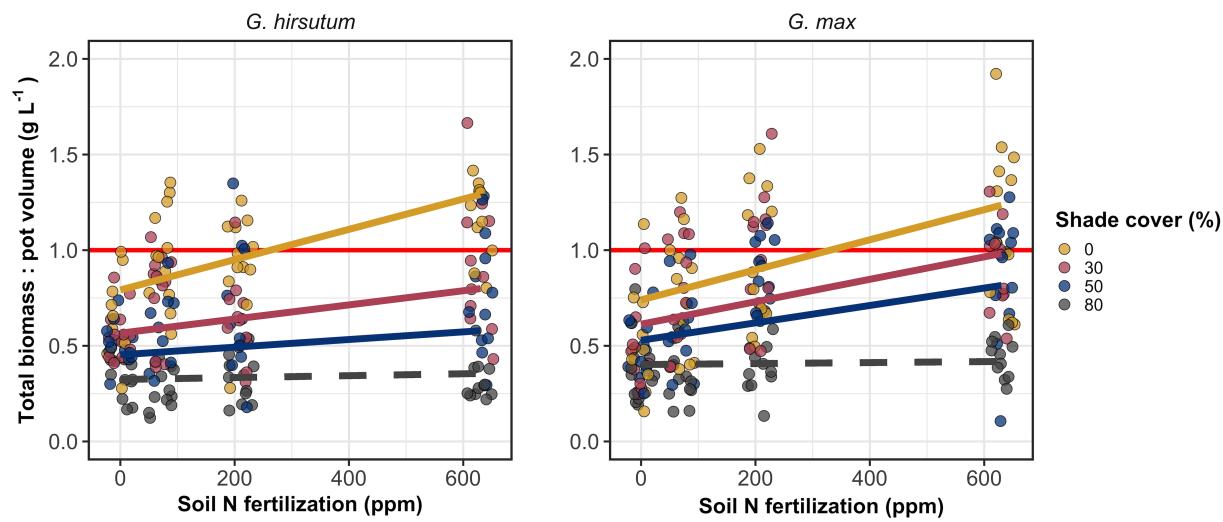


Figure A1. Effects of shade cover and nitrogen fertilization on the ratio of plant biomass to rooting volume in *G. hirsutum* (left panel) and *G. max* (right panel). The red horizontal line indicates the recommended 1 g L^{-1} threshold for BVR recommended by Poorter et al. (2012) to avoid pot size-induced growth limitation. Nitrogen fertilization treatments are represented on the x-axis. Shade cover treatments are represented through colored points and trendlines. Points are jittered for visibility. Yellow points and trendlines represent the 0% shade cover treatment, blue points and trendlines represent the 30% shade cover treatment, green points and trendlines represent the 50% shade cover treatment, and purple points and trendlines represent the 80% shade cover treatment. Solid trendlines indicate slopes that are significantly different from zero (Tukey: $p < 0.05$), while dashed trendlines indicate slopes that are not statistically different from zero.

3648 Appendix B: Supplemental material for "Soil nitrogen availability
3649 modifies leaf nitrogen economies in mature temperate deciduous
3650 forests: a direct test of photosynthetic least-cost theory"

Table B1. Sample sizes of each species, abbreviated by their USDA NRCS PLANTS database code, within each plot at each site*

	ACRU	ACSA	FAGR	FRAM	QURU	N_{plot}
Bald Hill						
+N; +S	0	6	1	0	1	8
+N; -S	1	2	2	0	1	6
-N; +S	2	2	0	0	2	6
-N; -S	2	3	3	0	2	10
Carter Creek						
+N; +S	0	6	1	2	0	9
+N; -S	0	4	0	2	0	6
-N; +S	0	5	1	4	0	10
-N; -S	0	7	0	0	0	7
Mount Pleasant						
+N; +S	3	2	1	0	3	9
+N; -S	0	5	4	1	0	10
-N; +S	1	2	4	0	0	7
-N; -S	3	3	1	2	1	10
N_{spp}	12	47	18	11	10	98

3651 *Plots within each site are represented based on nitrogen and sulfur addition
3652 status. The final column on the right depicts total sample size per plot in each
3653 site (N_{plot}) and the final row on the bottom represents cumulative species sample
3654 size across all plots and all sites (N_{spp}). Key: ACRU=*A. rubrum*; ACSA=*A.*
3655 *saccharum*; FAGR=*F. grandifolia*; FRAM=*F. americana*; QURU=*Q. rubra*

Table B2. Analysis of variance results exploring the linear effect of leaf temperature on net photosynthesis rate (A_{net} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) and stomatal conductance (g_{sw} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) measured at $400 \mu\text{mol mol}^{-1} \text{CO}_2$

	df	A_{net}		g_{sw}	
		χ^2	p	χ^2	p
Leaf temperature	1	1.287	0.257	1.716	0.190

3656 *Results detail linear mixed effects model where temperature was regressed against
3657 net photosynthesis or stomatal conductance, with site and species designated as
3658 random intercept terms. Significance was determined using Type II Wald χ^2 tests
3659 ($\alpha=0.05$).

Table B3. Second order log-polynomial regression coefficients that described the effect of leaf temperature on net photosynthesis (A_{net} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) and stomatal conductance (g_s ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) measured at $400 \mu\text{mol mol}^{-1} \text{CO}_2$ *

	a	b	c
A_{net}	9.422	-0.573	0.010
g_s	-0.170	-0.186	0.003

3660 *Net photosynthesis and stomatal conductance values were fit to the log-polynomial
3661 equation $\log(y) = a + bx + cx^2$, where x is leaf temperature in °C.

Table B4. Mean, standard error, and 95% confidence interval ranges of soil nitrogen availability estimates across all measured plots. All units are expressed as $\mu\text{g N g}^{-1}$ resin d^{-1}

Site	Treatment	Mean	SE	Lower 95% CI	Upper 95% CI
Bald Hill	Ammonium sulfate	27.11	6.14	15.08	39.13
Bald Hill	Control	14.41	5.02	4.56	24.26
Bald Hill	Sodium nitrate	20.65	3.15	14.46	26.83
Bald Hill	Sulfur	6.33	2.19	2.04	10.62
Carter Creek	Ammonium sulfate	26.94	5.36	16.43	37.44
Carter Creek	Control	19.87	1.92	16.10	23.64
Carter Creek	Sodium nitrate	15.51	4.16	7.36	23.65
Carter Creek	Sulfur	5.50	1.40	2.75	8.25
Mount Pleasant	Ammonium sulfate	8.02	2.31	3.49	12.56
Mount Pleasant	Control	2.00	0.57	0.89	3.11
Mount Pleasant	Sodium nitrate	2.52	0.68	1.19	3.85
Mount Pleasant	Sulfur	2.42	0.39	1.66	3.17

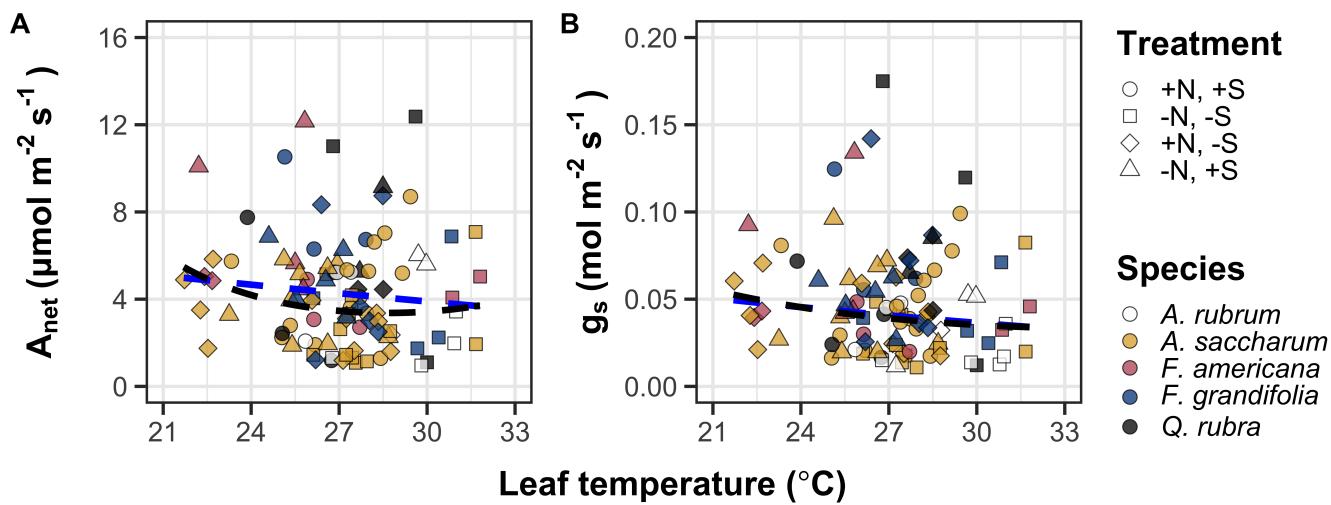


Figure B1. Effects of leaf temperature on net photosynthesis rate (A) and stomatal conductance (B) values when measured at $400 \mu\text{mol mol}^{-1} \text{CO}_2$. Leaf temperature is represented on the x-axis, while species are represented as colored points. Colored points and shapes are as explained in Figure 3.1. The dashed blue trendline describes the linear relationship between leaf temperature and each response variable, while the dashed black trendline describes the same relationship with a log-polynomial regression equation.

**3662 Appendix C: Supplemental material for "The relative cost of resource
3663 use for photosynthesis drives variance in leaf nitrogen content across a
3664 climate and soil resource availability gradient"**

3665 C.1 Calculations for soil water holding capacity

3666 Water holding capacity (θ_{WHC} ; mm) was calculated as a function of the volumetric
3667 soil water storage at field capacity (W_{FC} ; $m^3 m^{-3}$), and the volumetric soil water
3668 storage at wilting point (W_{PWP} ; $m^3 m^{-3}$):

$$\theta_{WHC} = (W_{FC} - W_{PWP})(1 - f_{gravel}) * \min(z_{bedrock}, z_{max}) \quad (C4.1)$$

3669 where f_{gravel} (%) is the fraction of gravel content in soil, $z_{bedrock}$ (mm) is the
3670 distance to bedrock, and z_{max} (mm) is the maximum allowable distance to bedrock,
3671 set to 2000mm. W_{FC} is calculated as:

$$\theta_{FC} = k_{fc} + (1.283 * (k_{fc})^2 - 0.374 * k_{fc} - 0.015) \quad (C4.2)$$

3672 where

$$\begin{aligned} k_{fc} = & -0.251 * f_{sand} + 0.195 * f_{clay} + 0.011 * f_{OM} \\ & + 0.006 * (f_{sand} * f_{OM}) - 0.027 * (f_{clay} \\ & * f_{OM}) + 0.452 * (f_{sand} * f_{clay}) + 0.299 \end{aligned} \quad (C4.3)$$

3673 W_{PWP} is calculated as:

$$W_{PWP} = k_{pwp} + (0.14 * k_{pwp} - 0.02) \quad (\text{C4.4})$$

3674 where

$$\begin{aligned} k_{pwp} = & -0.024 * f_{sand} + 0.487 * f_{clay} + 0.006 * f_{OM} \\ & + 0.005 * (f_{sand} * f_{OM}) - 0.013 * (f_{clay} \\ & * f_{OM}) + 0.068 * (f_{sand} * f_{clay}) + 0.031 \end{aligned} \quad (\text{C4.5})$$

3675 In Equations C4.4 and C4.5, f_{sand} (%) is the fraction of sand content in soil

3676 (%), f_{clay} (%) is the fraction of clay content in soil (%), and f_{OM} is the fraction of

3677 organic matter in soil (%). Organic matter in the soil was calculated by converting

3678 soil organic carbon data extracted from SoilGrids 2.0 to soil organic matter using

3679 the van Bemmelen factor (1.724 conversion factor).

Table C1. List of sampled species and their plant functional group assignment

Symbol	Species	Photo. pathway	Growth duration	Growth habit	N-fixer?	Plant functional group	Number sampled
ACAN11	<i>Acaciella angustissima</i>	c3	perennial	forb	yes	c3_legume	3
AMAR2	<i>Ambrosia artemisiifolia</i>	c3	annual	forb	no	c3_nonlegume	25
AMPS	<i>Ambrosia psilostachya</i>	c3	perennial	forb	no	c3_nonlegume	32
ARAL3	<i>Argemone albiflora</i>	c3	annual	forb	no	c3_nonlegume	3
ARPU9	<i>Aristida purpurea</i>	c4	perennial	graminoid	no	c4_nonlegume	2
ASAS	<i>Asclepias asperula</i>	c3	perennial	forb	no	c3_nonlegume	3
ASLA4	<i>Asclepias latifolia</i>	c3	perennial	forb	no	c3_nonlegume	3
ASSY	<i>Asclepias syriaca</i>	c3	perennial	forb	no	c3_nonlegume	18
BOIS	<i>Bothriochloa ischaemum</i>	c4	perennial	graminoid	no	c4_nonlegume	6
BOSA	<i>Bothriochloa saccharoides</i>	c4	perennial	graminoid	no	c4_nonlegume	6
CAPL3	<i>Carex planostachys</i>	c4	perennial	graminoid	no	c4_nonlegume	3
CAREX	<i>Carex</i> spp.	c4	perennial	graminoid	no	c4_nonlegume	16
CHFE3	<i>Chamaesyce fendleri</i>	c3	perennial	forb	no	c3_nonlegume	2
CHPI8	<i>Chrysopsis pilosa</i>	c3	annual	forb	no	c3_nonlegume	3
COCO13	<i>Conoclinium coelestinum</i>	c3	perennial	forb	no	c3_nonlegume	3
COER	<i>Commelina erecta</i>	c3	perennial	forb	no	c3_nonlegume	3
CRGLL	<i>Croton glandulosus</i>	c3	annual	forb	no	c3_nonlegume	22
CYDA	<i>Cynodon dactylon</i>	c4	perennial	graminoid	no	c4_nonlegume	15
DIAN	<i>Dichanthium annulatum</i>	c4	perennial	graminoid	no	c4_nonlegume	8
ENPE4	<i>Engelmannia peristenia</i>	c3	perennial	forb	no	c3_nonlegume	6
EUMA8	<i>Euphorbia marginata</i>	c3	annual	forb	no	c3_nonlegume	6
GAPU	<i>Gaillardia pulchella</i>	c3	annual	forb	no	c3_nonlegume	16
GLGO	<i>Glandularia gooddngii</i>	c3	perennial	forb	no	c3_nonlegume	2
HEAN3	<i>Helianthus annuus</i>	c3	annual	forb	no	c3_nonlegume	6

Table C2. List of sampled species and their plant functional group assignment (cont.)

Symbol	Species	Photo. pathway	Growth duration	Growth habit	N-fixer?	Plant functional group	Number sampled
HECA8	<i>Heterotheca canescens</i>	c3	perennial	forb	no	c3_nonlegume	2
HETE3	<i>Heliotropium tenellum</i>	c3	annual	forb	no	c3_nonlegume	3
IVAX	<i>Iva axillaris</i>	c3	perennial	forb	no	c3_nonlegume	4
LIAT	<i>Lilaeopsis attenuata</i>	c3	perennial	forb	no	c3_nonlegume	3
LIPU	<i>Liatris punctata</i>	c3	perennial	forb	no	c3_nonlegume	3
LOPE	<i>Lolium perenne</i>	c3	perennial	graminoid	no	c3_nonlegume	9
MIQU2	<i>Mimosa quadrivalvis</i>	c3	perennial	forb	yes	c3_legume	15
NALE3	<i>Nassella leucotricha</i>	c3	perennial	graminoid	no	c3_nonlegume	19
OECU2	<i>Oenothera curtiflora</i>	c3	annual	forb	no	c3_nonlegume	3
OENOT	<i>Oenothera</i> spp.	c3	annual	forb	no	c3_nonlegume	1
PAVI2	<i>Panicum virgatum</i>	c4	perennial	graminoid	no	c4_nonlegume	12
RACO3	<i>Ratibida columnifera</i>	c3	perennial	forb	no	c3_nonlegume	40
RHSET	<i>Rhynchosia senna</i>	c3	perennial	forb	yes	c3_legume	1
RUHI2	<i>Rudbeckia hirta</i>	c3	perennial	forb	no	c3_nonlegume	3
RUNU	<i>Ruellia nudiflora</i>	c3	perennial	forb	no	c3_nonlegume	15
RUTR	<i>Rubus trivialis</i>	c3	perennial	vine	no	c3_nonlegume	3
SAFA2	<i>Salvia farinacea</i>	c3	perennial	forb	no	c3_nonlegume	7
SCHIZ4	<i>Schizachyrium</i> spp.	c4	perennial	graminoid	no	c4_nonlegume	8
SCSC	<i>Schizachyrium scoparium</i>	c4	perennial	graminoid	no	c4_nonlegume	3
SODI	<i>Solanum dimidiatum</i>	c3	perennial	forb	no	c3_nonlegume	1
SOEL	<i>Solanum elaeagnifolium</i>	c3	perennial	forb	no	c3_nonlegume	53
SOHA	<i>Sorghum halapense</i>	c4	perennial	graminoid	no	c4_nonlegume	38
STTE3	<i>Stillingia texana</i>	c3	perennial	forb	no	c3_nonlegume	3
VEOC	<i>Verbesina occidentalis</i>	c3	perennial	forb	no	c3_nonlegume	3
VEST	<i>Verbena stricta</i>	c3	perennial	forb	no	c3_nonlegume	3

Table C3. Model selection results for soil moisture and vapor pressure deficit. Soil moisture was used in a bivariate regression against log-transformed β , while vapor pressure deficit was used in bivariate regressions against leaf $C_l:C_a$

Day	Soil moisture		VPD	
	AICc	RMSE	AICc	RMSE
1	1431.77	0.8400	-772.71	0.0853
2	1431.76	0.8400	-775.47	0.0849
3	1431.78	0.8400	-770.86	0.0854
4	1431.79	0.8401	-793.49	0.0839
5	1431.79	0.8401	-771.66	0.0853
6	1431.78	0.8401	-771.66	0.0853
7	1431.78	0.8401	-771.05	0.0854
8	1431.76	0.8401	-770.94	0.0854
9	1431.75	0.8401	-770.11	0.0854
10	1431.74	0.8401	-770.08	0.0855
15	1431.54	0.8401	-768.64	0.0856
20	1431.40	0.8401	-769.77	0.0855
30	1431.23	0.8400	-772.18	0.0853
60	1429.84	0.8391	-779.06	0.0848
90	1429.14	0.8385	-773.99	0.0852

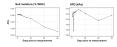


Figure C1. Model selection results exploring relevant timescales for soil moisture (left panel) and vapor pressure deficit (right panel). The x-axis indicates the number of days before each site visit and the y-axis notes the corrected Akaike Information Criterion value. The timescale with the lowest AICc value, and therefore most relevant timescale to include in statistical models, is noted as a red point.

**3680 Appendix D: Supplemental material for "Optimal resource investment
 3681 to photosynthetic capacity maximizes nutrient allocation to whole
 3682 plant growth under elevated CO₂"**

Table D1. Summary table containing volumes of compounds used to create modified Hoagland's solutions for each soil nitrogen fertilization treatment. All volumes are expressed as milliliters per liter (mL/L)

Compound	0 ppm N	35 ppm N	70 ppm N	105 ppm N	140 ppm N
1 M NH ₄ H ₂ PO ₄	0	0.165	0.33	0.5	0.67
2 M KNO ₃	0	0.335	0.67	1	1.33
2 M Ca(NO ₃) ₂	0	0.335	0.67	1	1.33
1 M NH ₄ NO ₃	0	0.165	0.33	0.5	0.67
8 M NH ₄ NO ₃	0	0	0	0	0
1 M KH ₂ PO ₄	1	0.85	0.67	0.5	0.33
1 M KCl	3	2.45	2	1.5	1
1 M CaCO ₃	4	3.33	2.67	2	1.33
2 M MgSO ₄	1	1	1	1	1
10% Fe-EDTA	1	1	1	1	1
Trace elements	1	1	1	1	1

Compound	210 ppm N	280 ppm N	350 ppm N	630 ppm N
1 M NH ₄ H ₂ PO ₄	1	1	1	1
2 M KNO ₃	2	2	2	2
2 M Ca(NO ₃) ₂	2	2	2	2
1 M NH ₄ NO ₃	1	3.5	0	0
8 M NH ₄ NO ₃	0	0	0.75	2
1 M KH ₂ PO ₄	0	0	0	0
1 M KCl	0	0	0	0
1 M CaCO ₃	0	0	0	0
2 M MgSO ₄	1	1	1	1
10% Fe-EDTA	1	1	1	1
Trace elements	1	1	1	1

Table D2. Summary of the daily growth chamber growing condition program

Time	Air temperature (°C)	Light (%)
09:00	21	25
09:45		50
10:30	25	75
11:15		100
22:45	21	75
23:30		50
00:15	17	25
01:00		0

Table D3. Effects of CO₂, fertilization, and inoculation on whole plant biomass: pot volume (BVR; g L⁻¹)*

	df	Coefficient	χ^2	p
(Intercept)	-	1.33E-01	-	-
CO ₂	1	1.53E-01	146.004	<0.001
Inoculation (I)	1	4.19E-01	19.320	<0.001
Fertilization (N)	1	1.90E-03	279.387	<0.001
CO ₂ *I	1	1.03E-01	0.007	0.934
CO ₂ *N	1	2.44E-03	49.725	<0.001
I*N	1	-6.90E-04	9.006	0.003
CO ₂ *I*N	1	-4.95E-04	0.640	0.424

3683 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less
3684 than 0.05 are in bold. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square
3685 test statistic.

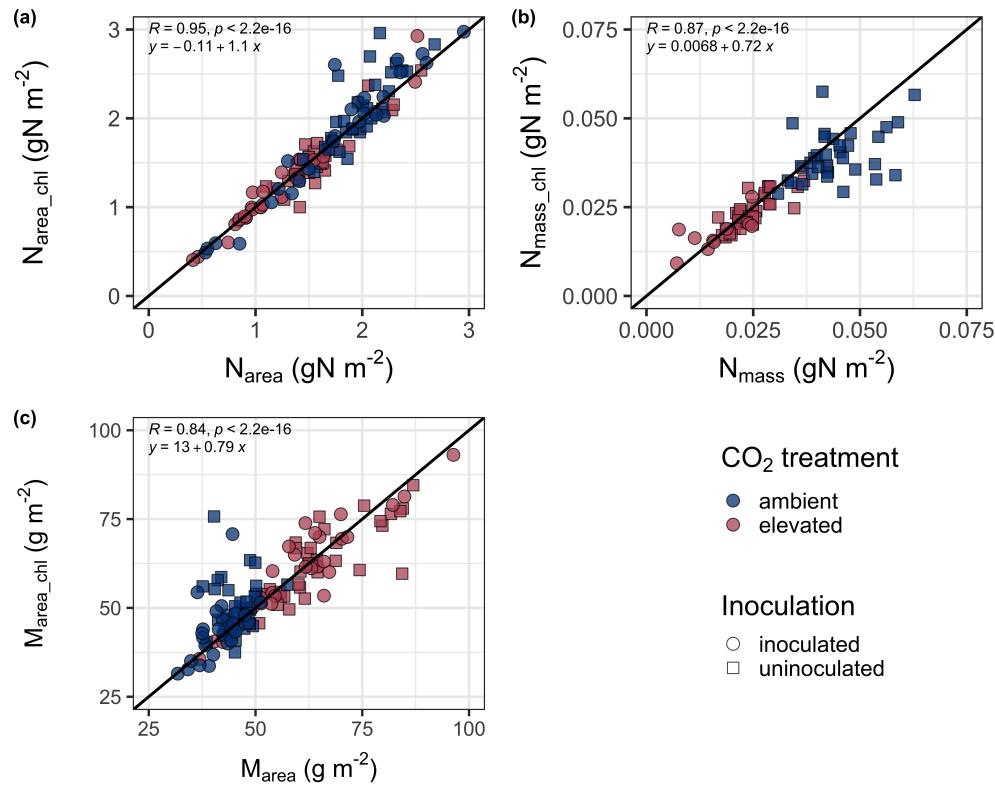


Figure D1. Relationships between area-based leaf nitrogen content (a), mass-based leaf nitrogen content (b), and leaf mass per unit leaf area (c) measured on the focal leaf used to generate A_{net}/C_i curves (x-axis) and leaf nitrogen content measured on the leaf used for chlorophyll extractions (y-axis). Blue points refer to leaves grown under ambient CO₂ and red points refer leaves grown under elevated CO₂. Square points indicate uninoculated pots and circular points indicate inoculated pots. Pearson's correlation coefficient, associated *p*-values, and the line of the regression line that described each bivariate are included in the top left corner of each plot. The solid black line visualizes the trend given a 1:1 bivariate relationship.

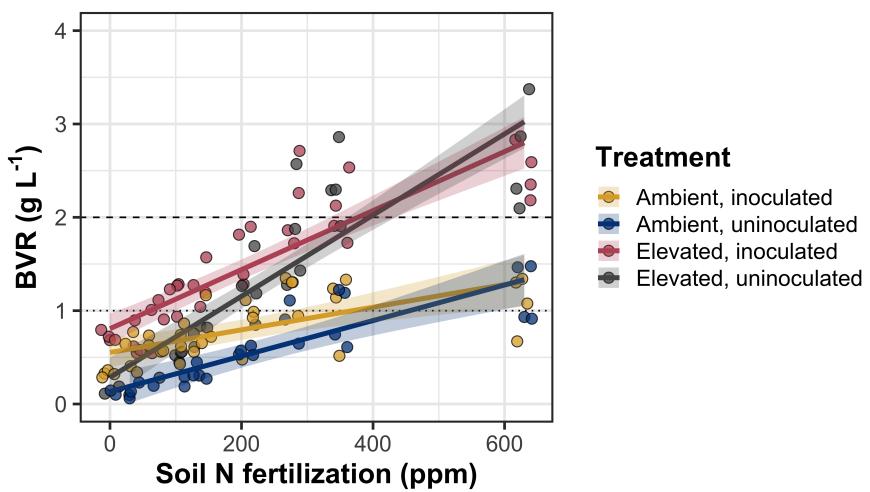


Figure D2. Effects of CO₂, fertilization, and inoculation on the ratio of whole plant biomass to pot volume. Soil nitrogen fertilization is represented on the x-axis in all panels. Yellow points and trendlines indicate inoculated individuals grown under ambient CO₂, blue points and trendlines indicate uninoculated individuals grown under ambient CO₂, red points and trendlines indicate inoculated individuals grown under elevated CO₂, and grey points indicate uninoculated individuals grown under elevated CO₂. Solid trendlines indicate regression slopes that are different from zero ($p<0.05$). The dotted horizontal line indicates the point where biomass:pot volume exceeds 1 g L⁻¹, and the dashed line indicates the point where biomass:pot volume exceeds 2 g L⁻¹.